Diversity of Tulasnellaceae mycorrhizal associations of Australian terrestrial orchids

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Research School of Biology

DECLARATION

I, Arild R. Arifin certify that:

This thesis does not contain material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution. All chapters are co-authored and the author contributions are declared.

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[Arild Ranlym Arifin]

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ABSTRACT

Orchid mycorrhizal fungi (OMF) is essential for orchid seed germination and survival due to the tiny size and lack of endosperm of orchid seeds. Studying the fungal relationships in orchids are important as they provide insights into understanding fungal biodiversity and ecology. My first chapter is a study of the OMF associations in the Australian *Cryptostylis* orchids, which are sexually-deceptive with several unusual features in relation to pollinator sharing and a mix of evergreen and leafless species. This chapter investigates the diversity of *Tulasnella* in *Cryptostylis*, finding that the five Australian *Cryptostylis* species associate with nine *Tulasnella* operational taxonomic units (OTUs)/species and an additional three Asiatic *Cryptostylis* associate with four *Tulasnella* OTUs. Interestingly, all the *Tulasnella* fungi are closely related despite the geographical distance among the orchid hosts. Furthermore, a small number of endophytic and ectomycorrhizal fungi also associate with *Cryptostylis* species and is not restricted to the putative mycoheterotrophic species, meaning the phenomena of also associating with ectomycorrhizal or endophytic fungi is not related with the orchid’s trophic status and unusual in orchids.

My second chapter is exploring whether closely related orchids also have closely related mycorrhizal fungi. To address this, we studied the mycorrhizal associations of sexually-deceptive orchids in the subtribes Drakaeinae and Cryptostylidinae. Drakaeinae (*Arthrochilus*, *Caleana*, *Paracaleana*, *Chiloglottis*, *Drakaea*, and *Spiculaea*) and Cryptostylidinae orchids associate with 20 closely related fungal OTUs/species, with four of them were shared between two orchid subtribes. Cophylogenetic analysis between Drakaeinae orchids and *Tulasnella* fungi shows both phylogenies are congruent, but no congruency between *Cryptostylis* and *Tulasnella*. The significant congruency between Drakaeinae and *Tulasnella* symbionts suggests a pattern of phylogenetic niche conservatism rather than coevolution since fungi can grow independently of orchids. Rapid diversification in *Chiloglottis* and *Drakaea* but not in *Cryptostylis* may reveal the different patterns of congruency in Drakaeinae and Cryptostylidinae.

While exploring *Tulasnella* from *Cryptostylis* as well as Drakaeinae, several undescribed species of *Tulasnella* were discovered. In Chapter Three we use multiple sequence locus phylogenetic analyses combined with morphological characteristics to delimit and describe six new *Tulasnella* species associated with Australian sexually-deceptive orchids from the subtribes Cryptostylidinae and Drakaeinae. Five of the new species, *Tulasnella australiensis, T. occidentalis, T. punctata, T. densa* and *T. concentrica*, all associate with *Cryptostylis* (Cryptostylidinae) orchids whereas *T. rosea* associates with *Spiculaea ciliata* (Drakaeinae). Despite the orchid hosts being distantly related they share phylogenetically closely related *Tulasnella* species with similar macro and micromorphological features, with one of the most distinctive features of having binucleate hyphal compartments.

In Chapter Four, I describe a further two *Tulasnella* species from *Rimacola elliptica* and *Pyrorchis nigricans,* both orchid hosts are members of subtribe Megastylidinae. These two fungal species belong to a two distantly related *Tulasnella* clades, which are also distantly related to those from Drakaeinae and Cryptostylidinae orchids as described in Chapter Three. One new *Tulasnella* species from Megastylidinae is characterised by multinucleate cell characteristics. This is the first report of a multinucleate *Tulasnella*, with only binucleate species reported, therefore this multinucleate species may harbour genetically diverse nuclei, making it more adaptable to e.g. environmental conditions, a subject worth further exploration. By delimiting and formally describing these *Tulasnella* species in Chapters Three and Four, it significantly contributes to the documentation of *Tulasnella* diversity and provides names and delimitations to underpin further research on the fungi and their relationships with orchids.

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AUTHORSHIP DECLARATION FOR CO-AUTHORED CHAPTERS

This thesis contains work that has been prepared for publications.

Chapter 1: *Cryptostylis* species (Orchidaceae) from a broad geographic and habitat range associate with a phylogenetically narrow lineage of Tulasnellaceae fungi

Arild R. Arifin (AR) performed the data collections and fieldwork, performed the research, analysed and interpreted the data and drafted the chapter. Alyssa M. Weinstein (AW) contributed to data collections, fieldwork and editing, Ryan D. Phillips (RDP) contributed to conceptual development of the chapter and editorial comments. Celeste C. Linde (CCL) contributed to research design, conceptual development and editorial comments.

Chapter 2: Evolutionary shifts in host association among Australian orchids: *Tulasnella* fungi associated with Drakaeinae and Cryptostylidinae orchids

AR performed the data collection, fieldwork, conducted the research, analysed and interpreted the data and drafted the chapter. RDP contributed to data collection, fieldwork and editorial comments. CCL contributed to research design, conceptual development and editorial comments.

Chapter 3: New species of *Tulasnella* associated with Australian terrestrial orchids in the Cryptostylidinae and Drakaeinae

AR performed the research and data collection, analysed and interpreted the data and drafted the chapter. Tom W. May (TM) contributed to conceptual development and editorial comments. CCL contributed to research design, conceptual development and editorial comments.

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Chapter 4: New species of *Tulasnella* associated with Megastylidinae orchids from Australia

AR performed the research and data collection, analysed and interpreted the data and drafted the chapter. TM contributed to conceptual development and editorial comments. CCL contributed to research design, conceptual development and editorial comments.

Chapter 5: Orchid specificity at subtribe level: Thelymitrinae and Megastylidinae

AR performed the research and data collection, analysed and interpreted the data and drafted the chapter, RDP contributed to data collection and fieldwork. CCL contributed to research design, conceptual development and editorial comments.

GENERAL INTRODUCTION

Orchidaceae is one of the largest plant families in the world (World Checklist of Selected Plant Families, 2019), and the mycorrhizal fungi form an inseparable part of the life cycle of all the orchid species. Orchid mycorrhizal fungi may have a significant impact on orchid ecology and at the individual level on plant establishment and survival. However, owing to the hidden nature of this interaction and difficulties with fungal species determination, progress in untangling the fungal-orchid interactions were relatively show before the advent of DNA barcoding. Australian orchids represent a unique study system due to frequently observed extreme specificity to a few very closely related fungal taxa, hardly seen in orchids from other continents. Thus, these phenomena make Australian orchids and their fungal partners worth of detailed studying and exploration.

Due to the microscopic size and lack of endosperm, orchid seeds need mutualistic interactions with orchid mycorrhizal fungi (OMF) to continue their life cycle (Rasmussen, 1995; Rasmussen et al., 2015; Rasmussen & Rasmussen, 2009). At the early stages of germination, orchids form a non-photosynthetic protocorm which requires colonization by OMF to develop. When the orchids reach maturity, the mutualistic interaction supplies nutrients and enables carbon exchange (Cameron et al., 2006; Dearnaley & Cameron, 2017; Fochi et al., 2017; Sommer et al., 2012).

Autotrophic orchids mainly associate with three main groups of OMF; Serendipitaceae, Tulasnellaceae and Ceratobasidiaceae (Warcup, 1981; Warcup & Talbot, 1967, 1971, 1980). The orchid-mycorrhizal fungi relationships show various degrees of associations, from broad fungal lineages (highly generalist) to highly specialist per orchid species. An example of a highly generalist orchid in Australia is *Microtis* which associate with various OMF lineages from Serendipitaceae, Tulasnellaceae and Ceratobasidiaceae (De Long et al., 2013). Some orchid genera show less generalist interactions with multiple fungal operational taxonomic units (OTUs) / species per orchid species, such as in *Dendrobium* (Chen et al., 2019; Xing et al., 2017), *Dactylorhiza* (Jacquemyn et al., 2012), *Cypripedium* (Shefferson et al., 2005), *Cryptostylis* (Nguyen et al., 2019) and *Orchis* (Jacquemyn et al., 2010; Jacquemyn et al., 2011). The orchid genera that are considered highly specific are more common in several Australian orchid genera *Caladenia* (Phillips et al., 2016; Whitehead et al., 2017; Wright et al., 2011) and one to two OTUs per genus in *Chiloglottis*

(Roche et al., 2010), *Drakaea* (Phillips et al., 2011), *Caleana* and *Paracaleana* (Linde et al., 2014).

Orchids which lack or have a reduced chlorophyll content, can be considered as mycoheterotrophic (Barrett & Davis, 2012; Barrett et al., 2014). Mycoheterotrophic orchids use a different strategy to obtain carbon, through association with a completely different set of mycorrhizal fungi to autotrophic orchids. In mycoheterotrophic orchids, which have fewer photosynthesis-related genes compared to autotrophic orchid counterparts, ectomycorrhizal fungi (ECM) are the main fungal associates (Dearnaley, 2006; Ogura-Tsujita et al., 2012; Yagame et al., 2016). The ECM fungi can obtain carbon from surrounding autotrophic plants, then forwarding the carbon to mycoheterotrophic plants nearby (Merckx, 2013; Merckx et al., 2009). However, several green (autotrophic) orchids also gain carbon from ECM. Such chlorophyllous orchids therefore utilise carbon from both primary photosynthesis and ECM fungi (Gebauer et al., 2016; Jacquemyn & Merckx, 2019). Australia only has a few known leafless or potentially mycoheterotrophic orchids, one of those is *Cryptostylis hunteriana*, which will be investigated for its OMF associations in Chapter One.

The associations between orchids and OMF raises questions whether it can be considered as a coevolutionary system because OMF can persist and survive independently of the orchid. Coevolution is the reciprocal evolutionary change between individuals in two populations (Janzen, 1980; Thompson, 2009). The evolutionary origins of orchids and their OMF appear to overlap with both evolving approximately a100 Mya before present (Garnica et al., 2016; Givnish et al., 2015; Li et al., 2019; Strullu-Derrien et al., 2018; Taylor & Berbee, 2006), which may suggest a coevolutionary process. Coevolutionary relationships not only occur in host-parasite interactions, when closely-related parasites associate with closely-related hosts and show a congruency in both phylogenetic trees (Legendre et al., 2002; Vermeij, 1994), but also can be found in mutualistic systems (Bascompte et al., 2003; Rezende et al., 2007). However, because OMF do not necessarily need the orchids, any evolutionary patterns in orchid and OMF interactions does not suggest a coevolutionary event. Only a few studies suggested closely-related orchids associate with closely-related fungi (Jacquemyn et al., 2011; Xing et al., 2017) with little known about their evolutionary process. In this thesis, I will investigate whether Australian sexually deceptive the orchid subtribes Drakaeinae, which show very high specificity for OMF associations (Linde et al., 2014; Phillips et al., 2011; Roche et al., 2010) and Cryptostylidinae (Nguyen et al., 2019), have an evolutionary relationship by using cophylogenetic analyses.

As the most common OMF in orchids worldwide (Oberwinkler et al., 2017; Yukawa et al., 2009), *Tulasnella* (Tulasnellaceae, Cantharellales, Basidiomycota) consists of approximately 100 described species around the world which was previously named *Epulorhiza* (Moore, 1987). *Tulasnella* grows in various habitats and can be found as saprotrophs (Cruz et al., 2011; Roberts, 1994), as a symbiont in orchids (Warcup, 1971, 1973, 1981) and liverworts (Kottke et al., 2008), and in some cases play a role as ectomycorrhizal fungi (Bidartondo et al., 2003; Solis et al., 2017). Before the development of molecular biology, fungal identification relied on morphological features such as examining sexual spores and microscopic techniques to distinguish their hyphae and basidiomata (Currah, 1997; Roberts, 1992, 1993). However, several fungi can be considered cryptic species because they have few distinctive morphological features, making it impossible to differentiate them morphologically (Bickford et al., 2007). Molecular biology provided a breakthrough for fungal identification and characterization by using several mitochondrial and nuclear DNA sequence markers (loci) (Schoch et al., 2012; Taylor et al., 2000). While using multilocus markers to delimit cryptic fungal taxa is essential (Taylor et al., 2000), a multilocus comparison of seven nuclear and mitochondrial loci in *Tulasnella* showed that the internal transcribed spacer (ITS) region locus alone provide similar species delimitation resolution compared to using all seven loci in a multilocus dataset (Linde et al., 2017; Linde et al., 2014). However, even though molecular DNA phylogenetic studies provide a high accuracy in delimiting fungal taxa, morphological studies should not be abandoned completely when describing new fungi as it often provides some distinctive information or features that support the molecular data.

This thesis deals with evolution of association between mycorrhizal fungi from Tulasnellaceae and several orchid subtribes in the Diurideae. Furthermore, formal descriptions of several new *Tulasnella* species, including detailed investigation of molecular and morphological characteristics are provided. Chapter One explores the *Tulasnella* associations in five Australian *Cryptostylis*, their specificity and OMF diversity, as well as comparing fungal diversity between autotrophic and a partially mycoheterotrophic species. To broaden the scope, I examine the OMF in three species of Asiatic *Cryptostylis* species. Chapter Two focuses on whether we can detect phylogenetic congruency between orchids and their OMF. The orchids from the subtribes Drakaeinae and Cryptostylidinae and their

Tulasnella symbionts are examined. Cophylogenetic analyses were conducted to test if closely related orchids associate with closely related *Tulasnella* fungi and show congruency between phylogenies. Chapter Three and Four are formal species descriptions, where in total eight new *Tulasnella* species obtained from orchids in my first two chapters are described. In Chapter Three, six new *Tulasnella* from Cryptostylidinae and Drakaeinae are described based on morphological and molecular techniques. All of these *Tulasnella* belong to phylogenetic group IV based on classification by Cruz et al. (2014) and are closely-related to *Tulasnella* obtained from *Chiloglottis* and *Drakaea* orchids in Australia (Linde et al., 2017). In Chapter Four, two new *Tulasnella* species, one from phylogenetic group III and the other from a proposed new phylogenetic group V. These two new *Tulasnella* species were obtained from Megastylidinae orchids, which are the first *Tulasnella* species described from this subtribe of orchids in Australia. Chapter five is a preliminary investigation of OMF diversity in Thelymitrinae and Megastylidinae; both are members of tribe Diurideae.

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CHAPTER ONE

Cryptostylis species (Orchidaceae) from a broad geographic and habitat range associate with a phylogenetically narrow lineage of Tulasnellaceae fungi

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Cryptostylis species (Orchidaceae) from a broad geographic and habitat range associate with a phylogenetically narrow lineage of Tulasnellaceae fungi

ABSTRACT

Here we explore the mycorrhizal diversity of *Cryptostylis* species (Orchidaceae) of Australasian and Asiatic origin. While many Australian terrestrial orchids have highly specialized mycorrhizal assocations, we tested the hypothesis that this geographically widespread orchid genus associates with a diverse range of mycorrhizal fungi. We investigated in detail the mycorrhizal associations of five Australian *Cryptostylis* species and included limited sampling from three Asiatic *Cryptostylis* species. All Australian *Cryptostylis* associated with three to seven *Tulasnella* operational taxonomic units (OTUs), except for *C. hunteriana* where only one *Tulasnella* OTU was detected. In total, eleven *Tulasnella* OTUs associate with Australian *Cryptostylis*. The Asiatic *Cryptostylis* associated with four different *Tulasnella* OTUs belonging to the same lineage as the Australian species, suggesting phylogenetic niche conservatism. Associations with non-*Tulasnella* fungi were infrequent. While five *Tulasnella* OTUs were used by multiple species of Australian *Cryptostylis,* the most commonly used OTU differed between species. There was no overlap in the OTUs used between the leafless *C. hunteriana* and three other Australian *Cryptostylis* growing in the same region. The association with different fungi by *Cryptostylis* species co-occuring at the same site suggests that in any given environmental condition, *Cryptostylis* species may intrinsically favour different fungal OTUs.

Keywords: *Cryptostylis* – fungal sharing – fungi – orchid – mycorrhizae – *Tulasnella* – DNA sequencing – specialization.

INTRODUCTION

Orchids have dust-like seeds that lack an endosperm, and therefore are completely dependent on mycorrhizal fungi for seed germination and subsequent seedling establishment (Rasmussen *et al.*, 2015; Rasmussen & Rasmussen, 2009). Terrestrial orchids continue to rely on mycorrhizal fungi into adulthood, with the extent of this reliance varying between species (Clements *et al.*, 1987; Jacquemyn *et al.*, 2011; McCormick *et al.*, 2004; Rasmussen, 1995). In photosynthetic orchids, this orchid-fungus relationship facilitates bidirectional carbon exchange, with carbon transfer occurring from the plant to the fungus (Alexander & Hadley, 1985) or *vice versa* (Cameron *et al.*, 2006). Alternatively, nutrients, especially phosphorous and nitrogen, are taken up by the fungus and transferred to the plant (Cameron *et al.*, 2006; Dearnaley & Cameron, 2017; Fochi *et al.*, 2017).

Photosynthetic orchids are commonly associated with three main groups of orchid mycorrhizal fungi (OMF); Serendipitaceae (formerly Sebacinales Group B) (Weiß *et al.*, 2016), Tulasnellaceae (Warcup, 1973; Warcup & Talbot, 1971), and Ceratobasidiaceae (Dearnaley, 2007; Warcup, 1981). Members of these fungal genera are typically saprophytic and not dependent on the orchid for survival. Mycoheterotrophic orchids, which do not capture their own carbon via photosynthesis, often associate with completely different fungal families to photosynthetic orchids (Merckx, 2013; Smith & Read, 2008).

In orchid-mycorrhizal relationships, orchids vary between highly generalist (i.e. many fungal lineages per orchid species) and highly specialist (i.e. few fungal lineages per orchid species) in their fungal associations. Outside of Australia, terrestrial orchid species typically associate with numerous fungal operational taxonomic units (OTUs) (hereafter referred to as OTUs even in cases where both OTUs and named species are involved) , but with an apparent preference for one or two main fungal genera (Phillips *et al.*, 2020). Within the predominantly Australian orchid tribe Diurideae, there are some examples of mycorrhizal generalists such as *Microtis media* – which associates with fungi *Piriformospora indica*, *Serendipita vermifera*, *Tulasnella calospora* and *Ceratobasidium* (De Long *et al.*, 2013), and *Caladenia flava* – which associates with at least four *Serendipita* OTUs (Phillips *et al.*, 2016; Whitehead *et al.*, 2017). However, most Diurideae orchid species show a remarkably high specificity in their fungal associations (Phillips *et al.*, 2020). Specialization on one or a few fungal OTUs per orchid species appears to be typical in members of the diurid genera *Chiloglottis* (Roche *et al.*, 2010; Ruibal *et al.*, 2017), *Drakaea* (Linde *et al.*, 2014; Phillips *et al.*, 2011), *Rhizanthella* (Bougoure *et al.*, 2009), *Thelymitra* (Reiter *et al.*, 2018), *Pheladenia* (Davis *et al.*, 2015), *Diuris* (Smith *et al.*, 2010) and *Caladenia* (Oktalira *et al.*, 2019; Reiter *et al.*, 2020; Whitehead *et al.*, 2017). In general, in the Diuridae only a few OTUs of fungi associate with a given orchid genus, with most of the fungal OTUs shared between related orchid species (Otero *et al.*, 2011; Phillips *et al.*, 2016; Phillips *et al.*, 2011; Roche *et al.*, 2010). However, in some genera, orchid species associate with different fungal taxa, with the orchids incapable of seed germination using fungi from different host species (Swarts *et al*. 2010). Nonetheless, based on these observations, we expect most Australian Diurideae orchids to have specialized mycorrhizal fungal associations but with extensive sharing of fungal OTUs among close relatives.

The genus *Cryptostylis* (tribe Diurideae, subtribe Cryptostylidinae) is unusual among members of the Diuirideae in that instead of having a centre of diversity in temperate parts of southern Australia, only five of the 23 recognised species (KEW Plant Checklist 2019) are native to Australia (Jones, 2006). Instead, most species occur in south-east Asia and New Guinea, with a lesser diversity in the south Pacific (Pridgeon *et al.*, 2001). Further, most of the *Cryptostylis* species are exceptional among the Diurideae in that a leaf is maintained year round (Jones, 2006), while one of the five Australian species, *Cryptostylis hunteriana* Nicholls, is a leafless saprophyte (Bell, 2001; Pridgeon *et al.*, 2001).

Mycorrhizal associations in *Cryptostylis* are of particular interest due to the unusual life history and biogeography of *Cryptostylis* compared to other Diurideae orchids. Thus far the only detailed study of mycorrhizal associations in *Cryptostylis* was in the Western Australian species *Cryptostylis ovata* R.Br. (Nguyen *et al.*, 2019). This study showed that *C. ovata* associates with a small number of *Tulasnella* OTUs, with one OTU shared with *Chiloglottis* (Linde *et al.*, 2017; Roche *et al.*, 2010), a member of the Drakaeinae orchids (subtribe Diurideae). Another study formally described five *Tulasnella* species associated with Australian *Cryptostylis* species (Arifin *et al.*, 2020). In this study, we aim to explore the mycorrhizal diversity in all five Australian *Cryptostylis* species, as well as three Asiatic *Cryptostylis* species. Specifically, we will determine (i) the identity and diversity of fungi associating with Australian and Asiatic *Cryptostylis*, and (ii) whether Australian *Cryptostylis* species associate with different fungal OTUs.

MATERIALS AND METHODS

Study species

Cryptostylis subulata (Labill.) Rchb.f., *C. erecta* R.Br., *C. hunteriana* Nicholls and *C. leptochila* F.Muell. ex Benth occur sympatrically in eastern Australia, while *C. ovata* occurs in allopatry in south-west Western Australia (Jones, 2006). Australian *Cryptostylis* grow in a variety of habitats, but most commonly occur in eucalypt forests (Backhouse, 2019; Brundrett, 2014; Jones, 2006). However, populations are also known from coastal sand dunes, granite outcrops, and the margins of swamps (Pridgeon *et al.*, 2001). A population of *C. ovata* that we sampled in southwestern Australia includes some individuals growing epiphytically on *Allocasuarina decussata* (Casuarinaceae) (Brundrett, 2014). While most Australian *Cryptostylis* are common, the leafless *C. hunteriana* is considered vulnerable in the state of NSW and endangered in Victoria (Bell, 2001), and is federally listed as vulnerable. All Australian *Cryptostylis* orchids are all pollinated by sexual deception of the same species of ichneumonid wasp, *Lissopimpla excelsa* (Coleman 1930a, 1928, 1929, 1930b), which contrasts with most sexually-deceptive orchids, where co-occuring species typically use different pollinator species (Paulus & Gack, 1990; Peakall *et al.*, 2010; Phillips *et al.*, 2017; Phillips *et al.*, 2014).

In addition to the five Australian species, a further 18 species of *Cryptostylis* occur in Asia and the south Pacific, spanning India, Sri Lanka, Taiwan, southeast Asia, Solomon Islands, New Guinea, New Caledonia, Fiji, and Samoa (Pridgeon *et al.*, 2001). The ecology of Asiatic *Cryptostylis* is poorly known compared to the Australian counterparts, but Asiatic species tend to grow in mountainous regions mostly at mid-elevations, from 800 - 2,700 metres a.s.l, typically in humus-rich areas including rainforests (O'Byrne & Schneider, 2015; Robinson *et al.*, 2016).

Sampling of fungi from orchids

Root samples were collected from mature plants of all five Australian *Cryptostylis* species (Figure 1) between October to March (Table 1). Root samples were collected from no more than one individual per clump. All sampled root material was wrapped in damp paper towel and kept in plastic bags at 5 °C until processing within five days. All Australian *Cryptostylis* species were sampled from a minimum of five populations, with the exception of the rare *C. hunteriana*, which was sampled from only three populations to minimise impact in accordance with our permit conditions. Roots of *C. ovata* plants were collected in Western Australia from nine populations. *Cryptostylis subulata*, *C. leptochila*, and *C. erecta* were collected from populations (seven, five, and nine respectively) in New South Wales (NSW). In addition, *C. leptochila* was collected from three populations in Victoria. Two to three species of *Cryptostylis* grew in sympatry in nine of the NSW populations (Table 1). We aimed to collect five individual plants per population, however, the extremely dry weather conditions during this study necessitated the collection of a lesser number of plants at some populations. To ensure correct identification of species, we only sampled flowering individuals of the sympatric eastern species, which cannot be confidently distinguished by their leaf morphology alone. For each individual plant, a 5-mm-long peloton rich root fragment was stored at -20 °C for DNA extraction and direct sequencing.

To assess the phylogenetic diversity of fungi from Asiatic *Cryptostylis* species, we also collected roots from *C. acutata* J.J.Sm. (four plants, voucher specimen SAN 165625 deposited at the Forest Research Center Herbarium (SAN)) and *C. filiformis* Blume (one plant, voucher specimen SAN 165626) from Sabah, Malaysia, and *C. javanica* J.J.Sm. (one plant, voucher specimen SW Gale SG1380, lodged at Kadoorie Farm and Botanic Garden) from Lantau Island, Hong Kong (Figure 2). Root samples were placed in silica gel immediately after sampling to preserve the tissue for DNA extraction and direct sequencing. We aimed to collect sufficient numbers of species and populations from Asiatic *Cryptostylis* to make a fair comparison with Australian species. Unfortunately, due to limited available information about their phenology and habitats, we only successfully sampled three Asiatic *Cryptostylis* with limited numbers of plants and populations. To account for the possibility that Australian *Cryptostylis* associate with unculturable fungi, we employed both fungal culturing and direct sequencing. For the Asiatic samples, only direct sequencing was undertaken as no *in situ* facilites were available for culturing.

Isolation of fungal cultures

Two types of growth media were used for the isolation of OMF from *Cryptostylis*: Fungal Isolation Media (FIM) (Clements & Ellyard, 1979) and a modified Melin-Norkrans medium (Wright *et al.*, 2010) plus A-Z multivitamins (Centrum “Balanced Formula”, Wyeth Consumer Healthcare, Baulkham Hills, NSW, Australia) (3MN + A-Z) following Ruibal *et al.* (2017), both of which were supplemented with 50 mg mL^{-1} streptomycin sulfate. Root pieces were washed under running tap water, then dissected in sterile water to locate pelotons. In *Cryptostylis*, mycorrhizal colonization occurs at irregular intervals along the root system (Ramsay *et al.*, 1986). Pelotons or peloton rich tissues were serially rinsed in three changes of sterile distilled water before plating onto FIM and/or 3MN + A-Z plates and incubated at 22 ^{o}C in the dark. To obtain pure, single cultures, hyphal tips growing from pelotons were transferred onto new plates. We aimed to isolate 10-20 germinating pelotons per plant, but in practice the succes rate was lower in several plants and populations.

To obtain sufficient mycelia for DNA extraction, cultures were grown in FIM or 3MN + A-Z liquid media. Following Linde *et al.* (2017), small blocks of agar from the isolates were macerated and inoculated into the liquid media and incubated for 3 to 4 weeks at 22 ºC in the dark. After incubation, mycelia were harvested and lyophilized before being stored at -20 °C. Fungal genomic DNA was extracted from the lyophilized mycelium using Qiagen DNeasy Plant Mini Kit (Qiagen, Germany), following the manufacturer's protocol. If the fungal isolates did not grow on liquid media, genomic DNA was extracted directly from fungi on agar plates. A QG buffer (Qiagen, Germany) was used to dissolve the agar at 65 °C. The mycelia were then rinsed three times with sterile water, before DNA extraction with the same method as above (Qiagen DNeasy Plant Mini Kit, Qiagen, Germany).

For DNA sequencing, the internal transcribed spacer (ITS) region was amplified for 1-2 isolates from each plant with the following primers: ITS5/ITS 4, ITS1/ITS 4 (Gardes & Bruns, 1993). Since the ITS region provides the same or higher level of resolution as a combination of loci in *Tulasnella* (Linde *et al.* 2014), only amplification of the ITS locus was attempted.

Direct sequencing of fungi from roots

For direct sequencing of fungi from roots, DNA isolation from lyophilized Australian *Cryptostylis* root tissues, and the Asiatic samples dried in silica gel, were conducted using DNeasy Plant Mini Kit (Qiagen, Germany). Because no *Serendipita* or *Ceratobasidium* was cultured from either of the five species in this study or a previous study on *C. ovata* (Nguyen *et al.*, 2019), clone library construction was attempted using the *Tulasnella* specific primers ITS4-Tul (Jacquemyn *et al.*, 2012) or ITS4-Tul2 (Oja *et al.*, 2015) to eliminate other fungi as contaminants, or plant DNA being amplified. ITS4-Tul or ITS4-Tul2 were paired with ITS1 or ITS5 (Taylor & McCormick, 2008); in this study we ultimately used ITS5 and ITS4-Tul because this combination resulted in more consistent PCR success. Because *Tulasnella* specific primers may not detect other genera of Basidiomycetes fungi, 12 root samples of the leafless *C. hunteriana* and six each for the leafy Australian *Cryptostylis* species were also amplified using the ITS 1F / ITS 4 and ITS 1F / ITS 4B (Gardes & Bruns, 1993; White *et al.*, 1990) primer combinations with five clones being sequenced for each plant-primer combination.

PCR amplification and sequencing

PCR conditions were as follows: 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 55 s. The final cycle was followed by 72 °C in 7 min. The PCR reaction mix contained 3-5 µL of fungal DNA as a template, 5 µL of 5X polymerization buffer, 1 µL of each ITS primer (10 mM), 12.85 µL of sterile H_2O, and 0.15 µL of MyTaq Polymerase (Bioline, USA). Amplification products were visualized using electrophoresis on 1.2 % agarose gels for 30 minutes at 110 V. PCR products obtained from *Tulasnella*-specific primers were purified using Wizard® SV Gel and PCR Clean-Up system (Promega, USA). Because one individual sample (plant) might associate with one or more *Tulasnella* OTU, purified PCR products from root tissue were cloned using pCR4-TOPO cloning vector (ThermoFisher, USA) and transformed using competent *Escherichia coli* DH10B. Five to eight white colonies from each sample were picked and diluted in 100 µL nuclease-free water as M13 PCR templates. We attempted to sequence fungi directly from peloton-rich roots for all plants, unless no root material was left over after fungal isolation. Colony PCR of clone libraries was performed using 2 µL template and M13F-M13R primers in 20 µL reactions. Clone libraries were purified using EXO-CIP (New England Biolabs, USA), and precipitated with ethanol following the BigDye Terminator v3.1 Sequencing Kit protocols (Applied Biosystems, Foster City, California, USA). Sequencing was performed bidirectionally using M13 forward and reverse primers with ABI PRISM BigDye Terminator v3.1 sequencing kit (Applied Biosystems) on an ABI-3130 automated sequencer.

Phylogenetic analysis

Fungal ITS sequences were edited and aligned using SEQUENCHER 5.4.1 (Gene Codes Corp.) and manually checked for any base ambiguities. BLAST searches using consensus sequences were performed to find closely related accessions from GenBank (http://www.ncbi.nlm.nih.gov/BLAST). The top BLAST hits from GenBank were downloaded (Table S1) and added to a multiple sequence alignment using ClustalW in GENEIOUS 10.2.6 (Kearse *et al.*, 2012) and adjusted manually. *Tulasnella* phylogenetic trees were inferred with Bayesian inference analyses and constructed using MRBAYES 3.2.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Two parallel runs of four chains each were run for 2 million generations and trees sampled every 200 generations after a 25 % burn-in with a GTR+G substitution model. To verify that the burn-in was sufficient, likelihood profiles were examined. Convergence of runs was confirmed when the average standard deviation was < 0.02 with effective sample sizes > 200. In addition, a maximum likelihood analysis was conducted using RAxML as implemented in GENEIOUS 10.2.6 (Kearse *et al.*, 2012) with 1,000 pseudoreplicates of nonparametric bootstrapping (Stamatakis *et al.*, 2008). Trees were viewed in FIGTREE 1.4.3 (Rambaut, 2016) and rooted to *Tulasnella eichleriana* based on previous studies (Cruz *et al.*, 2014; Linde *et al.*, 2017). Operational Taxonomic Units (OTUs) were identified using a combination of branch support and a maximum sequence divergence threshold of 4 % (Linde *et al.*, 2017; Linde *et al.*, 2014). Pairwise sequence divergence of the ITS sequences among lineages were estimated in GENEIOUS 10.2.6 (Kearse *et al.*, 2012).

Fungal diversity

The total number of *Tulasnella* OTUs obtained per population from fungal isolation and direct sequencing combined was calculated. Using populations as replicates, a Kruskal-Wallis test was conducted in R 3.5.1 (R Core Team, 2019) to test for differences in the number of OTUs detected per orchid species. This test was only conducted for the Australian *Cryptostylis* species.

Phylogenetic Diversity (PD) is defined as ‘the minimum total length of all the phylogenetic branches required to span a given set of taxa on the phylogenetic tree’ (Faith, 1992). The PD of the *Tulasnella* associated with each Australian *Cryptostylis* species in each population was calculated using the R package ‘PICANTE’ (Kembel *et al.*, 2010). Because PD values can be affected by the number of fungal sequences represented for each OTU, to standardize for sampling effort we also calculated corrected PD values where only one randomly chosen individual of each OTU per orchid population was included. A one-way ANOVA was then used to determine whether the corrected and uncorrected PD values differed significantly among species in R 3.5.1 (R Core Team 2019). Phylogenetic diversity analyses was only conducted for fungi from Australian *Cryptostylis* species as sample sizes for the Asiatic species were too low.

RESULTS

Diversity of orchid-mycorrhizal associations at the species level

In total, 185 fungal isolates (Table S2) representing 167 plants from five *Cryptostylis* species in Australia were sequenced. In addition, 307 sequences were obtained from direct sequencing of *Cryptostylis* roots (264 from Australian plants, 43 from Asiatic plants). The majority of isolates and clone sequences were identified as *Tulasnella* (Table S3), with a small number of clone sequences identified as Ascomycota, endophytes or ectomycorrhizal fungi (Table S4). Together with isolates from GenBank, based on a sequence divergence threshold of a maximum of 4 %, 15 *Tulasnella* OTUs (some of which are described species) were identified with high bootstrap and posterior probability support, including 11 OTUs from Australian *Cryptostylis* species (Figure 3). The 11 *Tulasnella* OTUs identified from Australian *Cryptostylis* included *Tulasnella prima*, *T. warcupii*, *T. sphagneti*, five recently described *Tulasnella* species (*Tulasnella australiensis*, *T. occidentalis*, *T. punctata*, *T. densa*, and *T. concentrica*) (Arifin *et al*., 2020), a taxonomically undescribed OTU (OTU 1) and two OTUs previously detected from *C. ovata* (Jarrahdale 1 and 2, Nguyen *et al*. (2019)) (Figures 3 & 4, Figure S1), all of which belong to *Tulasnella* phylogenetic group IV as assigned by Cruz *et al.* (2014). Sequences similar to those identified by Nguyen *et al.* (2019) from *C. ovata* (Jarrahdale 1 (MK430451 – 2) and Jarrahdale 2 (MK430461 and MK430468)) in Western Australia were not detected in the present study. However, Nguyen *et al.* (2019) reported several *Tulasnella* isolates from *C. ovata* as a “Southern group”, which we found clustered with *T. prima*, and a “Bertram” clade, which we found clustered with *T. occidentalis* (Figure 3, Figure S1).

Ninety-three percent of plants (108) associated with only one OTU, six percent of plants (7) associated with two OTUs, and one percent of plants (1 *C. ovata*) associated with three *Tulasnella* OTUs (Figure S2). *Cryptostylis erecta* associated with three *Tulasnella* species, of which *T. australiensis* was the most frequently detected (> 95 % of sequences, Figure 4, Table S3). One isolate from *C. erecta* was identified as *T. punctata*, and three clone sequences were identified as *T. densa*. *Cryptostylis subulata*, the species most frequently co-occuring with *C. erecta,* also associated with three *Tulasnella* species, with *T. punctata* being the most common fungal partner (65 % of sequences). The leafless *C. hunteriana* only associated with a single *Tulasnella* species, *T. densa*. *Cryptostylis leptochila* was the most generalist of the eastern Australian species, associating with four *Tulasnella* species. *T. concentrica* was the most frequently detected fungal symbiont (56 % of sequences), being found in eight *C. leptochila* populations from NSW and Victoria. Five *Tulasnella* OTUs were isolated from *C. ovata*, mainly *T. occidentalis* (57 % of sequences) and *T. prima* (29 % of sequences), but also OTU 1, *T. warcupii* and *T. australiensis* (Figure 4). Using direct cloning of root samples, two plants of *C. ovata* growing epiphytically on *Allocasuarina decussata* were found to associate with OTU 1 (Figure 3). One fungal clone from *C. erecta* and one clone from *C. ovata* showed *Serendipita* as their closest BLASTn result from GenBank (GenBank accession number KF061288 and JX138547, respectively).

The three studied Asiatic *Cryptostylis* species associated with four *Tulasnella* OTUs (Figure S3). *Cryptostylis filiformis* and *C. acutata* were each recorded associating with one *Tulasnella* OTU (OTU 3 and OTU 2, respectively), while *C. javanica* associated with two *Tulasnella* OTUs (OTU 4 and OTU 5) (Figure S3). These were all closely related to *Tulasnella* from Australian *Cryptostylis* species (Figure 3).

Diversity of orchid-mycorrhizal associations at the population level

A mean of 1.34 ± 0.46 (SD) OTUs were found per population across the *Cryptostylis* species, ranging from one to three; *C. erecta* (mean 1.5 ± 0.53, N = 8 populations), *C. subulata* (mean 1.14 ± 0.38, N = 7 populations), *C. leptochila* (mean 1.75 ± 0.71, N = 8 populations), *C. ovata* (mean = 1.3 ± 0.67, N = 10 populations), and *C. hunteriana* (mean 1 ± 0.00, N = 3 populations) (Figure 5). The number of *Tulasnella* OTUs per population did not differ significantly among species (Kruskal Wallis χ^2 = 6.84, P = 0.145). Uncorrected PD values of fungal diversity for each *Cryptostylis* species ranged between 0.158 and 0.403 and did not differ significantly among orchid species at the population level (Figure 6A, Table 2, PDobs, Kruskal Wallis χ^2 = 4.79, P = 0.31, df = 4). The corrected *Tulasnella* PD values also did not differ significantly among Australian *Cryptostylis* species (Figure 6B, Table 2, PDnull, ANOVA P = 0.849).

Is there fungal sharing among Australian* Cryptostylis*?

All Australian *Cryptostylis* species, except *C. hunteriana*, share *T. australiensis* as a fungal symbiont. Three other *Tulasnella* fungi were shared between *Cryptostylis* species: *T. warcupii* and *T. prima* are shared between *C. leptochila* and *C. ovata*, and *T. densa* is shared between *C. erecta* and *C. hunteriana* (Figure 4, Table S3). However, in some cases, co-occurring *Cryptostylis* species used different *Tulasnella* species to one another (Table S5). Three *Cryptostylis* species co-occurred at the Erowal Bay site, each using a single different *Tulasnella* species; *C. erecta*: *T. australiensis* (N = 5 plants), *C. subulata*: *T. punctata* (N = 5 plants) and *C. hunteriana*: *T. densa* (N = 6 plants). Similarly, at the Fitzroy Falls population, the co-occurring *C. erecta* (N = 1 plant), *C. leptochila* (N = 4 plants) and *C. subulata* (N = 6 plants) each associated with a different single *Tulasnella* species (*T. australiensis*, *T. concentrica*, and *T. punctata* respectively).

Cryptostylis *non*-Tulasnella *endophytes*

Some endophytes belonging to fungal genera not known to be mycorrhizal, as well as ectomycorrhizal fungi, were detected from the cloned sequences. In all Australian *Cryptostylis* except *C. ovata* and *C. subulata*, a variety of ectomycorrhizal (ECM) fungi were detected such as *Russula* (*C. hunteriana, C. leptochila*, and *C. erecta*; closest GenBank accession numbers KP012769, DQ398092 and GU222292 respectively), Thelephoraceae (*C. hunteriana*, closest GenBank match KF679822), *Cortinarius* (*C. hunteriana* - KT875188 and NR1563000 and *C. leptochila* - KT875181), and *Phellodon* sp. (*C. erecta*). Not all plants analysed for the presence of Basidiomycota fungi contained ECM fungi. Closest matches on GenBank suggest that ECM fungi in *C. hunteriana* were present in only six clones from 12 plants analysed (i.e. six out of 120 clones), whereas in *C. leptochila* and *C. erecta,* four clones (i.e. four out of 60 clones) from the six plants analysed for each species contained ECM fungi (Table S4). Furthermore, some clone sequences with closest GenBank matches to ericoid mycorrhizal and saprophytic fungi were also detected; *Oidiodendron* sp. (*C. hunteriana*), *Helotiales* sp. (*C. leptochila* and *C. subulata*), *Phialocephala* (*C. hunteriana* and *C. subulata*), *Cladophialophora* and *Exophiala* (*C. ovata*) (see Table S4 for detail descriptions of closest GenBank matches).

DISCUSSION

***Fungal diversity in* Cryptostylis**

This is the first comprehensive study of the fungal associations across the Australian species of *Cryptostylis*. Previously, fungal associations were investigated for *C. ovata* only, initially finding unidentified members of *Tulasnella* and *Cladophialophora* based on direct sequencing of roots from five individuals (Sommer *et al.*, 2012). Recently, Nguyen *et al.* (2019) revealed four *Tulasnella* OTUs associated with *Cryptostylis ovata* from five populations in Western Australia, with one of those fungi (*T. prima*) previously described from *Chiloglottis* orchids (Linde *et al.*, 2017). Here we find a similar, but larger diversity of fungi in *C. ovata*. In our study, all five Australian species of *Cryptostylis* were found to associate predominanty with *Tulasnella*. All *Tulasnella* OTUs that associate with *Cryptostylis* are closely related and belong to *Tulasnella* phylogenetic group IV (Cruz *et al.*, 2014). In total, we found nine *Tulasnella* OTUs associated with Australian *Cryptostylis* (excluding the two Jarrahdale OTUs reported by Nguyen *et al.* (2019) but not found in this study) and four *Tulasnella* OTUs associated with the three Asiatic study species. Some of the *Tulasnella* fungi from *Cryptostylis* represent previously described species (*T. prima, T. sphagneti* and *T. warcupii* (Linde *et al.*, 2017) that are known to associate with orchids in the subtribe Drakaeinae within the Diuridae (Linde *et al.*, 2014; Ruibal *et al.*, 2017).

This study is the first to identify OMF associated with Asiatic *Cryptostylis,* with four *Tulasnella* OTUs identified across three *Cryptostylis* species (Figure S2). These *Tulasnella* OTUs were related to, but differed from the *Tulasnella* species/OTUs identified from Australian *Cryptostylis* species. However, the *Tulasnella* diversity associated with Asiatic *Cryptostylis* is likely broader than reported here because of the low numbers of plants analysed. Because the species of OMF that orchid species associate with can vary between habitats (Jacquemyn *et al.*, 2016; Reiter *et al.*, 2018; Ruibal *et al.*, 2017) and across the orchid's geographic range (Xing *et al.*, 2019), it is not unexpected that the Asiatic *Cryptostylis* species associate with a different suite of *Tulasnella* to Australian species. Nevertheless, it is interesting to note that despite growing in different habitats and biogeographic regions, these four OTUs belong to the same major *Tulasnella* phylogenetic lineage as their Australian counterparts (Figure 3).

Fungal isolation versus direct sequencing

Both fungal isolation from culture and direct sequencing identified almost all of the *Tulasnella* OTUs in Australian *Cryptostylis* orchids. However, *T. warcupii* (four clones) and OTU 1 (six clones) were only detected by direct sequencing in this study (Table 3). Failing to culture these *Tulasnella* species in this study may have more to do with their growth rate (slow growing species could be outcompeted by fast growing species in culture) rather than culturability, because *T. warcupii* has been successfully isolated from *Arthrochilus* orchids in the past (Linde *et al.*, 2017). Direct sequencing returned a much larger range of fungi that are likely to occur as endophytes, but without functioning as mycorrhizae (see other examples in orchids (Chen *et al.*, 2011; Han *et al.*, 2016; Stark *et al.*, 2009). In other orchids these endophytic fungi are not known to contribute to plant germination and growth, nor do they cause detrimental effects to their host plant (Selosse *et al.*, 2009). Interestingly, in the present study two sequence clones identified as two *Serendipita* OTUs were detected in both *C. erecta* and *C. ovata*, belonging to *Serendipita* OTU D and OTU G respectively when compared to sequences from (Whitehead *et al.*, 2017) (data not shown). *Serendipita* is well known as a mycorrhizal fungus of many orchid species (Bonnardeaux *et al.*, 2007; Reiter *et al.*, 2020; Těšitelová *et al.*, 2015; Yagame *et* *al.*, 2016), but germination studies are required to confirm whether it is mycorrhizal in *Cryptostylis*.

Interestingly, a number of ectomycorrhizal or saprotrophic fungi often associated with mycoheterotrophic orchids (Jacquemyn & Merckx, 2019; Smith & Read, 2008) were identified in a small number of cloned sequences of *Cryptostylis*, especially the leafless *C. hunteriana*. A recent review indicated that the evolution from autotrophy towards mycoheterotrophy is often accompanied by either losses or shifts in mycorrhizal partners and thus that a full or partial loss of photosynthesis selects for different mycorrhizal communities (Jacquemyn & Merckx, 2019). *Cryptostyis hunteriana*, which has a green stem indicative of photosynthesis, may be at the very early stage of this evolution towards mycoheterotrophy. However, stable isotope studies to confirm carbon transfer are required to confirm a shift towards mycoheterotrophy or whether these orchids simply infrequently associate with non-*Tulasnella* fungi.

***Specificity of orchid-fungus associations in* Cryptostylis**

The number of *Tulasnella* OTUs associated with the five species of Australian *Cryptostylis* in this study ranged between one (*C. hunteriana*) and five (*C. ovata*), with four of the species associating with multiple *Tulasnella* OTUs. However, all Australian *Cryptostylis* species analysed showed a similar phylogenetic diversity of OMF (Table 2). Generally, for a given orchid species the dominant OTUs were similar between sites, but in *C. subulata* there was evidence of different fungal associations among populations. Despite up to five OTUs being detected in some Australian *Cryptostylis* species, most individual plants only associated with a single OTU, a similar result to that reported in a study focusing on *C. ovata* (Nguyen *et al.*, 2019).

Some Australian *Cryptostylis* species (e.g. *C. ovata* and *C. leptochila*) associate with more fungal lineages than has been observed to date in some other Australian sexually deceptive orchid genera such as *Chiloglottis* (Roche *et al.*, 2010), *Drakaea* (Phillips *et al.*, 2011), many members of *Caladenia* (Reiter *et al.*, 2020), and *Pterostylis* (Otero *et al.*, 2011). Of the orchid genera studied to date, *Drakaea* is the most host-specific, with nine species associating with *Tulasnella secunda* (Linde *et al.*, 2017; Phillips *et al.*, 2020). Similarly, 18 species of *Chiloglottis* were found to associate with only two *Tulasnella* species, *T. prima* and *T. sphagneti* (Linde *et al.*, 2017; Roche *et al.*, 2010). The similar pattern of narrow specificity also occurs in other members of the Diurideae, such as *Caladenia* (Reiter *et al.*, 2020), *Pheladenia* (Davis *et al*. 2015), *Thelymitra* (Reiter *et al*.

2018), and *Rhizanthella* (Bougoure *et al.*, 2009), with each species typically associating with one to two OTUs. In constrast, across the five species of Australian *Cryptostylis*, a total of 11 *Tulasnella* OTUs were identified (9 in this study, two more from Nguyen *et al.* (2019)), which is the greatest OTU to species ratio observed within an Australian sexually deceptive genus.

While most *Cryptostylis* species have a lower degree of specificity for fungal associations than many other Australian terrestrial orchids, their specificity remains high relative to many species of European orchids (Phillips *et al.*, 2020). For example, *Cryptostylis* species associated with slightly fewer *Tulasnella* OTUs (range 1-7) than did other European terrestrial orchids such as *Dactylorhiza* (5-9 OTUs) (Jacquemyn *et al.*, 2012), *Orchis* (2-15 OTUs) (Girlanda *et al.*, 2011; Jacquemyn *et al.*, 2011; Pellegrino *et al.*, 2016), *Ophrys* (4-13 OTUs) (Pellegrino *et al.*, 2016) and *Gymnadenia conopsea* (28 OTUs) (Těšitelová *et al.*, 2013). Therefore, in comparison, *Cryptostylis* associates with a much narrower breadth of fungi, although more OTUs may be detected with further sampling to detect uncommon fungal associates. Although Ruibal *et al.* (2017) provided possible explanations for this high specificity in Australian orchids such as phylogenetically constrained heritable traits in the plants or orchids retaining an ancestral ecological niche, further investigations are required to tease apart some of those suggestions.

Mycorrhizal associations and rarity of **Cryptostylis hunteriana**

While *C. hunteriana* has been adversely affected by habitat clearing across much of its geographic range (Bell, 2001), even within the remaining habitat this species is much rarer than other Australian *Cryptostylis*. Pollinator availability is unlikely to be contributing to rarity as suggested in some other sexually deceptive orchids (Phillips *et al.*, 2014), as its pollinator *L. excelsa* is common, geographically widespread (Tomlinson & Phillips, 2012), and persists in degraded habitats (Weinstein *et al.*, 2016). Alternatively, the high degree of fungal host specificity observed in *C. hunteriana* (only associating with *T. densa*) might have contributed to its rarity. *T. densa*'s geographic range is likely much larger than that of *C. hunteriana*, given the potentially long distance aerial dispersal ability of basidiomycota fungi and the broad geographic range (500 km between *C. hunteriana* sampling sites in this study) over which *T. densa* has already been observed. Indeed, most OMF that have been studied in detail in Australia have broad geographic ranges and occur in a range of habitats (Davis *et al.*, 2015; Linde *et al.*, 2014; Otero *et al.*, 2011; Phillips *et al.*, 2016; Reiter *et al.*, 2020), including some of the *Tulasnella* that associate with other *Cryptostylis*. However, orchid rarity could also be due to the fungus being uncommon in the landscape, leading to fewer opportunities for fungal recruitment, or fewer sites with sufficient fungus (McCormick *et al.*, 2012). Other factors that could contribute to the rarity of *C. hunteriana* include its poorly developed root system (personal observation, also see Bell, 2001) and thereby restricted ability to spread vegetatively. The leaflessness of *C. hunteriana* may also contribute to its rarity – a likely increased dependency on fungi for carbon may result in a lower growth rate. Bushfires may further impact the persistance of *C. hunteriana*, both through summer fires burning inflorescences and preventing recruitment (Bell, 2001), and by fires reducing the amount of organic litter in the soil. A reduced amount of organic litter may affect the OMF that *C. hunteriana* associates with by reducing their nutrient source, which would in turn affect the nutrient uptake and survival of the orchid.

CONCLUSIONS

This study of the mycorrhizal associations in adult Australian and Asiatic *Cryptostylis* showed that Australian *Cryptostylis* associate with 11 *Tulasnella* OTUs, while four were identified in three Asiatic *Cryptostylis* species. Australian *Cryptostylis* has lower specificity compared to other genera of Australian sexually deceptive orchids but still have higher specificity compared to most European terrestrial orchids. All *Cryptostylis* sampled from a large geographic range associated with closely related *Tulasnella* fungi as their main fungal symbionts. The association with different fungi by species co-occuring at the same site suggests that in any given environmental conditions, different *Cryptostylis* species may intrinsically favour different fungal OTUs. Six new *Tulasnella* OTUs from Australian *Cryptostylis* were detected and, given the effectiveness of ITS for discriminating between fungal taxa in this group of *Tulasnella* (Linde *et al.*, 2017), should be formally described. Furthermore, studies employing Next-Generation Sequencing may reveal a further abundance and diversity of OMF as well as further non-*Tulasnella* associations in *Cryptostylis*. Carbon-isotopes studies also may indicate which fungal symbionts are contributing most carbon to the orchids, which would aid in elucidating whether fungi from Australian *Cryptostylis* orchids with and without leaves differ in their ability to contribute carbon and nutrients to the plants.

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Table 1. Australian and Asiatic *Cryptostylis* species sampled in this study, collection locations, and number of plants where DNA sequencing of orchid mycorrhizal fungi was undertaken.

Cryptostylis species	State/Country	Locality	Latitude	Longitude	Date sampled	No. of plants sampled
Cryptostylis erecta	New South Wales/Australia	**Fitzroy Falls**	-34.6474	150.4806	Apr-16	1
	New South Wales/Australia	**Frenchs Forest**	-33.7476	151.2358	Jun-17	4
	New South Wales/Australia	**Bulahdelah**	-32.4097	152.2149	Nov-17	8
	New South Wales/Australia	**Nowra**	-34.9787	150.6751	Dec-17	7
	New South Wales/Australia	**Erowal Bay**	-35.0934	150.6573	Jan-18	5
	New South Wales/Australia	**Wogamia Nature Reserve**	-34.8665	150.5267	Jan-18	5
	New South Wales/Australia	**Carls Mountain Rd**	-35.6758	150.2328	Jan-18	5
	New South Wales/Australia	**Meroo National Park**	-35.4710	150.389	Dec-17	2
	New South Wales/Australia	**Ulladulla**	-35.3655	150.4892	Dec-17	5
Cryptostylis subulata	New South Wales/Australia	**French Forest**	-33.7476	151.2358	Jun-17	4
	New South Wales/Australia	**Ulladulla**	-35.3655	150.4892	Dec-17	8
	New South Wales/Australia	**Fitzroy Falls**	-34.6472	150.4805	Nov-17	6
	New South Wales/Australia	Karuah	-32.5878	151.9212	Nov-17	2
	New South Wales/Australia	**Nowra**	-34.9787	150.6752	Dec-17	6
	New South Wales/Australia	**Erowal Bay**	-35.0934	150.6573	Jan-18	5

	New South Wales/Australia	**Wogamia Nature Reserve**	-34.8663	150.5244	Jan-18	5
Cryptostylis leptochila	New South Wales/Australia	Bundanoon	-34.6750	150.2973	Oct-17	2
	New South Wales/Australia	**Fitzroy Falls**	-34.6469	150.4807	Nov-17	4
	New South Wales/Australia	**Kangaloon**	-34.5309	150.5741	Feb-17	7
	New South Wales/Australia	Penrose State Forest	-34.6597	150.1954	Jan-18	5
	New South Wales/Australia	**Carls Mountain Rd**	-35.6758	150.2328	Jan-18	5
	Victoria/Australia	Cabbage Tree Creek	-37.7516	148.6548	Mar-18	5
	Victoria/Australia	Cape Conran	-37.7412	148.7163	Mar-18	5
	Victoria/Australia	Bunyip State Park	-37.9839	145.5891	Mar-18	5
Cryptostylis ovata	Western Australia/Australia	Boyanup	-33.4754	115.7576	Sep-18	5
	Western Australia/Australia	Rockingham	-33.4757	115.7578	Dec-17	4
	Western Australia/Australia	Frosty Rd	-34.3398	116.4221	Oct-17	3
	Western Australia/Australia	Boronia Reserve	N/A	N/A	Oct-17	3
	Western Australia/Australia	Bridgetown Jarrah Park	N/A	N/A	Oct-17	2
	Western Australia/Australia	Johnston Rd	-32.9621	115.8327	Oct-17	3
	Western Australia/Australia	Muja Conservation Park	-33.5635	116.4373	Oct-17	5
	Western Australia/Australia	Granite Peaks State Forest	-34.7585	116.5008	Nov-17	2
	Western Australia/Australia	Capel	-32.5904	115.5445	Dec-17	2
	Western Australia/Australia	Margaret River	N/A	N/A	Dec-17	5

	Western Australia/Australia	Walpole*	-34.9787	116.8946	Sep-18	2
Cryptostylis hunteriana	New South Wales/Australia	Bulahdelah	N/A	N/A	Nov-17	2
	New South Wales/Australia	Meroo National Park	N/A	N/A	Dec-17	7
	New South Wales/Australia	Erowal Bay	N/A	N/A	Jan-18	6
Cryptostylis javanica	Hong Kong	Lantau Island	N/A	N/A	Sep-17	1
Cryptostylis filiformis	Malaysia	Alab Mountain	N/A	N/A	Jun-17	1
Cryptostylis acutata	Malaysia	Alab Mountain	N/A	N/A	Jun-17	4

*Epiphytic population of *Cryptostylis ovata*.

Bold localites indicate populations where two or more Australian *Cryptostylis* occur sympatrically.

Table 2. The number of operational taxonomic units (OTUs) and phylogenetic diversity (PD) from each population of *Cryptostylis* species. At some sites *Cryptostylis* species occurred sympatrically.

Orchid species	Population	Number of OTUs	Pd_{obs}	Pd_{null}
Cryptostylis erecta	Fitzroy Falls	1	0.158	0.172
	Bulahdelah	2	0.291	0.174
	Nowra	2	0.291	0.174
	Erowal Bay	1	0.158	0.173
	Wogamia Nature Reserve	2	0.291	0.169
	Carls Mountain Rd	1	0.158	0.175
	Ulladulla	1	0.158	0.189
Cryptostylis subulata	Frenchs Forest	1	0.225	0.174
	Ulladulla	1	0.225	0.18
	Fitzroy Falls	1	0.203	0.165
	Karuah	1	0.225	0.175
	Nowra	2	0.346	0.178
	Erowal Bay	1	0.225	0.18
	Wogamia Nature Reserve	1	0.203	0.172
Cryptostylis leptochila	Bundanoon	2	0.302	0.166
	Fitzroy Falls	1	0.158	0.175
	Kangaloon	3	0.403	0.175
	Penrose	2	0.217	0.167
	Carls Mountain Rd	2	0.302	0.177
	Cabbage Tree Creek	2	0.302	0.186
	Cape Conran	1	0.181	0.177
	Bunyip State Park	1	0.181	0.178
Cryptostylis ovata	Boyanup	1	0.166	0.169
	Rockingham	3	0.332	0.176
	Frosty Rd	1	0.191	0.172
	Bridgetown Jarrah Park	1	0.191	0.177
	Johnston Rd	1	0.166	0.196
	Muja Conservation Park	1	0.166	0.181
	Granite Peaks State Forest	1	0.166	0.165
	Capel	2	0.3	0.175
	Margaret River	1	0.191	0.183
	Walpole	1	0.242	0.176
Cryptostylis hunteriana	Bulahdelah	1	0.17	0.18
	Meroo National Park	1	0.17	0.182
	Erowal Bay	1	0.17	0.175
Cryptostylis javanica	Lantau Island	2	0.365	0.171
Cryptostylis filiformis	Alab Mountain	1	0.182	0.179
Cryptostylis acutata	Alab Mountain	1	0.193	0.168

Table 3. Comparisons of *Tulasnella* OTUs recovered from isolates and as well as from the DNA sequencing approach (clones).

	Total *Tulasnella* species/OTU(s) recovered	
	Isolates	Clones
Cryptostylis erecta	2 (*T. australiensis, T. punctata*)	2 (*T. australiensis, T. densa*)
Cryptostylis subulata	3 (*T. sphagneti, T. australiensis, T. punctata*)	3 (*T. sphagneti, T. australiensis, T. punctata*)
Cryptostylis leptochila	3 (*T. prima, T. australiensis, T. concentrica*)	4 (*T. prima, T. warcupii, T. australiensis, T. concentrica*)
Cryptostylis ovata	2 (*T. prima, T. occidentalis*)	5 (*T. prima, T. warcupii, T. australiensis, T. occidentalis,* OTU 1)
Cryptostylis hunteriana	1 (*T. densa*)	1 (*T. densa*)

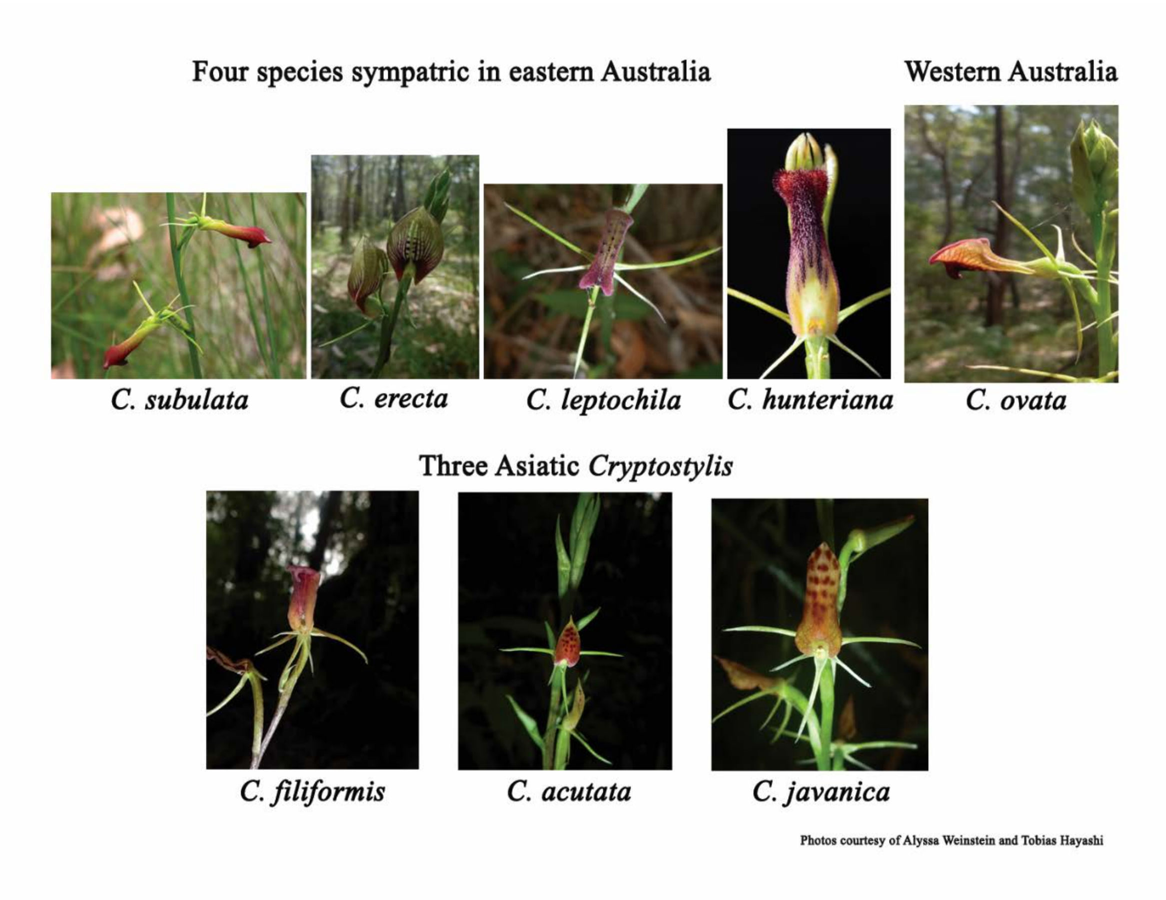

Figure 1. Australian and Asiatic *Cryptostylis* species used in this study.

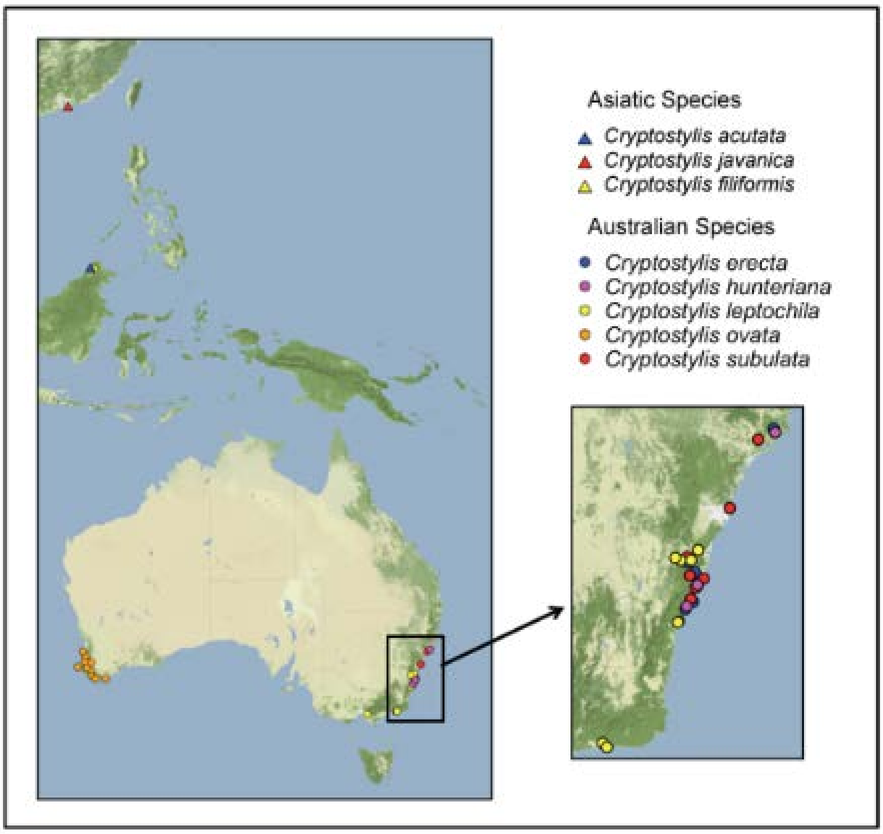

Figure 2. Map of locations where Australian and Asiatic *Cryptostylis* were sampled.

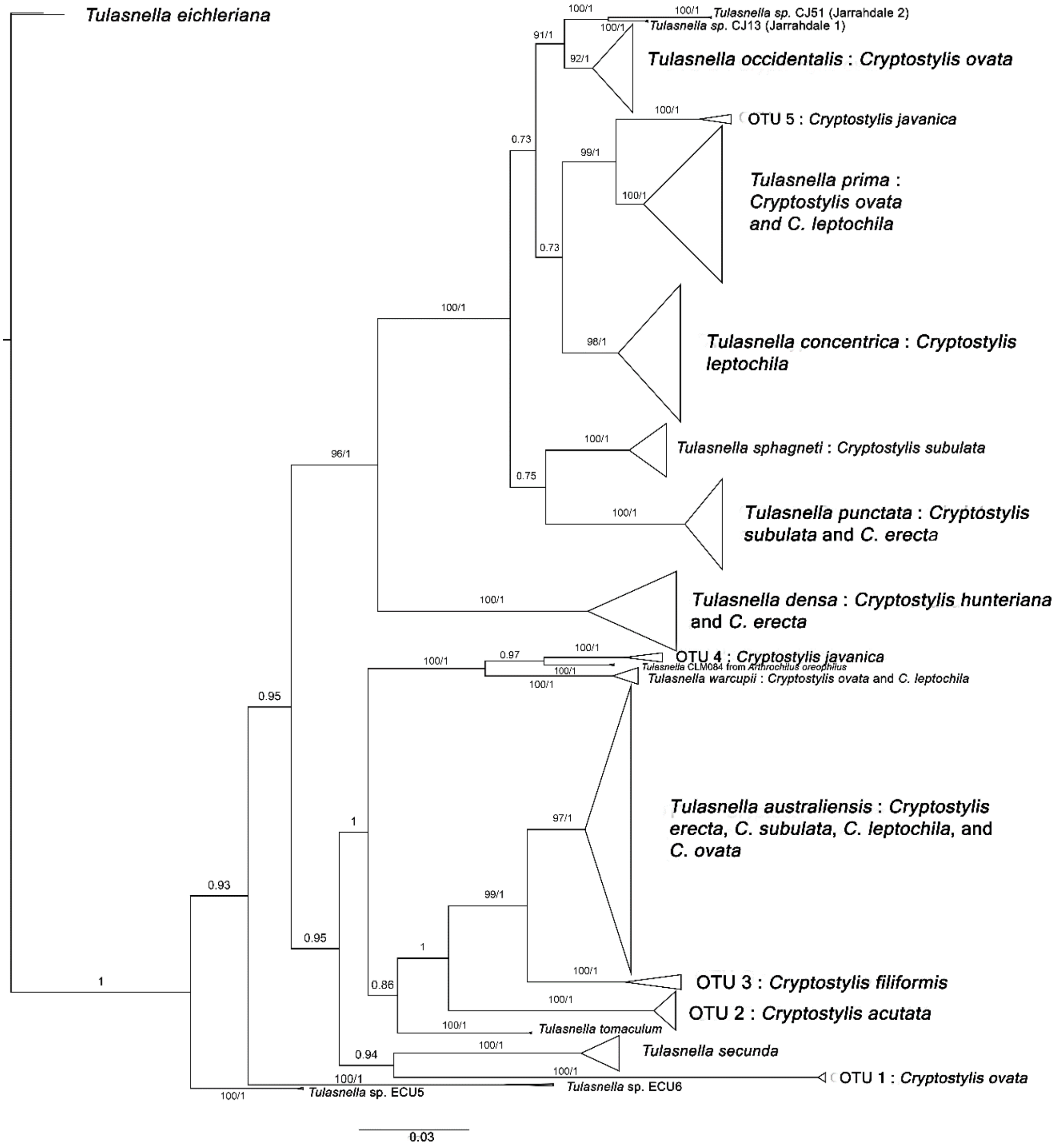

Figure 3. Rooted MrBayes phylogenetic tree for *Tulasnella* associated with *Cryptostylis* orchids, obtained for the Internal Transcribed Spacer (ITS) region. OTU = Operational Taxonomic Unit. The numbers above/under the branches are maximum likelihood bootstrap values / Bayesian posterior probabilities. Only Bootstrap values ≥ 70 and Bayesian posterior probabilities ≥ 0.7 are shown.

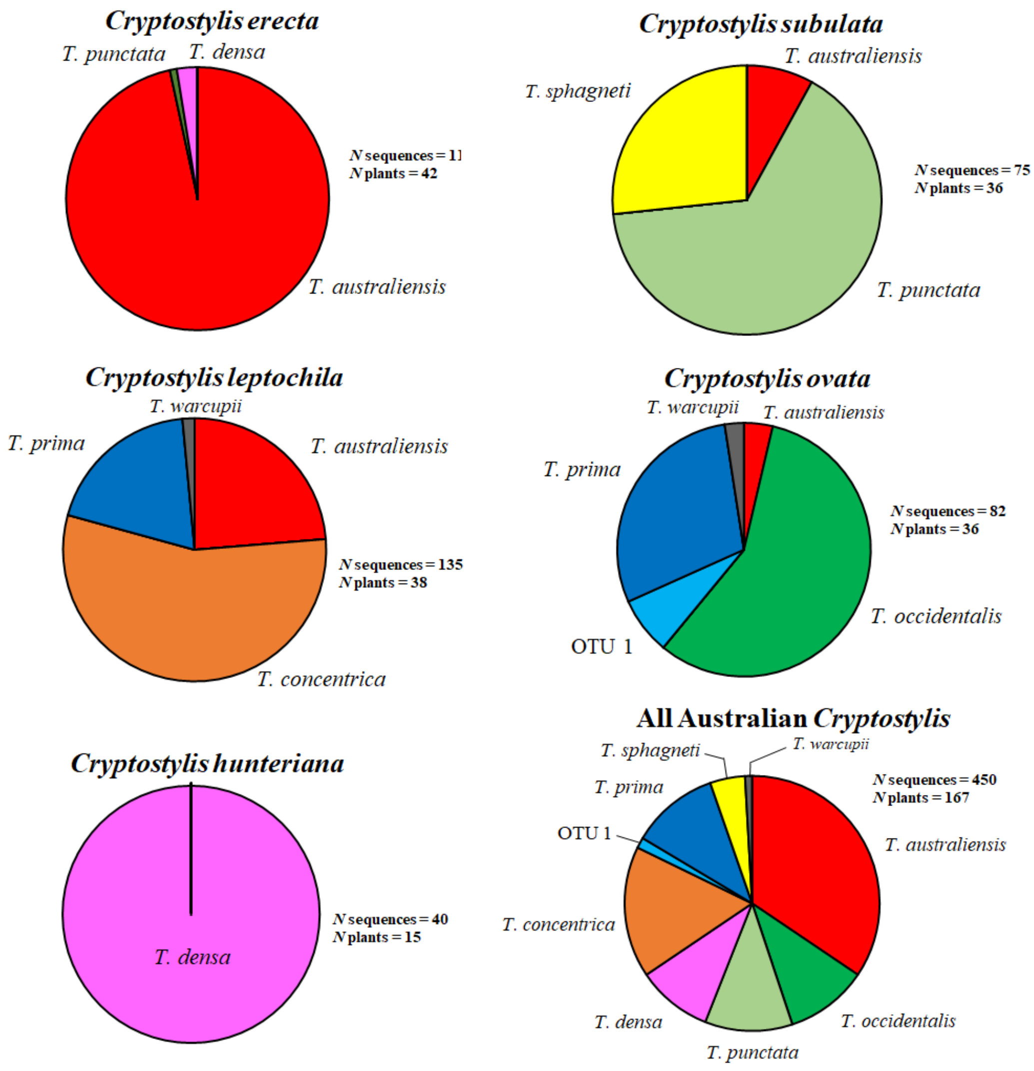

Figure 4. Frequency of the observed *Tulasnella* operational taxonomic units (OTUs)/species among five Australian *Cryptostylis*, as well as in all Australian *Cryptostylis* species combined. *N* sequences: the number of sequences obtained; *N* plants: the number of *Cryptostylis* plants sampled in this study for a given species.

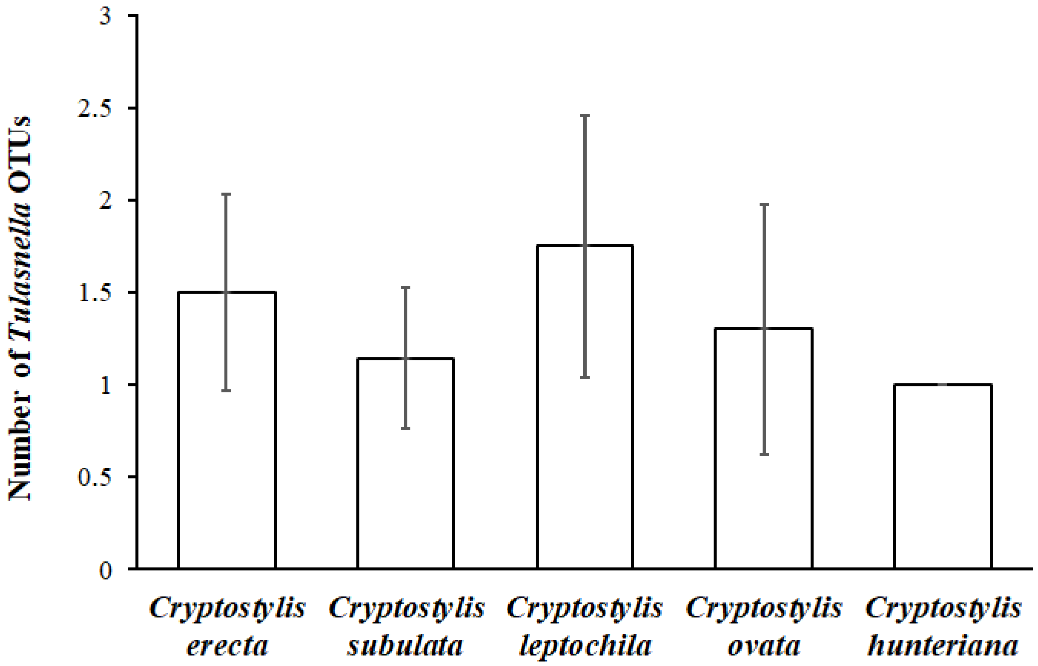

Figure 5. The average number of *Tulasnella* Operational Taxonomic Units (OTUs) per population of five Australian *Cryptostylis* species (± SD).

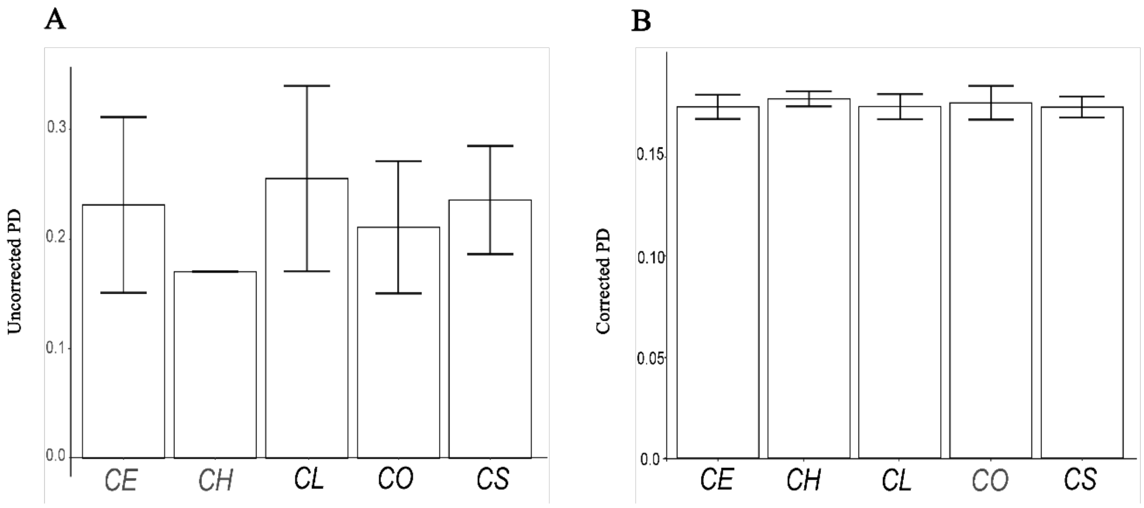

Figure 6. (A) Uncorrected Phylogenetic Diversity (Uncorrected PD ± SD) per orchid species and (B) corrected Phylogenetic Diversity (Corrected PD ± SD) for species richness in five Australian *Cryptostylis* species (CE: *Cryptostylis erecta*; CH: *C. hunteriana*; CL: *C. leptochila*; CO: *C. ovata*; CS: *C. subulata*).

SUPPLEMENTARY MATERIALS

Table S1. Reference sequences used in phylogenetic analysis of *Tulasnella* mycorrhizal fungi from *Cryptostylis* orchids.

GenBank Number	Fungal species	Country of origin	Host Species / substrate	Seq. length	References
AY373292	*Tulasnella eichleriana*	United States	Unknown	780 bp	McCormick et al. 2004
KC152389	*Tulasnella eichleriana*	Germany	Decayed wood	768 bp	Cruz et al. 2014
KC152397	*Tulasnella* sp. ECU5	Ecuador	Fallen branch	700 bp	Cruz et al. 2014
KC152398	*Tulasnella* sp. ECU5	Ecuador	Fallen branch	716 bp	Cruz et al. 2014
JN015192	*Tulasnella densa*	Australia	Unknown	781 bp	Howard & Clements 2011, unpublished
KC152401	*Tulasnella* sp. ECU6	Ecuador	Fallen branch	699 bp	Cruz et al. 2014
KC152402	*Tulasnella* sp. ECU6	Ecuador	Fallen branch	774 bp	Cruz et al. 2014
KY095117*	*Tulasnella sphagneti*	Australia	*Chiloglottis aff. valida*	738 bp	Linde et al. 2017
KY445922	*Tulasnella sphagneti*	Australia	*Chiloglottis turfosa*	728 bp	Linde et al. 2017
KY445923	*Tulasnella sphagneti*	Australia	*Chiloglottis turfosa*	728 bp	Linde et al. 2017
KY445924	*Tulasnella sphagneti*	Australia	*Chiloglottis turfosa*	728 bp	Linde et al. 2017
KY445925	*Tulasnella sphagneti*	Australia	*Chiloglottis* sp.	728 bp	Linde et al. 2017
KY445926	*Tulasnella sphagneti*	Australia	*Chiloglottis* sp.	728 bp	Linde et al. 2017
KY445927	*Tulasnella sphagneti*	Australia	*Chiloglottis* sp.	728 bp	Linde et al. 2017
KY445928	*Tulasnella sphagneti*	Australia	*Chiloglottis* sp.	728 bp	Linde et al. 2017
KY445929	*Tulasnella sphagneti*	Australia	*Chiloglottis* sp.	728 bp	Linde et al. 2017
KF476575*	*Tulasnella secunda*	Australia	*Drakaea elastica*	736 bp	Linde et al. 2014
KF476593	*Tulasnella secunda*	Australia	*Drakaea elastica*	736 bp	Linde et al. 2014
KF476579	*Tulasnella secunda*	Australia	*Drakaea confluens*	738 bp	Linde et al. 2014

KF476592	*Tulasnella secunda*	Australia	*Drakaea confluens*	736 bp	Linde et al. 2014
KF476588	*Tulasnella secunda*	Australia	*Drakaea concolor*	736 bp	Linde et al. 2014
KF476591	*Tulasnella secunda*	Australia	*Drakaea gracilis*	736 bp	Linde et al. 2014
KF476578	*Tulasnella secunda*	Australia	*Drakaea gracilis*	736 bp	Linde et al. 2014
KF476590	*Tulasnella secunda*	Australia	*Drakaea livida*	736 bp	Linde et al. 2014
HQ386778	*Tulasnella secunda*	Australia	*Drakaea livida*	852 bp	Linde et al. 2014
KF476576	*Tulasnella secunda*	Australia	*Drakaea livida*	736 bp	Linde et al. 2014
KF476583	*Tulasnella secunda*	Australia	*Drakaea glyptodon*	736 bp	Linde et al. 2014
KF476586	*Tulasnella secunda*	Australia	*Drakaea glyptodon*	736 bp	Linde et al. 2014
HQ386743	*Tulasnella secunda*	Australia	*Drakaea glyptodon*	804 bp	Linde et al. 2014
KF476577	*Tulasnella secunda*	Australia	*Drakaea glyptodon*	736 bp	Linde et al. 2014
KF476585	*Tulasnella secunda*	Australia	*Drakaea isolata*	736 bp	Linde et al. 2014
KF476580	*Tulasnella secunda*	Australia	*Paracaleana triens*	736 bp	Linde et al. 2014
KF476584	*Tulasnella secunda*	Australia	*Paracaleana hortiorum*	736 bp	Linde et al. 2014
KF476574	*Tulasnella secunda*	Australia	*Paracaleana terminalis*	736 bp	Linde et al. 2014
KF476573	*Tulasnella secunda*	Australia	*Paracaleana lyonsii*	735 bp	Linde et al. 2014
KF476568	*Tulasnella secunda*	Australia	*Paracaleana minor*	736 bp	Linde et al. 2014
KF476602	*Tulasnella australiensis*	Australia	*Arthrochilus oreophilus*	735 bp	Linde et al. 2014
KF476594	*Tulasnella* sp. CLM084	Australia	*Arthrochilus oreophilus*	731 bp	Linde et al. 2014
KF476595	*Tulasnella* sp. CLM085	Australia	*Arthrochilus oreophilus*	731 bp	Linde et al. 2014
KF476596*	*Tulasnella warcupii*	Australia	*Arthrochilus oreophilus*	734 bp	Linde et al. 2014
KF476599	*Tulasnella warcupii*	Australia	*Arthrochilus oreophilus*	732 bp	Linde et al. 2014
KF476597	*Tulasnella warcupii*	Australia	*Arthrochilus oreophilus*	733 bp	Linde et al. 2014
KF476598	*Tulasnella warcupii*	Australia	*Arthrochilus oreophilus*	733 bp	Linde et al. 2014
KF476600	*Tulasnella warcupii*	Australia	*Arthrochilus oreophilus*	735 bp	Linde et al. 2014
KF476601	*Tulasnella warcupii*	Australia	*Arthrochilus oreophilus*	735 bp	Linde et al. 2014

KF476556*	*Tulasnella prima*	Australia	*Chiloglottis trilabra*	736 bp	Linde et al. 2014
HM196805	*Tulasnella prima*	Australia	*Chiloglottis reflexa*	862 bp	Linde et al. 2014
KF476543	*Tulasnella prima*	Australia	*Chiloglottis formicifera*	736 bp	Linde et al. 2014
HM196791	*Tulasnella prima*	Australia	*Chiloglottis* aff. *jeanesii*	863 bp	Linde et al. 2014
HM196783	*Tulasnella prima*	Australia	*Chiloglottis* aff. *jeanesii*	863 bp	Linde et al. 2014
HM196784	*Tulasnella prima*	Australia	*Chiloglottis* aff. *jeanesii*	863 bp	Linde et al. 2014
HM196788	*Tulasnella prima*	Australia	*Chiloglottis* aff. *jeanesii*	863 bp	Linde et al. 2014
HM196786	*Tulasnella prima*	Australia	*Chiloglottis* aff. *jeanesii*	864 bp	Linde et al. 2014
HM196779	*Tulasnella prima*	Australia	*Chiloglottis* aff. *jeanesii*	864 bp	Linde et al. 2014
HM196785	*Tulasnella prima*	Australia	*Chiloglottis* aff. *jeanesii*	863 bp	Linde et al. 2014
HM196782	*Tulasnella prima*	Australia	*Chiloglottis* aff. *jeanesii*	863 bp	Linde et al. 2014
HM196795	*Tulasnella prima*	Australia	*Chiloglottis* aff. *jeanesii*	862 bp	Linde et al. 2014
HM196787	*Tulasnella prima*	Australia	*Chiloglottis* aff. *jeanesii*	863 bp	Linde et al. 2014
HM196792	*Tulasnella prima*	Australia	*Chiloglottis* aff. *jeanesii*	863 bp	Linde et al. 2014
HM196804	*Tulasnella prima*	Australia	*Chiloglottis valida*	863 bp	Linde et al. 2014
HM196801	*Tulasnella prima*	Australia	*Chiloglottis valida*	862 bp	Linde et al. 2014
HM196810	*Tulasnella prima*	Australia	*Chiloglottis trapeziformis*	848 bp	Linde et al. 2014
HM196789	*Tulasnella prima*	Australia	*Chiloglottis trapeziformis*	863 bp	Linde et al. 2014
HM196796	*Tulasnella prima*	Australia	*Chiloglottis trapeziformis*	862 bp	Linde et al. 2014
HM196790	*Tulasnella prima*	Australia	*Chiloglottis trapeziformis*	862 bp	Linde et al. 2014
HM196809	*Tulasnella prima*	Australia	*Chiloglottis trapeziformis*	856 bp	Linde et al. 2014
HM196794	*Tulasnella prima*	Australia	*Chiloglottis trapeziformis*	863 bp	Linde et al. 2014
HM196806	*Tulasnella prima*	Australia	*Chiloglottis trapeziformis*	862 bp	Linde et al. 2014
HM196793	*Tulasnella prima*	Australia	*Chiloglottis trapeziformis*	863 bp	Linde et al. 2014
HM196807	*Tulasnella prima*	Australia	*Chiloglottis trapeziformis*	862 bp	Linde et al. 2014
HM196799	*Tulasnella prima*	Australia	*Chiloglottis trapeziformis*	862 bp	Linde et al. 2014

KF476552	*Tulasnella prima*	Australia	*Chiloglottis diphylla*	736 bp	Linde et al. 2014
HM196803	*Tulasnella prima*	Australia	*Chiloglottis diphylla*	862 bp	Linde et al. 2014
HM196798	*Tulasnella prima*	Australia	*Chiloglottis seminuda*	862 bp	Linde et al. 2014
HM196797	*Tulasnella prima*	Australia	*Chiloglottis seminuda*	862 bp	Linde et al. 2014
HM196800	*Tulasnella prima*	Australia	*Chiloglottis seminuda*	863 bp	Linde et al. 2014
MK430521	*Tulasnella prima* (Southern group)	Australia	*Cryptostylis ovata*	563 bp	Nguyen et al. 2019
MK430470	*Tulasnella prima* (Southern group)	Australia	*Cryptostylis ovata*	727 bp	Nguyen et al. 2019
MK430473	*Tulasnella prima* (Southern group)	Australia	*Cryptostylis ovata*	705 bp	Nguyen et al. 2019
AY373296	*Tulasnella tomaculum*	United States	Unknown	800 bp	McCormick et al. 2004
KC152380	*Tulasnella tomaculum*	United Kingdom	Unknown	714 bp	Cruz et al. 2014
MK430461	*Tulasnella* sp. CJ51 (Jarrahdale 2)	Australia	*Cryptostylis ovata*	639 bp	Nguyen et al. 2019
MK430468	*Tulasnella* sp. CJ51 (Jarrahdale 2)	Australia	*Cryptostylis ovata*	574 bp	Nguyen et al. 2019
MK430451	*Tulasnella* sp. CJ11 (Jarrahdale 1)	Australia	*Cryptostylis ovata*	710 bp	Nguyen et al. 2019
MK430452	*Tulasnella sp.* CJ13 (Jarrahdale 1)	Australia	*Cryptostylis ovata*	730 bp	Nguyen et al. 2019
MK430445	*Tulasnella occidentalis* (Bertram)	Australia	*Cryptostylis ovata*	731 bp	Nguyen et al. 2019
MK430439	*Tulasnella occidentalis* (Bertram)	Australia	*Cryptostylis ovata*	696 bp	Nguyen et al. 2019
MK626568	*Tulasnella* sp. JM 2019a	Canada	Rotten wood	775 bp	Mack & Seifert 2019, unpublished
MK626687	*Tulasnella* sp. JM 2019a	Canada	Rotten wood	775 bp	Mack & Seifert 2019, unpublished
MK626686	*Tulasnella* sp. JM 2019a	Canada	Rotten wood	775 bp	Mack & Seifert 2019, unpublished

*holotype

Table S2. *Tulasnella* isolates obtained from the five Australian *Cryptostylis* species in each population. Fungal Operational Taxonomic Unit/species of each isolate was determined with ITS sequencing and phylogenetic analyses.

No.	GenBank ITS accession	Isolate	Fungal species	Orchid host	Location sampled
1	MT003766	CLM1647	*Tulasnella australiensis*	*Cryptostylis erecta*	Fitzroy Falls, NSW
2	MT003765	CLM1648	*Tulasnella australiensis*	*Cryptostylis erecta*	Fitzroy Falls, NSW
3	MT003764	CLM1649	*Tulasnella australiensis*	*Cryptostylis erecta*	Fitzroy Falls, NSW
4	MT003763	CLM1650	*Tulasnella australiensis*	*Cryptostylis erecta*	Fitzroy Falls, NSW
5	MT003762	CLM1651	*Tulasnella australiensis*	*Cryptostylis erecta*	Fitzroy Falls, NSW
6	MT003761	CLM1652	*Tulasnella australiensis*	*Cryptostylis erecta*	Fitzroy Falls, NSW
7	MT003760	CLM1656	*Tulasnella australiensis*	*Cryptostylis erecta*	Fitzroy Falls, NSW
8	MT003759	CLM1657	*Tulasnella australiensis*	*Cryptostylis erecta*	Fitzroy Falls, NSW
9	MT003758	CLM1660	*Tulasnella australiensis*	*Cryptostylis erecta*	Fitzroy Falls, NSW
10	MT003757	CLM1661	*Tulasnella australiensis*	*Cryptostylis erecta*	Fitzroy Falls, NSW
11	MT003756	CLM1662	*Tulasnella australiensis*	*Cryptostylis erecta*	Fitzroy Falls, NSW
12	MT003755	CLM1663	*Tulasnella australiensis*	*Cryptostylis erecta*	Fitzroy Falls, NSW
13	MT003754	CLM1664	*Tulasnella australiensis*	*Cryptostylis erecta*	Fitzroy Falls, NSW
14	MT003753	CLM1667	*Tulasnella australiensis*	*Cryptostylis erecta*	Fitzroy Falls, NSW
15	MT003752	CLM1668	*Tulasnella australiensis*	*Cryptostylis erecta*	Fitzroy Falls, NSW
16	MT003751	CLM1670	*Tulasnella australiensis*	*Cryptostylis leptochila*	Fitzroy Falls, NSW
17	MT003750	CLM1685	*Tulasnella australiensis*	*Cryptostylis leptochila*	Fitzroy Falls, NSW
18	MT003749	CLM1692	*Tulasnella australiensis*	*Cryptostylis leptochila*	Fitzroy Falls, NSW
19	MT003748	CLM1693	*Tulasnella australiensis*	*Cryptostylis leptochila*	Fitzroy Falls, NSW
20	MT008104	CLM1901	*Tulasnella punctata*	*Cryptostylis subulata*	Ulladulla, NSW
21	MW145114	CLM1933	*Tulasnella prima*	*Cryptostylis leptochila*	Kangaloon, NSW
22	MW145115	CLM1934	*Tulasnella prima*	*Cryptostylis leptochila*	Kangaloon, NSW
23	MW145116	CLM1935	*Tulasnella prima*	*Cryptostylis leptochila*	Kangaloon, NSW
24	MW145117	CLM1936	*Tulasnella prima*	*Cryptostylis leptochila*	Kangaloon, NSW
25	MW145118	CLM1937	*Tulasnella prima*	*Cryptostylis leptochila*	Kangaloon, NSW
26	MT008096	CLM1938	*Tulasnella occidentalis*	*Cryptostylis ovata*	Boyanup, WA
27	MT008095	CLM1939	*Tulasnella occidentalis*	*Cryptostylis ovata*	Boyanup, WA
28	MT008094	CLM1940	*Tulasnella occidentalis*	*Cryptostylis ovata*	Boyanup, WA
29	MT008093	CLM1941	*Tulasnella occidentalis*	*Cryptostylis ovata*	Boyanup, WA
30	MT008092	CLM1942	*Tulasnella occidentalis*	*Cryptostylis ovata*	Boyanup, WA
31	MT008091	CLM1943	*Tulasnella occidentalis*	*Cryptostylis ovata*	Boyanup, WA
32	MT003731	CLM1944	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
33	MT003730	CLM1945	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
34	MT003729	CLM1946	*Tulasnella australiensis*	*Cryptostylis subulata*	Nowra, NSW
35	MT003728	CLM1947	*Tulasnella australiensis*	*Cryptostylis subulata*	Nowra, NSW

36	MT214493	CLM1952	*Tulasnella sphagneti*	*Cryptostylis subulata*	Fitzroy Falls, NSW
37	MT003738	CLM1953	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
38	MT003737	CLM1954	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
39	MT003734	CLM1955	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
40	MT003733	CLM1956	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
41	MT003736	CLM1957	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
42	MT003732	CLM1958	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
43	MT003715	CLM2004	*Tulasnella australiensis*	*Cryptostylis erecta*	Frenchs Forest, NSW
44	MT003714	CLM2005	*Tulasnella australiensis*	*Cryptostylis erecta*	Frenchs Forest, NSW
45	MT003713	CLM2006	*Tulasnella australiensis*	*Cryptostylis erecta*	Frenchs Forest, NSW
46	MT003712	CLM2007	*Tulasnella australiensis*	*Cryptostylis erecta*	Frenchs Forest, NSW
47	MT003711	CLM2008	*Tulasnella australiensis*	*Cryptostylis erecta*	Frenchs Forest, NSW
48	MT003710	CLM2009	*Tulasnella australiensis*	*Cryptostylis erecta*	Frenchs Forest, NSW
49	MT003709	CLM2010	*Tulasnella australiensis*	*Cryptostylis erecta*	Frenchs Forest, NSW
50	MT003708	CLM2011	*Tulasnella australiensis*	*Cryptostylis erecta*	Frenchs Forest, NSW
51	MT008124	CLM2012	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
52	MT003727	CLM2013	*Tulasnella australiensis*	*Cryptostylis erecta*	Frenchs Forest, NSW
53	MT003726	CLM2014	*Tulasnella australiensis*	*Cryptostylis erecta*	Frenchs Forest, NSW
54	MW145113	CLM2015	*Tulasnella australiensis*	*Cryptostylis erecta*	Frenchs Forest, NSW
55	MT008123	CLM2016	*Tulasnella punctata*	*Cryptostylis erecta*	Frenchs Forest, NSW
56	MT008122	CLM2017	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
57	MT008121	CLM2018	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
58	MT008120	CLM2019	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
59	MT008119	CLM2020	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
60	MT008118	CLM2021	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
61	MT008117	CLM2022	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
62	MT008116	CLM2023	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
63	MT008115	CLM2024	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
64	MT008114	CLM2025	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
65	MT008113	CLM2026	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
66	MT008112	CLM2027	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
67	MT008111	CLM2028	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
68	MT008110	CLM2029	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
69	MT008109	CLM2030	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
70	MT008108	CLM2031	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
71	MT008107	CLM2032	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
72	MT008106	CLM2033	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
73	MT008105	CLM2034	*Tulasnella punctata*	*Cryptostylis subulata*	Frenchs Forest, NSW
74	MT036547	CLM2071	*Tulasnella concentrica*	*Cryptostylis leptochila*	Bundanoon, NSW
75	MT036546	CLM2072	*Tulasnella concentrica*	*Cryptostylis leptochila*	Bundanoon, NSW

76	MT003747	CLM2073	*Tulasnella australiensis*	*Cryptostylis leptochila*	Bundanoon, NSW
77	MT008090	CLM2074	*Tulasnella occidentalis*	*Cryptostylis ovata*	Johnston Rd, WA
78	MT008089	CLM2075	*Tulasnella occidentalis*	*Cryptostylis ovata*	Johnston Rd, WA
79	MT008088	CLM2076	*Tulasnella occidentalis*	*Cryptostylis ovata*	Johnston Rd, WA
80	MT008087	CLM2077	*Tulasnella occidentalis*	*Cryptostylis ovata*	Johnston Rd, WA
81	MT008086	CLM2078	*Tulasnella occidentalis*	*Cryptostylis ovata*	Johnston Rd, WA
82	MT008085	CLM2079	*Tulasnella occidentalis*	*Cryptostylis ovata*	Johnston Rd, WA
83	MT008084	CLM2080	*Tulasnella occidentalis*	*Cryptostylis ovata*	Johnston Rd, WA
84	MT008083	CLM2081	*Tulasnella occidentalis*	*Cryptostylis ovata*	Muja Conservation Park, WA
85	MT008082	CLM2082	*Tulasnella occidentalis*	*Cryptostylis ovata*	Muja Conservation Park, WA
86	MT008081	CLM2083	*Tulasnella occidentalis*	*Cryptostylis ovata*	Muja Conservation Park, WA
87	MT008080	CLM2084	*Tulasnella occidentalis*	*Cryptostylis ovata*	Muja Conservation Park, WA
88	MT008079	CLM2085	*Tulasnella occidentalis*	*Cryptostylis ovata*	Muja Conservation Park, WA
89	MW145119	CLM2086	*Tulasnella prima*	*Cryptostylis ovata*	Frosty Rd, WA
90	MW145120	CLM2087	*Tulasnella prima*	*Cryptostylis ovata*	Frosty Rd, WA
91	MW145121	CLM2088	*Tulasnella prima*	*Cryptostylis ovata*	Frosty Rd, WA
92	MW145122	CLM2089	*Tulasnella prima*	*Cryptostylis ovata*	Frosty Rd, WA
93	MW145123	CLM2090	*Tulasnella prima*	*Cryptostylis ovata*	Frosty Rd, WA
94	MW145124	CLM2091	*Tulasnella prima*	*Cryptostylis ovata*	Frosty Rd, WA
95	MT003746	CLM2092	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
96	MT003743	CLM2093	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
97	MT003742	CLM2094	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
98	MT003735	CLM2095	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
99	MT003767	CLM2096	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
100	MT003745	CLM2097	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
101	MT003744	CLM2098	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
102	MT003741	CLM2100	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
103	MT003740	CLM2101	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
104	MT003739	CLM2102	*Tulasnella australiensis*	*Cryptostylis erecta*	Nowra, NSW
105	MT008077	CLM2103	*Tulasnella occidentalis*	*Cryptostylis ovata*	Granite Peaks, WA
106	MT008078	CLM2104	*Tulasnella occidentalis*	*Cryptostylis ovata*	Granite Peaks, WA
107	MT008076	CLM2105	*Tulasnella occidentalis*	*Cryptostylis ovata*	Granite Peaks, WA
108	MT008075	CLM2106	*Tulasnella occidentalis*	*Cryptostylis ovata*	Granite Peaks, WA
109	MT036529	CLM2107	*Tulasnella densa*	*Cryptostylis hunteriana*	Bulahdelah, NSW
110	MT036528	CLM2108	*Tulasnella densa*	*Cryptostylis hunteriana*	Bulahdelah, NSW
111	MT036527	CLM2109	*Tulasnella densa*	*Cryptostylis hunteriana*	Bulahdelah, NSW
112	MT036526	CLM2110	*Tulasnella densa*	*Cryptostylis hunteriana*	Bulahdelah, NSW
113	MT036525	CLM2111	*Tulasnella densa*	*Cryptostylis hunteriana*	Meroo National Park, NSW
114	MT036524	CLM2112	*Tulasnella densa*	*Cryptostylis hunteriana*	Meroo National Park, NSW
115	MT036523	CLM2114	*Tulasnella densa*	*Cryptostylis hunteriana*	Meroo National Park, NSW

116	MT036522	CLM2115	*Tulasnella densa*	*Cryptostylis hunteriana*	Meroo National Park, NSW
117	MT036521	CLM2116	*Tulasnella densa*	*Cryptostylis hunteriana*	Meroo National Park, NSW
118	MT036520	CLM2117	*Tulasnella densa*	*Cryptostylis hunteriana*	Meroo National Park, NSW
119	MT036519	CLM2118	*Tulasnella densa*	*Cryptostylis hunteriana*	Meroo National Park, NSW
120	MT003725	CLM2119	*Tulasnella australiensis*	*Cryptostylis leptochila*	Fitzroy Falls, NSW
121	MT003724	CLM2120	*Tulasnella australiensis*	*Cryptostylis leptochila*	Fitzroy Falls, NSW
122	MT003723	CLM2121	*Tulasnella australiensis*	*Cryptostylis leptochila*	Fitzroy Falls, NSW
123	MT008103	CLM2123	*Tulasnella punctata*	*Cryptostylis subulata*	Karuah, NSW
124	MT008099	CLM2124	*Tulasnella punctata*	*Cryptostylis subulata*	Karuah, NSW
125	MT008098	CLM2125	*Tulasnella punctata*	*Cryptostylis subulata*	Karuah, NSW
126	MT008097	CLM2126	*Tulasnella punctata*	*Cryptostylis subulata*	Karuah, NSW
127	MT008069	CLM2127	*Tulasnella occidentalis*	*Cryptostylis ovata*	Johnston Rd, WA
128	MT008068	CLM2128	*Tulasnella occidentalis*	*Cryptostylis ovata*	Muja Conservation Park, WA
129	MT036514	CLM2129	*Tulasnella densa*	*Cryptostylis hunteriana*	Meroo National Park, NSW
130	MT214494	CLM2130	*Tulasnella sphagneti*	*Cryptostylis subulata*	Fitzroy Falls, NSW
131	MT214495	CLM2131	*Tulasnella sphagneti*	*Cryptostylis subulata*	Fitzroy Falls, NSW
132	MT214496	CLM2132	*Tulasnella sphagneti*	*Cryptostylis subulata*	Fitzroy Falls, NSW
133	MW145125	CLM2133	*Tulasnella prima*	*Cryptostylis leptochila*	Kangaloon, NSW
134	MW145126	CLM2134	*Tulasnella prima*	*Cryptostylis leptochila*	Kangaloon, NSW
135	MW145127	CLM2135	*Tulasnella prima*	*Cryptostylis leptochila*	Kangaloon, NSW
136	MT008074	CLM2136	*Tulasnella occidentalis*	*Cryptostylis ovata*	Rockingham, WA
137	MT008073	CLM2137	*Tulasnella occidentalis*	*Cryptostylis ovata*	Rockingham, WA
138	MT008072	CLM2138	*Tulasnella occidentalis*	*Cryptostylis ovata*	Rockingham, WA
139	MT008071	CLM2139	*Tulasnella occidentalis*	*Cryptostylis ovata*	Rockingham, WA
140	MT008070	CLM2140	*Tulasnella occidentalis*	*Cryptostylis ovata*	Rockingham, WA
141	MT036545	CLM2142	*Tulasnella concentrica*	*Cryptostylis leptochila*	Bundanoon, NSW
142	MW145128	CLM2157	*Tulasnella prima*	*Cryptostylis leptochila*	Penrose Nature Reserve, NSW
143	MW145129	CLM2158	*Tulasnella prima*	*Cryptostylis leptochila*	Penrose Nature Reserve, NSW
144	MW145130	CLM2159	*Tulasnella prima*	*Cryptostylis leptochila*	Penrose Nature Reserve, NSW
145	MT036544	CLM2160	*Tulasnella concentrica*	*Cryptostylis leptochila*	Penrose Nature Reserve, NSW
146	MT036518	CLM2161	*Tulasnella densa*	*Cryptostylis hunteriana*	Erowal Bay, NSW
147	MT036517	CLM2162	*Tulasnella densa*	*Cryptostylis hunteriana*	Erowal Bay, NSW
148	MT036516	CLM2163	*Tulasnella densa*	*Cryptostylis hunteriana*	Erowal Bay, NSW
149	MT036515	CLM2164	*Tulasnella densa*	*Cryptostylis hunteriana*	Erowal Bay, NSW
150	MT036552	CLM2165	*Tulasnella concentrica*	*Cryptostylis leptochila*	Carls Mountain Rd, NSW
151	MT036551	CLM2166	*Tulasnella concentrica*	*Cryptostylis leptochila*	Carls Mountain Rd, NSW
152	MT036550	CLM2167	*Tulasnella concentrica*	*Cryptostylis leptochila*	Carls Mountain Rd, NSW
153	MT036549	CLM2168	*Tulasnella concentrica*	*Cryptostylis leptochila*	Carls Mountain Rd, NSW
154	MT036548	CLM2169	*Tulasnella concentrica*	*Cryptostylis leptochila*	Carls Mountain Rd, NSW
155	MT036555	CLM2170	*Tulasnella concentrica*	*Cryptostylis leptochila*	Carls Mountain Rd, NSW

156	MT036554	CLM2171	*Tulasnella concentrica*	*Cryptostylis leptochila*	Carls Mountain Rd, NSW
157	MT036553	CLM2172	*Tulasnella concentrica*	*Cryptostylis leptochila*	Carls Mountain Rd, NSW
158	MT036543	CLM2173	*Tulasnella concentrica*	*Cryptostylis leptochila*	Carls Mountain Rd, NSW
159	MT036542	CLM2174	*Tulasnella concentrica*	*Cryptostylis leptochila*	Carls Mountain Rd, NSW
160	MT008102	CLM2175	*Tulasnella punctata*	*Cryptostylis subulata*	Erowal Bay, NSW
161	MT008101	CLM2176	*Tulasnella punctata*	*Cryptostylis subulata*	Erowal Bay, NSW
162	MT008100	CLM2177	*Tulasnella punctata*	*Cryptostylis subulata*	Erowal Bay, NSW
163	MT003722	CLM2178	*Tulasnella australiensis*	*Cryptostylis erecta*	Erowal Bay, NSW
164	MT003721	CLM2179	*Tulasnella australiensis*	*Cryptostylis erecta*	Erowal Bay, NSW
165	MT003720	CLM2180	*Tulasnella australiensis*	*Cryptostylis erecta*	Erowal Bay, NSW
166	MT003719	CLM2181	*Tulasnella australiensis*	*Cryptostylis erecta*	Erowal Bay, NSW
167	MT003718	CLM2182	*Tulasnella australiensis*	*Cryptostylis erecta*	Erowal Bay, NSW
168	MT003717	CLM2183	*Tulasnella australiensis*	*Cryptostylis erecta*	Erowal Bay, NSW
169	MT003716	CLM2184	*Tulasnella australiensis*	*Cryptostylis erecta*	Erowal Bay, NSW
170	MT003707	CLM2185	*Tulasnella australiensis*	*Cryptostylis erecta*	Carls Mountain Rd, NSW
171	MT003706	CLM2186	*Tulasnella australiensis*	*Cryptostylis erecta*	Carls Mountain Rd, NSW
172	MT003705	CLM2187	*Tulasnella australiensis*	*Cryptostylis erecta*	Carls Mountain Rd, NSW
173	MT036541	CLM2188	*Tulasnella concentrica*	*Cryptostylis leptochila*	Cabbage Tree Creek, Vic
174	MT036531	CLM2189	*Tulasnella concentrica*	*Cryptostylis leptochila*	Cabbage Tree Creek, Vic
175	MT036530	CLM2190	*Tulasnella concentrica*	*Cryptostylis leptochila*	Cabbage Tree Creek, Vic
176	MT036540	CLM2191	*Tulasnella concentrica*	*Cryptostylis leptochila*	Cabbage Tree Creek, Vic
177	MT036539	CLM2192	*Tulasnella concentrica*	*Cryptostylis leptochila*	Cabbage Tree Creek, Vic
178	MT036538	CLM2193	*Tulasnella concentrica*	*Cryptostylis leptochila*	Cabbage Tree Creek, Vic
179	MT036537	CLM2194	*Tulasnella concentrica*	*Cryptostylis leptochila*	Cape Conran, Vic
180	MT036536	CLM2195	*Tulasnella concentrica*	*Cryptostylis leptochila*	Cape Conran, Vic
181	MT036535	CLM2196	*Tulasnella concentrica*	*Cryptostylis leptochila*	Bunyip State Park, Vic
182	MT036534	CLM2197	*Tulasnella concentrica*	*Cryptostylis leptochila*	Bunyip State Park, Vic
183	MT036533	CLM2198	*Tulasnella concentrica*	*Cryptostylis leptochila*	Bunyip State Park, Vic
184	MT036532	CLM2199	*Tulasnella concentrica*	*Cryptostylis leptochila*	Bunyip State Park, Vic

Table S3. Distribution of *Tulasnella* isolates and clones in each population of Australian and Asiatic *Cryptostylis*, with total plants 167 Australian and 6 Asiatic *Cryptostylis* sampled. Total *Tulasnella* sequences recovered: 492.

***Cryptostylis* species**	**Population**	***N* plants**	**Number of isolates (Species/OTU)**									**Number of clones (Species/OTU)**													
			T. prima	***T. warcupii***	***T. sphagneti***	***T. australiensis***	***T. occidentalis***	***T. punctata***	***T. densa***	***T. concentrica***	**OTU Isolates**	***T. prima***	***T. warcupii***	***T. sphagneti***	***T. australiensis***	***T. occidentalis***	***T. punctata***	***T. densa***	***T. concentrica***	**OTU 1**	**OTU 2**	**OTU 3**	**OTU 4**	**OTU 5**	**OTU Clones**
C. erecta	Fitzroy Falls	1				15					1														1
	Frenchs Forest	4				11		1			2				17										2
	Bulahdelah	8									0				4			1							2
	Nowra	7				18					1				13			1							2
	Erowal Bay	5				7					1				3										1
	Wogamia	5									0				7			1							2
	Carls Mountain	5				3					1				7										1
	Meroo	2									0														0
	Ulladulla	5									0				9										1
	Subtotal	**42**				**54**		**1**							**60**			**3**							118
C. subulata	Frenchs Forest	4						19			1						14								1
	Ulladulla	8									0						1								1
	Fitzroy Falls	6			4						1			10											1
	Karuah	2						4			1														1
	Nowra	6				2		1			2				3		4								2
	Erowal Bay	5						3			1						3								1
	Wogamia	5									0			6											1
	Subtotal	**36**			**4**	**2**		**27**						**16**	**3**		**22**								74
C. leptochila	Bundanoon	2				2				3	2				2				2						2
	Fitzroy Falls	4				7					1				8										1
	Kangaloon	7	8								1	10	2		7										3
	Penrose	5	3							1	2	5							5						2
	Carls Mountain	5								10	1				4				12						2
	Cabbage Tree	5								6	1				2				9						2
	Cape Conran	5								2	1								11						1
	Bunyip	5								4	1								10						1
	Subtotal	**38**	**11**			**9**				**26**		**15**	**2**		**23**				**49**						135
C. ovata	Boyanup	5					6				1					4									1
	Rockingham	4					5				1	1			3	2									3
	Frosty Rd	3	6								1	8													1
	Boronia	3									failed														0
	Bridgetown Jarrah Park	2									0	2													1
	Johnston Rd	3					8				1					6									1
	Muja	5					6				1					4									1
	Granite Peaks	2					4				1														1
	Capel	2									0		2			2									2
	Margaret River	5									0	7													1
	Walpole	2									0									6					1
	Subtotal	**36**	**6**				**29**					**18**	**2**		**3**	**18**				**6**					82
C. hunteriana	Bulahdelah	2							4		1							4							1
	Meroo	7							8		1							10							1
	Erowal Bay	6							4		1							10							1
	Subtotal	**15**							**16**									**24**							40
C. javanica	Lantau, HK	1	isolation not attempted																				6	6	2
	Subtotal	**1**																					**6**	**6**	12
C. filiformis	Alab Mountain, MY	1	isolation not attempted																			9			1
	Subtotal	**1**																				**9**			9
C. acutata	Alab Mountain, MY	4	isolation not attempted																		22				1
	Subtotal	**4**																			**22**				22

Table S4. Closest GenBank matches of non-*Tulasnella* clones obtained with ITS primer pair combinations. Mycorrhizal status of fungi as suggested from each study, is also indicated.

Orchid species	**Plant code**	**Clone number**	**Primers for cloning**	**GenBank Match**							
				Accession number	**Identity of closest matching sequence**	**Substrate / host**	**Country**	**Sequence identity**	**Phylum/Order/Class**	**Mycorrhizal status#**	**References**
Cryptostylis hunteriana	CL046	Clone 1	ITS1F-ITS4	MF161310	*Athelia* sp. isolate BHI-F636b	Bark of dead arrowwood	USA	94%	Basidiomycota/Atheliales	Saprotroph	Haelewaters *et al.* (2018)
Cryptostylis hunteriana	CL046	Clone 2	ITS1F-ITS4B	KP012865	*Phylloporus* sp. GMB-2014	Unknown	Australia	99%	Basidiomycota/Boletales	Mycorrhizal/Saprotroph	Bonito et al. (2014)*
Cryptostylis hunteriana	CL046	Clone 3	ITS1F-ITS4	KF679822	*Thelephoraceae* sp. MH01 MHR291689	*Pinus wallichiana* (root)	Pakistan	94%	Basidiomycota/Thelephorales	Ectomycorrhizal	Hanif & Khalid (2013)*
Cryptostylis hunteriana	CL140.1	Clone 4	ITS1F-ITS4B	AF354081	*Ceratobasidium* sp. *CAG4*	Soybean	USA	96%	Basidiomycota/Cantharellales	Plant pathogen	Gonzalez *et al.* (2011)
Cryptostylis hunteriana	CL140.1	Clone 5	ITS1F-ITS4	HQ667799	Uncultured Ceratobasidiaceae clone 207	*Hexalectris grandiflora* (root)	USA	97%	Basidiomycota/Cantharellales	OMF	Kennedy *et al.* (2011)
Cryptostylis hunteriana	CL140.1	Clone 6	ITS1F-ITS4	DQ398091	Uncultured fungus isolate EC2.1	*Erythrorchis cassythoides* (root)	Australia	99%	Basidiomycota/Cantharellales	OMF	Dearnaley (2006)
Cryptostylis hunteriana	CL140.1	Clone 7	ITS1F-ITS4	NR_155992	*Vanrija longa* CBS 5920	Unknown	Netherlands	100%	Basidiomycota/Trichosporonales	Yeast – non-mycorrhizal	Vu *et al.* (2016)
Cryptostylis hunteriana	CL140.2	Clone 8	ITS1F-ITS4	MH268056	*Phialocephala* sp. AMB14_7A	*Micrandra spruceana* (inner bark)	Peru	99%	Ascomycota	Endophytic	Skaltsas *et al.* (2019)
Cryptostylis hunteriana	CL140.2	Clone 9	ITS1F-ITS4B	KT875188	*Cortinarius mycenarum*	Unknown	NZ	96%	Basidiomycota/Agaricales	Ectomycorrhizal	Johnston *et al.* (2019)
Cryptostylis hunteriana	CL140.2	Clone 10	ITS1F-ITS4	NR_156300	*Cortinarius mycenarum* PDD 107715	Unknown	NZ	96%	Basidiomycota/Agaricales	Ectomycorrhizal	Johnston *et al.* (2019)

Cryptostylis hunteriana	CL140.4	Clone 11	ITS1F-ITS4	EU677757	Root-associated fungal sp. LP1-1	*Leptospermum polygalifolium*	Australia	100%	Basidiomycota/Agaricales	Ericoid/Ectomycorrhizal	Curlevski *et al.* (2009)
Cryptostylis hunteriana	CL140.4	Clone 12	ITS1F-ITS4	KF359578	*Oidiodendron* sp. CC 04-26	Eastern Hemlock	USA	98%	Ascomycota	Ericoid/Ectomycorrhizal	Baird *et al.* (2014)
Cryptostylis hunteriana	CL140.4	Clone 13	ITS1F-ITS4	AY627814	Root associated fungus EP51	*Epacris pulchella* (root)	Australia	98%		Ericoid mycorrhizal	Bougoure and Cairney (2005)
Cryptostylis hunteriana	CL140.4	Clone 14	ITS1F-ITS4	HF912261	*Coniophora prasinoides*	Softwood	Germany	99%	Basidiomycota/Boletales	Saprotroph	Huckfeldt & Schmidt (2013)*
Cryptostylis hunteriana	CL148.1	Clone 15	ITS1F-ITS4B	KP012769	*Russula* sp. 7 GMB-2014	Unknown	Australia	87%	Basidiomycota/Russulales	Ectomycorrhizal	Bonito *et al.* (2014)
Cryptostylis hunteriana	CL148.1	Clone 16	ITS1F-ITS4B	KY102928	*Saitozyma podzolica* culture CBS:9351	Soil	Costa Rica	99%	Basidiomycota/Tremellales	Yeast – non-mycorrhizal	Vu *et al.* (2016)
Cryptostylis leptochila	CL049	Clone 1	ITS1F-ITS4	EU273558	*Bionectria ochroleuca* isolate XSD-B42	Unknown	China	100%	Ascomycota/Hypocreales	Plant pathogen	Jiang *et al.* (2007)*
Cryptostylis leptochila	CL049	Clone 2	ITS1F-ITS4	NR_121495	*Ilyonectria cyclaminicola* CBS 302.93	*Cyclamen* sp. (bulb)	Netherlands	99%	Ascomycota/Hypocreales	Plant pathogen	Cabral *et al.* (2011)
Cryptostylis leptochila	CL049	Clone 3	ITS1F-ITS4	NR_121495	*Ilyonectria cyclaminicola* CBS 302.93	*Cyclamen* sp. (bulb)	Netherlands	100%	Ascomycota/Hypocreales	Plant pathogen	Cabral *et al.* (2011)
Cryptostylis leptochila	CL049	Clone 4	ITS1F-ITS4	EU273558	*Bionectria ochroleuca* isolate XSD-B42	Unknown	China	100%	Ascomycota/Hypocreales	Plant pathogen	Jiang *et al.* 2007*
Cryptostylis leptochila	CL049	Clone 5	ITS1F-ITS4	KM678355	*Helotiales* sp. Glum770	*Erica glumiflora* (root)	South Africa	99%	Ascomycota/Helotiales	Endophyte/Ericoid	Bizabani and Dames (2015)
Cryptostylis leptochila	CL136.1	Clone 6	ITS1F-ITS4B	KT875181	*Cortinarius elaiops* voucher PDD:107732	Unknown	NZ	94%	Basidiomycota/Agaricales	Ectomycorrhizal	Johnston *et al.* (2019)

Cryptostylis leptochila	CL136.2	Clone 7	ITS1F-ITS4	KM678355	*Helotiales* sp. Glum770	*Erica glumiflora* (root)	South Africa	99%	Ascomycota/Helotiales	Endophyte/Ericoid mycorrhizal	Bizabani and Dames (2015)
Cryptostylis leptochila	CL136.2	Clone 8	ITS1F-ITS4	KM678355	*Helotiales* sp. Glum770	*Erica glumiflora* (root)	South Africa	99%	Ascomycota/Helotiales	Endophyte/Ericoid mycorrhizal	Bizabani and Dames (2015)
Cryptostylis leptochila	CL141.1	Clone 9	ITS1F-ITS4	MN128966	*Leotiomycetes* sp. ECRU210	*Agathis australis* (root)	NZ	99%	Ascomycota/Helotiales	Endophyte/Ericoid mycorrhizal	Bizabani and Dames (2015)
Cryptostylis leptochila	CL141.1	Clone 10	ITS1F-ITS4	DQ398092	Uncultured fungus EC2.2 (*Russula*)	*Erythrorchis cassythoides* (root)	Australia	99%	Basidiomycota/Russulales	Ectomycorrhizal	Dearnaley (2006)
Cryptostylis leptochila	CL141.1	Clone 11	ITS1F-ITS4	DQ398092	Uncultured fungus EC2.2 (*Russula*)	*Erythrorchis cassythoides* (root)	Australia	99%	Basidiomycota/Russulales	Ectomycorrhizal	Dearnaley (2006)
Cryptostylis leptochila	CL141.2	Clone 12	ITS1F-ITS4B	KU973892	*Trechisporales* sp.isolate AD3TN	Cork Oak	Tunisia	95%	Basidiomycota/Trechisporales	Endophytic	Ali (2016)*
Cryptostylis leptochila	CL141.2	Clone 13	ITS1F-ITS4B	DQ398092	Uncultured fungus EC2.2 (*Russula*)	*Erythrorchis cassythoides* (root)	Australia	99%	Basidiomycota/Russulales	Ectomycorrhizal	Dearnaley (2006)
Cryptostylis leptochila	CL141.2	Clone 14	ITS1F-ITS4	JF414168	*Mucoromycotina* sp. MIB 8354	*Treubia lacunosa*	NZ	90%	Zygomycota	Endophytic	Bidartondo *et al.* (2011)
Cryptostylis subulata	CL048	Clone 2	ITS1F-ITS4	KT222442	*Hyalorbilia inflatula* voucher H.B. 9080	*Tilia* (branch)	Germany	81%	Ascomycota/Orbiliales	Saprotroph	Weiss (2015)*
Cryptostylis subulata	CL048	Clone 3	ITS1F-ITS4	JF273533	*Leotiomycetes* sp. EMF31	Evergreen forest (soil sample)	China	99%	Ascomycota/Leotiomycetes	Endophytic	Ding *et al.* (2011)*
Cryptostylis subulata	CL048	Clone 4	ITS1F-ITS4	KP814299	*Athelia bombacina* voucher UC2023122	Leaf litter	USA	95%	Basidiomycota/Atheliales	Saprotroph	Rosenthal & Bruns (2015)*

Cryptostylis subulata	CL128.1	Clone 5	ITS1F-ITS4	KF359595	*Herpotrichiellaceae* sp. 1 CC 13-06	Eastern Hemlock	USA	92%	Ascomycota/Chaetothyriales	Saprotroph	Baird *et al.* (2014)
Cryptostylis subulata	CL128.1	Clone 6	ITS1F-ITS4	MF101382	*Ilyonectria* sp. isolate Cg07	*Cymbidium goeringii* (root)	Korea	100%	Ascomycota/Hypocreales	Endophytic	Lee & Choi (2017)*
Cryptostylis subulata	CL128.1	Clone 7	ITS1F-ITS4	KF359595	*Herpotrichiellaceae* sp. 1 CC 13-06	Eastern Hemlock	USA	92%	Ascomycota/Chaetothyriales	Saprotroph	Baird *et al.* (2014)
Cryptostylis subulata	CL128.2	Clone 8	ITS1F-ITS4	JQ272347	*Verrucariales* sp. RB-2011	Unknown	USA	93%	Ascomycota/Verrucariales	Saprotroph	Baird *et al.* (2014)
Cryptostylis subulata	CL128.3	Clone 9	ITS1F-ITS4	JF414168	*Mucoromycotina* sp. MIB 8354	*Treubia lacunosa*	NZ	91%	Zygomycota	Endophytic	Bidartondo *et al.* (2011)
Cryptostylis subulata	CL128.3	Clone 10	ITS1F-ITS4	MN128999	*Helotiales* sp. 9-NN-2017	*Agathis australis* (root)	NZ	95%	Ascomycota/Helotiales	Endophyte/Ericoid mycorrhizal	Bizabani and Dames (2015)
Cryptostylis subulata	CL128.4	Clone 11	ITS1F-ITS4	MH268056	*Phialocephala* sp. AMB14_7A	*Micrandra spruceana* (root)	Peru	100%	Ascomycota/	Endophytic	Skaltsas *et al.* (2019)
Cryptostylis erecta	CL047	Clone 3	ITS1F-ITS4	KF061288	*Serendipita* sp. MAFF 305833	*Caladenia catenata*	Australia	98%	Basidiomycota/Sebacinales	Endophyte	Riess *et al.* (2014)
Cryptostylis erecta	CL047	Clone 4	ITS1F-ITS4	KM678367	*Hyaloscyphaceae* sp. D40	*Erica demissa*	South Africa	99%	Ascomycota/Helotiales	Endophyte/Ericoid	Bizabani and Dames (2015)
Cryptostylis erecta	CL047	Clone 7	ITS1F-ITS4	KJ508308	*Leotiomycetes* sp. *genotype 933*	*Brachythecium fendleri*	USA	97%	Ascomycota/Leotiomycetes	Endophyte	U'Ren *et al.* (2014)
Cryptostylis erecta	CL047	Clone 8	ITS1F-ITS4	NR_137772	*Penidiella aggregata* CBS 128772	*Phaenocoma prolifera* (leaf)	South Africa	90%	Ascomycota/Capnodiales	Endophyte	Crous and Groenewald (2011)
Cryptostylis erecta	CL116.2	Clone 9	ITS1F-ITS4B	KY774242	*Phellodon* sp. isolate CY13_154_1	Rainforest Soil	New Caledonia	95%	Basidiomycota/Thelephorales	Ectomycorrhizal	Carriconde *et al.* (2019)
Cryptostylis erecta	CL116.2	Clone 10	ITS1F-ITS4B	AY702070	Uncultured fungus clone 1 (*Russula*)	*Dipodium variegatum* (root)	Australia	94%	Basidiomycota/Russulales	Ectomycorrhizal	Bougoure and Dearnaley (2005)

Cryptostylis erecta	CL127.2	Clone 11	ITS1F-ITS4B	GU222292	*Russula* sp. PDD 89034	Unknown	NZ	95%	Basidiomycota/Russulales	Ectomycorrhizal	Johnston *et al.* (2019)
Cryptostylis erecta	CL127.2	Clone 12	ITS1F-ITS4B	GU222292	*Russula* sp. PDD 89034	Unknown	NZ	96%	Basidiomycota/Russulales	Ectomycorrhizal	Johnston *et al.* (2019)
Cryptostylis ovata	CL044	Clone 1	ITS5-ITS4	HE605213	*Exophiala moniliae*	Cave sediment	France	97%	Ascomycota/Chaetothyriales	Saprotroph	Martin-Sanchez *et al.* (2012)
Cryptostylis ovata	CL044	Clone 2	ITS5-ITS4	EU035403	*Cladophialophora chaetospira*	*Phyllostachys bambusoides* (wood)	China	96%	Ascomycota/Chaetothyriales	Saprotroph	Crous *et al.* (2007)
Cryptostylis ovata	CL044	Clone 3	ITS5-ITS4	JX138547	*Sebacinaceae* sp.4MB-2012	*Caladenia flava*	Australia	99%	Basidiomycota/Sebacinales	OMF	Sommer *et al.* (2012)
Cryptostylis ovata	CL132.1	Clone 4	ITS1F-ITS4	AB488490	*Exophiala* sp. NH1238	Bathroom	Japan	95%	Ascomycota/Chaetothyriales	Saprotroph	Hamada and Abe (2010)
Cryptostylis ovata	CL132.1	Clone 5	ITS1F-ITS4	AB488490	*Exophiala* sp. NH1238	Bathroom	Japan	98%	Ascomycota/Chaetothyriales	Saprotroph	Hamada and Abe (2010)
Cryptostylis ovata	CL135.2	Clone 6	ITS1F-ITS4	JQ272383	*Herpotrichiellaceae* sp. RB-2011	Unknown	USA	95%	Ascomycota/Chaetothyriales	Saprotroph	Baird *et al.* (2014)
Cryptostylis ovata	CL135.2	Clone 7	ITS1F-ITS4	JQ272383	*Herpotrichiellaceae* sp. RB-2011	Unknown	USA	95%	Ascomycota/Chaetothyriales	Saprotroph	Baird *et al.* (2014)

#OMF = orchid mycorrhizal fungus

*unpublished

Table S5. *Tulasnella* fungal associates of *Cryptostylis* where the orchid species are growing sympatrically. OTUs refer to operational taxonomic units of *Tulasnella* fungi.

Population	Orchid host	*N* plants	Fungal partner(s)*
Fitzroy Falls	*C. erecta*	1	*T. australiensis*
	C. subulata	6	*T. sphagneti*
	C. leptochila	4	*T. australiensis*
Frenchs Forest	*C. erecta*	4	*T. australiensis* (*N* = 4), *T. punctata* (*N* = 1)
	C. subulata	4	*T. punctata*
Nowra	*C. erecta*	7	*T. australiensis* (*N* = 7), *T. densa* (*N* = 1)
	C. subulata	6	*T. australiensis* (*N* = 6), *T. punctata* (*N* = 1)
Bulahdelah	*C. erecta*	8	*T. australiensis* (*N* = 8), *T. densa* (*N* = 1)
	C. hunteriana	2	*T. densa*
Erowal Bay	*C. erecta*	5	*T. australiensis*
	C. subulata	5	*T. punctata*
	C. hunteriana	6	*T. densa*
Wogamia	*C. erecta*	5	*T. australiensis* (*N* = 5), *T. densa* (*N* = 1)
	C. subulata	5	*T. sphagneti*
Carls Mountain	*C. erecta*	5	*T. australiensis*
	C. leptochila	5	*T. australiensis* (*N* = 1), *T. concentrica* (*N* = 4)
Ulladulla	*C. erecta*	5	*T. australiensis*
	C. subulata	8	*T. punctata*

*N shows the number of plants the OTU was successfully sampled from.

Figure S1. Rooted MrBayes phylogenetic tree for *Tulasnella* associated with *Cryptostylis* orchids, using the ITS region. *Tulasnella* isolate and clone sequences generated in this study and Arifin et al (2020) are shown in bold. Bootstrap (>70%) / BPP (>0.70) support values are shown above branches.

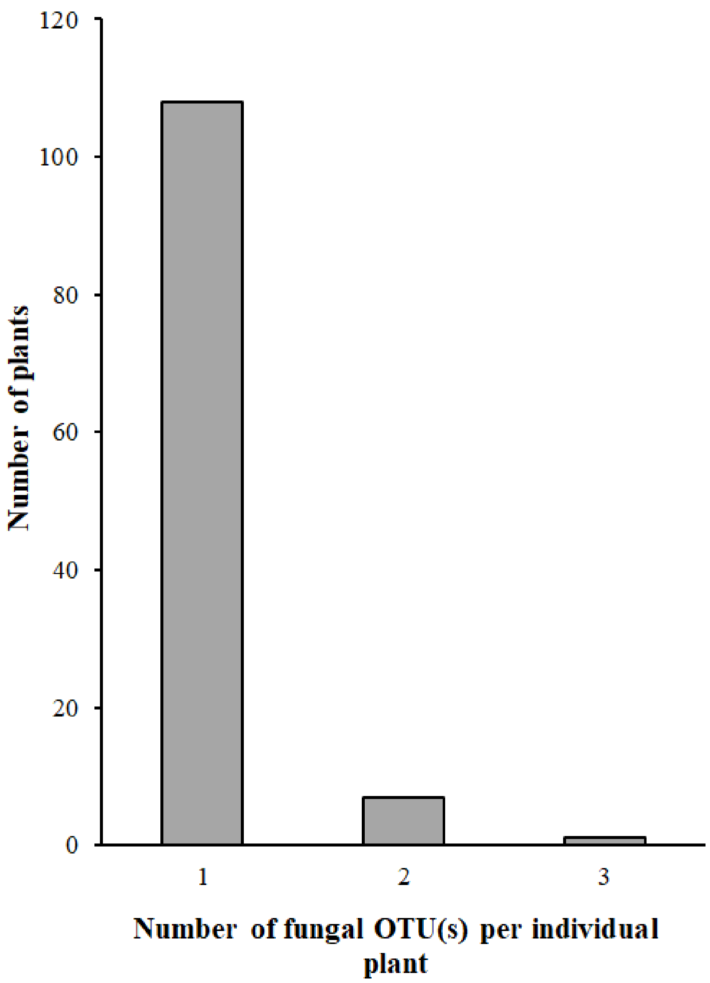

Figure S2. Frequency of the number of *Tulasnella* operational taxonomic units (OTUs) per individual plant combined across five Australian *Cryptostylis*.

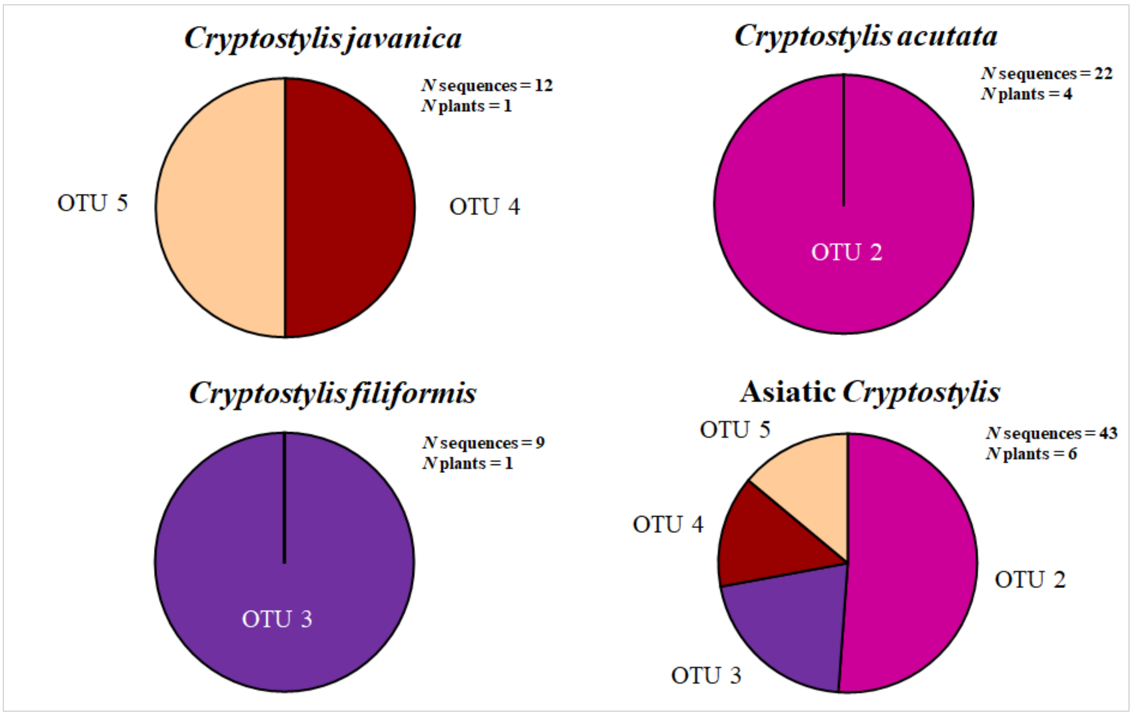

Figure S3. Frequency distribution of the observed *Tulasnella* operational taxonomic units (OTUs) among three Asiatic *Cryptostylis*, as well as in all Asiatic *Cryptostylis* species combined.

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CHAPTER TWO

Evolutionary shifts in host association among Australian orchids: *Tulasnella* fungi associated with Drakaeinae and Cryptostylidinae orchids

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Evolutionary shifts in host association among Australian orchids: *Tulasnella* fungi associated with Drakaeinae and Cryptostylidinae orchids

ABSTRACT

The study of congruency between phylogenies of interacting species can provide evidence of an ongoing evolutionary association. Orchid mycorrhizal fungi can grow and survive independently of orchids, thus testing for an evolutionary pattern between the interacting species will not reveal coevolution, but evolutionary interactions such as phylogenetic niche conservatism, adaptation and host shifts may be identified. The Australian orchid subtribe Drakaeinae is an iconic group of sexually-deceptive orchids that consists of approximately 66 species. In this study, we investigated the evolutionary relationships between all six Drakaeinae orchid genera (39 species) and their mycorrhizal fungi. Furthermore, we also included five Australian and three Asiatic species of *Cryptostylis*, which belong to the orchid subtribe Cryptostylidinae. A total of 20 closely related *Tulasnella* Operational Taxonomic Units (OTUs) or already described species, were isolated from the Drakaeinae and Cryptostylidinae orchids. Four of them were shared between the two orchid subtribes, with each genus associating with 2-9 *Tulasnella* lineages. Cophylogenetic analyses show Drakaeinae orchids and their *Tulasnella* associates have a similar phylogenetic tree topology, i.e. are congruent ($P < 0.0001$). However, for the orchid genera *Drakaea* and *Chiloglottis* multiple species were analysed, all associating with one or two *Tulasnella* species only. To remove a potential effect of pseudoreplication, only one species of each of these genera were included in the analyses. However, a significant cophylogenetic signal ($P = 0.0001$) was still observed. No significant congruency between Cryptostylidinae and fungus phylogenetic trees ($P = 0.61$) was found. For the Drakaeinae-*Tulasnella* interaction, a pattern of phylogenetic niche conservatism rather than coevolution likely lead to the observed phylogenetic congruency in orchid and fungal phylogenies. Rapid diversification in *Chiloglottis* and *Drakaea* but not *Cryptostylis*, may explain the discrepancy in congruency of orchid-*Tulasnella* interactions.

Keywords: Drakaeinae, *Cryptostylis*, cophylogeny, orchid mycorrhizal fungi (OMF), sexually-deceptive orchids, *Tulasnella*.

INTRODUCTION

An integral question in orchid-mycorrhizal research is whether there is a genetic basis for these interactions. In plant-pathogen associations, the interaction is controlled by genetic factors, with closely related pathogens often showing an evolutionary sharing of effectors (fungal proteins involved in the interaction) (Dodds & Rathjen, 2010). Although the genetic basis for orchid-mycorrhizal interactions is poorly understood (but see papers showing upregulation of plant defense genes when interacting with orchids (Fochi et al., 2017b; Perotto et al., 2014)), it is expected that closely related orchids should also associate with closely related mycorrhizal fungi. Here we test this hypothesis by examining cophylogenetic structure of orchid mycorrhizal associations in two orchid subtribes, the Drakaeinae and Cryptostylidinae.

Coevolution is “an evolutionary change in a trait of the individuals in one species in response to a trait of the individuals of a second species, followed by an evolutionary response by the second species to the change in the first” (Janzen, 1980). The interaction between hosts and parasites often shows the dependency of parasites to their host and indicates coevolution when both host and parasite phylogenetic trees shows a degree of phylogenetic similarity or congruency (Brooks, 1988a; Legendre et al., 2002a, 2002b; Vermeij, 1994). Coevolutionary associations are not restricted to those where there one is a detrimental (i.e. parasitic/pathogenic) interaction, but is also possible in beneficial (mutualistic) relationships between hosts (e.g. plants and algae) and symbionts (e.g. fungi and pollinators) (Adams & Nason, 2018; Singh et al., 2017a).

In coevolution, there are several evolutionary events that may occur; cospeciation (host and symbiont speciate simultaneously), duplication (the symbionts diverge independently and both new symbionts remain on the same host), host-switch (a duplication event where one of the new symbionts moves to associate with a different host), loss (the symbiont is extinct or does not speciate when the host does) and failure to diverge (symbiont associates with both diverged hosts) (Charleston, 1998; Conow et al., 2010). A cophylogenetic study is a comparative analysis that maps the evolutionary relationships between host and symbiont interactions, and is often visualised by a tanglegram consisting of both phylogenies (Avino et al., 2019; Page, 1993).

Several cophylogenetic studies such as those on plant hosts and fungal pathogens (Navaud et al., 2018), bacterial hosts and phages (Pratama et al., 2018), bats and coronaviruses (Joffrin et al., 2020), mammals and their zoonotic bacteria (Lei & Olival, 2014; McKee et al., 2019), as well as avian *Plasmodium* and African sunbirds (Lauron et al., 2015) showed coevolutionary relationships between the parasites/pathogens and their hosts. Coevolution was also shown in mutualistic interactions e.g. between fungi and algae (Singh et al., 2017a), ectomycorrhizal fungi and their hosts (Kretzer et al., 1996; Murata et al., 2013) and between arbuscular mycorrhizal fungi and their host plants (Reinhart & Anacker, 2014). Only a limited number of studies have investigated cophylogenetic evolution/network architecture between orchids and their mycorrhizal fungi, e.g. for *Dendrobium* (Xing et al., 2017) and *Orchis* (Jacquemyn et al., 2015; Jacquemyn et al., 2011a) closely related orchids tend to associate with closely related fungi. Furthermore, (Martos et al., 2012) compared mycorrhizal networks from terrestrial and epiphytic tropical orchids and showed that epiphytic orchids tend to associate with more closely related fungal partners compared to their terrestrial orchid counterparts. However, little is known about whether this pattern arises from coevolution between orchids and their mycorrhizal symbionts, or if the orchids adapt to the fungi without imposing strong selection on the fungi.

This ecological interaction among similar or closely related partners is called phylogenetic conservatism and is not only found in mutualistic and antagonistic relationships (Gomez et al., 2010). Mutualistic relationships between orchids and their mycorrhizal fungi is particularly interesting because the orchid is completely dependent on the fungus for seed germination, with the interaction maintained into orchid adulthood, although the plants may not be completely dependent at adulthood (Rasmussen et al., 2015; Rasmussen & Rasmussen, 2009). In contrast, the orchid mycorrhizal fungi (OMF) can exist independently of the orchids on dead organic material as saprophytes (Cruz et al., 2014; Rasmussen & Rasmussen, 2009). Therefore, the orchids obtain more benefit than the fungi in the mutualistic relationship (Alexander & Hadley, 1985; Fochi et al., 2017a), perhaps leading to a “skewed” evolutionary relationship. This condition is slightly different to other mycorrhizal counterparts such as arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EM), which in most cases are dependent on live hosts (Imhof, 2009).

Cryptostylidinae (tribe Diurideae) consists of a monotypic genus *Coilochilus* as well as *Cryptostylis*. The genus *Cryptostylis* can be found in Southeast Asia, Hongkong, India, Pacific, Australia and New Zealand (Jones, 2006; Pridgeon et al., 2001) with approximately 23 recognised species (KEW Plant Checklist 2019). Five species are native in Australia; *Cryptostylis subulata, C. erecta, C. hunteriana,* and *C. leptochila* occur sympatrically in eastern Australia, and *C. ovata* occurs in south-west Western Australia (Brundrett, 2014; Jones, 2006). Unusually for sexually deceptive orchids, the five Australian *Cryptostylis* species all share the same sexually-deceived pollinator, the ichneumonid wasp *Lissopimpla excelsa* (Coleman, 1927, 1929, 1930a, 1930b). Non-Australian *Cryptostylis* species are confined to Asia. Compared to Australian *Cryptostylis* which grow at relatively low altitudes and in sandy soils, Asiatic species grow in mountainous regions, from 400 metres a.s.l to 1,200 metres a.s.l and the preferable habitat is moist humus in deep forest (Cheng, 1979; Pridgeon et al., 2001; Wood et al., 1993).

Drakaeinae (tribe Diurideae) is a group of terrestrial orchids that consists of approximately 66 species. The members of this group are *Arthrochilus, Caleana, Chiloglottis, Drakaea*, *Paracaleana*, *Thynninorchis* and *Spiculaea.* Despite the number of species recorded in Drakaeinae, it is not being considered particularly species rich compared to other Australian orchid tribes e.g. Caladeniinae that consists of more than 300 species (Clements et al., 2015). Drakaeinae orchids are not restricted to Australia, but can also be found in New Zealand and Papua New Guinea (Jones, 2006; Pridgeon et al., 2001). They can grow in various habitats; including woodlands, open forests, heathlands, savannah, as well as in moist subalpine areas (Backhouse, 2019; Brundrett, 2014; Jones, 2006; Pridgeon et al., 2001). All Drakaeinae orchids are sexually deceptive and include well studied genera with regards to their pollinators. Typically, the orchids in these genera are specialized one or a few pollinator species (Bower & Brown, 2009; Mant et al., 2005b; Peakall et al., 2010), with some cases of pollinator sharing (Mant et al., 2005a; Phillips et al., 2014). However, there is no evidence for coevolution between orchids and pollinators - because the pollinators are not dependent on the orchids (Mant et al., 2005a). Phylogenetic analysis of pollinators and their orchid hosts (*Caladenia*, *Chiloglottis* and *Drakaea*) showed that closely related orchids tend to have closely related pollinators (Mant et al., 2002; Phillips et al., 2017), indicating that ecological and evolutionary effects play an important role for their interactions. Similarly, orchid - fungus interactions may be crucial in shaping their evolutionary dynamics in a phylogenetic context.

Most orchids are dependent on OMF for continuing their life cycle (Rasmussen, 1995; Rasmussen & Rasmussen, 2009), and there are three main fungal groups that associate with photosynthetic orchids: Ceratobasidiaceae, Tulasnellaceae and Serendipitaceae (Warcup, 1971; Warcup & Talbot, 1967, 1971, 1980). *Tulasnella* can be found worldwide and many orchids associate with *Tulasnella* as their symbionts (Jacquemyn et al., 2012;

Oberwinkler et al., 2017; Suarez et al., 2006; Yukawa et al., 2009). In Australia, the orchids that associate with Tulasnellaceae symbionts include e.g. *Dendrobium* (Warcup & Talbot, 1967; Xing et al., 2017), *Diuris* (Tedersoo et al., 2010), *Cryptostylis* (Nguyen et al., 2019 and Chapter 1) and *Thelymitra* (Reiter et al., 2018)), as well as Drakaeinae orchids (e.g. *Chiloglottis* (Roche et al., 2010; Ruibal et al., 2017), *Drakaea* (Phillips et al., 2011), *Arthrochilus, Caleana* and *Paracaleana* (Linde et al., 2017; Linde et al., 2014).

This study investigates the orchid-fungal interactions among the Drakaeinae and Cryptostylidinae orchids, providing us with information on fungal specificity, evolutionary shifts and phylogenetic conservatism for two orchid subtribes. In this study we tested the hypotheses that; (i) mycorrhizal fungi are shared among Drakaeinae and Cryptostylidinae; and (ii) closely related orchids tend to associate with closely related OMF. Here we use cophylogenetic studies of fungal symbionts in Drakaeinae and Cryptostylidinae. These two subtribes are distantly related (Figure S7, Weston et al., 2014) and provide useful evolutionary comparisons since Drakaeinae is a diverse species-rich subtribe consisting of six genera, whereas Cryptostylidinae only has two genera, of which we analyse only one. Drakaeinae and Cryptostylidinae also provide many examples of orchids living in various habitats and environments, have a wide geographical distribution in Australia as well as the unique relationships with pollinators as sexually deceptive orchids. As such, we have an extensive dataset using molecular phylogenetic approaches to test whether the evolutionary interaction between mycorrhizal fungi and orchid hosts represent topological congruency since fungi can grow independently from the orchids.

MATERIALS AND METHODS

Study species and orchid sampling

To determine the phylogenetic congruency between OMF and Drakaeinae as well as Cryptostylidinae orchids, it is essential to represent most of the orchid genera in these subtribes. Each genus may have different diversification rate that contributes to the congruency. Fungal symbiont sequences of Australian and Asiatic Cryptostylidinae were obtained from Chapter 1 as well as some sequences from Nguyen et al. (2019). Drakaeinae orchids were collected across Australia for OMF isolation. *Tulasnella* symbionts of some species of Drakaeinae from previous studies were included in this study; *Chiloglottis* (Roche et al., 2010; Ruibal et al., 2017), *Drakaea* (Phillips et al., 2011), *Caleana, Paracaleana* and a species of *Arthrochilus* (Linde et al., 2014). Additionally, the species from the monotypic *Spiculea*, as well as eight species from *Arthrochilus,* one *Caleana* and two *Paracaleana* species not previously studied, were sampled (Table 1). Note, the latest orchid nomenclature assigns *Caleana* and *Paracaleana* to separate genera (Hopper & Brown, 2019). *Arthrochilus irritabilis, A. prolixus* and *A. huntianus* were sampled in eastern Australia, whereas *A. latipes* was obtained from Kakadu National Park (Northern Territory). *Arthrochilus* species (*A. oreophilus, A. corinnae, A. rosulatus, A. dockrillii* and *A. stenophyllus*) were sampled from Queensland. At least 2-5 plants per population, and up to two population(s) were sampled for each analysed species (see Table 1 for details). In most cases, it was not possible to collect two populations per orchid species. Sampling was conducted during their flowering time to identify the orchid species, as without flowers it is sometimes impossible to distinguish among sympatric orchid species.

Fungal isolations

Fungal isolations and fungal ITS sequencing were conducted for all collected orchid samples. To increase the chance of obtaining culturable OMF, two types of growth media were used, half-strength Fungal Isolation Media (½ FIM) (Clements & Ellyard, 1979) and a Modified Melin-Norkrans medium/3MN (Wright et al., 2010) + A-Z vitamin (Centrum "Balanced Formula", Wyeth Consumer Healthcare, Baulkham Hills, NSW, Australia), both supplemented with 50 mg/mL streptomycin sulfate. Root pieces were washed under running tap water, then dissected in sterile water to locate the presence of pelotons. To reduce contamination, the root epidermis was peeled away as much as possible around peloton rich areas. Pelotons or peloton-rich tissues were serially rinsed in three changes of sterile distilled water before plating onto ½ FIM and/or 3MN + A-Z and plates were incubated at 22 ^{o}C in the dark. Any growth of *Rhizoctonia*-like fungi were transferred onto new ½ FIM plates after approximately one week. For each individual plant sampled, a 10-mm-long peloton-rich root fragment was stored at -20 °C for DNA extraction and direct sequencing.

Fungal DNA extraction, PCR amplification and sequencing

To obtain a fungal mycelium from cultures for DNA extraction, small pieces of agar blocks were cut from cultures and macerated following methods described in Linde et al. (2017), before inoculation into liquid ½ FIM and incubated at 22 °C in the dark. After 4 weeks, mycelium was harvested and lyophilized, then stored at -20 °C prior to DNA extraction. Fungal genomic DNA was extracted from lyophilized mycelium using Qiagen DNeasy Plant Mini Kit (Qiagen, Germany), following the manufacturer's protocol. In some cases when the fungal isolates did not grow in liquid ½ FIM medium, genomic DNA was extracted directly from mycelium growing on agar plates after treatment with QG buffer (Qiagen, Germany) to remove the agar medium. Genomic DNA was also isolated from lyophilized, orchid peloton-rich root tissues using the Qiagen DNeasy Plant Mini Kit, to identify possible unculturable fungal species.

PCR amplification of the Internal Spacer Region (ITS) was conducted for all fungal isolates using the ITS5 and ITS4 primer combinations (Gardes & Bruns, 1993; White et al., 1990). Initial fungal isolation and identification, as well as previous studies on Drakaeinae orchids, showed *Tulasnella* fungi as the main fungal partner. Therefore, we used *Tulasnella* specific primers (ITS5 and ITS4-Tul) to amplify the clone libraries of mycorrhizal fungi in Drakaeinae orchid roots (Taylor & McCormick, 2008). PCR conditions for ITS amplification were as follows: 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 55 s. The final cycle was followed by 72 °C for 7 min. The PCR reaction mix contained 3-5 µL of 10-20 ng of fungal DNA as template, 5 µL of 5X polymerization buffer, 1 µL each of ITS primer (10 mM), 12.85 µL sterile H_2O, and 0.15 µL of MyTaq Polymerase (Bioline, USA). Amplified products were visualized using electrophoresis on 1.2 % agarose gels for 30 minutes at 110 V and purified using Wizard® SV Gel and PCR Clean-Up system (Promega, USA). Purified PCR products were cloned using a pCR4-TOPO cloning vector (ThermoFisher, USA) and transformed using competent *Escherichia coli* DH10B. Five to eight white colonies from each sample were picked and diluted in 100 µL nuclease-free water as M13 PCR templates. Colony PCR of clone libraries was performed using 2 µL template and M13F-M13R primers in 20 µL reactions. Clone libraries were purified using EXO-CIP (New England Biolabs, USA), followed by ethanol precipitation following BigDye Terminator v3.1 Sequencing Kit protocols (Applied Biosystems, Foster City, California, USA). Sequencing was performed bidirectionally using M13 forward and reverse primers (for cloning from orchid roots) as well as ITS5 and ITS4 (for fungal isolates) using an ABI PRISM BigDye Terminator v3.1 sequencing kit (Applied Biosystems) on an ABI-3130xl automated sequencer.

Orchid DNA extraction, PCR amplification and sequencing

To generate a phylogenetic tree of the Drakaeinae and Cryptostylidinae orchids, leaf tissue of the sampled orchids were collected for DNA extraction. Genomic DNA was extracted from leaves using a DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. All of the analysed orchid species except *Arthrochilus* were sequenced using an exome-capture approach by co-workers in the Linde lab (Peakall et al., 2020 *in prep*). For this dataset, 200 single-copy orthologous genes (196,180 bp in total) developed for the Drakaeinae orchids, was used following the methodology of Peakall et. al., (2020 *in prep*). For the *Cryptostylis* species, 211 single-copy orthologous genes (186,827 bp) identified in Deng et al. (2015) was used following the methodology of Peakall et. al., (2020 *in prep*). All loci were manually checked in GENEIOUS 10.2.6 (Kearse et al., 2012). A phylogenetic tree for each subtribe was inferred using IQ-TREE 2.0 (Nguyen et al., 2014) after testing for compositional heterogeneity of each locus and concatenation of all loci (Linde et al., 2020 *in prep*). The best-fit substitution model was automatically selected by ModelFinder according to the Bayesian Information Criterion (Kalyaanamoorthy et al., 2017). Branch support were obtained with the built-in ultrafast bootstrap algorithm (Hoang et al., 2017) from 10,000 iterations. The phylogeny was visualised and rooted to *Pyrorchis* for the Drakaeinae, and midpoint rooted for the Cryptostylidinae in FIGTREE 1.4.3 (Rambaut, 2016). For the Drakaeinae orchid species that were not included in the exome-capture approach (*Arthrochilus* species except *A. oreophilus* and *A. huntianus*), ITS and *TrnL-F* sequences of all Drakaeinae orchids were either obtained from GenBank as generated in previous studies (Kores et al., 2001; Miller & Clements, 2014), or were sequenced in this study. The *TrnL-F* region from orchid genomic DNA was amplified following the method of Taberlet et al. (1991) and ITS region was obtained using universal ITS primers ITS1 and ITS4 (White et al., 1990). Orchid ITS and *TrnL-F* PCR products were purified using EXO-CIP (New England Biolabs, USA), followed by ethanol precipitation following BigDye Terminator v3.1 Sequencing Kit protocols (Applied Biosystems, Foster City, California, USA). Sequencing was conducted bidirectionally using ITS4 and ITS1 (for ITS) and two *TrnL-F* primers c and f on an ABI-3130xl automated sequencer. Any base ambiguities were edited using SEQUENCHER 5.4.1 (Gene Codes Corp.). The sequences for each locus were aligned using GENEIOUS 10.2.6 (Kearse et al., 2012) and concatenated with the exome-capture data set before phylogenetic tree inference in IQ-TREE 2.0 (Nguyen et al., 2014).

Fungal phylogenetic construction

Fungal sequences were edited manually for any base ambiguities using SEQUENCHER 5.4.1 (Gene Codes Corp.). BLAST searches using consensus sequences were performed to obtain the closest representative species from GenBank (http://www.ncbi.nlm.nih.gov/BLAST). Based on the BLAST result of each *Tulasnella* sequences, we separated phylogenetic trees constructions based on their phylogenetic *Tulasnella* group (Cruz et al., 2014). The top BLAST hits from GenBank were downloaded and included in a multiple sequence alignment. Multiple sequence alignments of *Tulasnella* were constructed using GENEIOUS 10.2.6 (Kearse et al., 2012) and adjusted manually. Because some *Tulasnella* spp. were shared between Cryptostylidinae and Drakaeinae orchids, a phylogenetic tree of all *Tulasnella* sequences were constructed to assign OTUs. Phylogenetic trees were constructed using maximum likelihood (ML) RAxML analysis (Stamatakis et al., 2008) with 1000 bootstrap replicates. Furthermore, Bayesian inference analyses were conducted to construct phylogenetic trees, with 10 % burn-in, 1,200,000 generations, Markov chain sampled every 120 generations with four chains, using GTR+G model (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) as implemented in GENEIOUS 10.2.6 (Kearse et al., 2012). *Tulasnella* phylogenetic trees were visualised in FIGTREE 1.4.3 (Rambaut, 2016) and midpoint rooted. We used a 4-6 % threshold to delimit *Tulasnella* based on Linde et al. (2017).

Cophylogenetic analyses

A cophylogenetic analysis was performed to test whether host and fungal phylogenies are congruent. Two methods were used in the cophylogenetic analyses, a global-fit method using Procrustean Approach to Cophylogeny / PACo (Balbuena et al., 2013) which was conducted in R version 3.5.1 (R Core Team, 2019) and an event-based method, JANE 4.0 (Conow et al., 2010). The Global-fit method determines the degree of congruence between two phylogenies of interacting species and detects the particular interactions that contribute most to their concordance (Balbuena et al., 2013; Desdevises, 2007; Legendre et al., 2002b). The level of cophylogenetic signal is calculated as the global goodness-of-fit statistic (m^2_{XY}) in the best-fit superimposition of two interacting phylogenies. The significance of the goodness-of-fit statistic is established by a randomization procedure. Null hypothesis (H_0) is when the host does not predict the symbiont ordination and the fungal clades are randomly associated to the host phylogeny. In contrast, the alternative hypothesis (H_1) is when the symbiont topology is congruent with the corresponding host topology (Balbuena et al., 2013). Furthermore, the contribution of a group of interactions that have stronger effect on the overall cophylogenetic signal can be measured based on comparing residuals of this interaction to those of remaining interactions (Welch's *t*-test). The agreement of the fit between orchid and *Tulasnella* phylogenies can be visualised with a Procrustes superimposition plot, which corresponds to patristic distances. Cophylogenetic analysis using PACo is suitable for orchid-fungi relationships because PACo can solve unresolved clades (Australian sexually deceptive orchids often cannot be phylogenetically resolved (Peakall et al., 2010), represent multiple host/symbiont interactions, and can have an unequal number of hosts and symbionts (Balbuena et al., 2013; Hutchinson et al., 2017b; Singh et al., 2017b).

We quantitatively assessed the relative contribution of each Drakaeinae or Cryptostylidinae orchid-*Tulasnella* association to the overall cophylogenetic congruence with PACo using the R package "PACO" (Hutchinson et al., 2017a), "VEGAN 2.5-3" (Oksanen et al., 2018) and "APE 5.2" (Paradis et al., 2004). For PACo analyses, both host and fungus phylogenetic trees in Nexus format were used as input data. Furthermore, a host-fungus interaction link matrix was prepared based on 'presence' (1) or 'absence' (0) of the interaction. Orchid and fungal network architecture were visualised as tanglegrams by PACo. Because all Drakaeinae and Cryptostylidinae orchids associate with *Tulasnella* phylogeny group IV (Cruz et al., 2014), the cophylogenetic analyses were only performed using *Tulasnella* group IV and the orchids they associated with in the subtribes Drakaeinae and Cryptostylidinae. To test whether "pseudoreplication" (multiple species within an orchid genus) of taxa within a genus affects cophylogenetic analyses between Drakaeinae orchids and *Tulasnella* fungi, we excluded all but one species of *Drakaea* and *Chiloglottis* and compared the result with the full set of taxa included.

In the event-based method, each event in the symbiont phylogeny is mapped onto the host phylogeny and a mapping is sought that minimizes the total cost with respect to a given cost metric (Conow et al., 2010). Mappings can examine sets of events that have the possibility to represent evolutionary events between two interacting phylogenies and statistical tests were used to assess the congruency (Conow et al., 2010). The Event-based method is useful to find the most probable evolutionary events responsible for congruency between phylogenies, by imposing some cost on each event. We used the default cost model (zero for cospeciation, one for duplication, two for duplication and host switch, one for symbiont losses, and one for failure to diverge). As data input, JANE 4.0 requires host and symbiont phylogenies in Nexus-based format showing the interactions between both phylogenies. The congruency between host and symbiont phylogenies is supported when the observed optimal cost is significantly lower than optimal costs computed from randomly generated trees.

RESULTS

Fungal symbionts and phylogenies

Overall, we successfully collected and sequenced 88 *Tulasnella* isolates and 89 clones from Drakaeinae orchids (Table 1). In total, 308 sequences were aligned to construct *Tulasnella* phylogenetic tree group IV (Figure 1, Table S1) — a combination of 86 *Tulasnella* ITS sequences from previous studies, 177 sequences of Drakaeinae orchids obtained in this study, 45 sequences of *Cryptostylis* orchids from Chapter One, Howard & Clements 2011 (unpublished), Nguyen et al. (2019). These *Tulasnella* OTUs/species are classified as *Tulasnella* phylogenetic group IV according to Cruz et al. (2014) and grouped into ten well-supported *Tulasnella* OTUs or previously described species (*T. prima*, *T. secunda*, *T. sphagneti* and *T. warcupii*). The OTUs K, L, M and P from Drakaeinae orchids are newly discovered in this study, whereas two OTUs have been reported previously; OTU N includes isolates from *A. oreophilus* (CLM084 and CLM085; GenBank accession numbers KF476594 and KF476595 respectively) and OTU A (from *A. oreophilus* CLM031, GenBank accession number KF476602) (Linde et al., 2014) (Figure 1). The OTUs B, C, D, E, F, G, H and I were obtained from previous research on Australian and Asiatic *Cryptostylis* orchids (Chapter One).

Fungal sharing among Drakaeinae and Cryptostylidinae orchids

All studied *Arthrochilus* species, except *A. corinnae*, share *T. warcupii* as their *Tulasnella* symbiont. *T. secunda* is shared among *Drakaea, Caleana* and *Paracaleana*. *T. prima* mainly associates with *Chiloglottis* and one *Paracaleana* species (Table 2). Four *Tulasnella* associated with *Drakaeinae* orchids —*T. prima*, *T. sphagneti*, *T. warcupii* and OTU A, also associated with Australian *Cryptostylis*.

Orchid phylogeny

Cryptostylis species could be distinguished with bootstrap support of 100. Two main *Cryptostylis* clades were identified, the first clade consisting of Australian species only (*C. hunteriana*, *C. ovata* and *C. subulata*). The second clade consists of two separate diversifications, one containing Australian species (*C. leptochila* and *C. erecta*) and the other containing Asiatic species (Figure S1).

All Drakaeinae genera were well supported with bootstrap support of 100. *Arthrochilus* contains a deep phylogenetic divergence with three main clades. *Spiculaea ciliata* is a monotypic species that is sister to *Arthrochilus*. *Caleana major* is the only species in this genus after being reinstated as separate from *Paracaleana* by Hopper and Brown (2019). *Paracaleana* sampled in this study consists of five species clustering together but *P. minor* and *P. lyonsii* form a separate clade. *Drakaea* and *Chiloglottis* represents two genera where all species have short branches and are not well supported with low bootstrap values – indicative of a rapid species diversification (Figure S2).

Cophylogenetic analyses

The cophylogenetic analyses for distance-based (PACo) and event-based methods (JANE 4.0) showed significant phylogenetic congruence between Drakaeinae orchid hosts and their *Tulasnella* fungi, with significant signal between the interacting phylogenies; m^2_{XY} = 42,673.91, $P < 0.001$. The orchid genus *Chiloglottis* showed the largest contribution to phylogenetic congruence within the subtribe (thickest lines in tanglegram of Figure 2) based on comparing residuals of the *Tulasnella-Chiloglottis* interaction to those of the remaining interactions (Welch's *t*-test; $t = -6.9$, $P = 7.41\text{e-}08$, Figure 3) and Procrustes superimposition plot (Figure 4). Here, we rejected the null hypothesis of random interactions between orchids and *Tulasnella* as both phylogenies can be considered phylogenetically congruent. The interaction of *Tulasnella-Drakaea* compared to those remaining interactions is less strong, but still significant (Welch's *t*-test; $t = -2.6$, $P = 0.01$, Figure S3), whereas the interaction of and *Tulasnella-Arthrochilus* was also significant (Welch's *t*-test; $t = 3.63$, $P = 0.003$) but showed larger Procrustes residuals indicating a less strong interaction. In contrast, the *Tulasnella-Paracaleana* interaction did not significantly contribute to the phylogenetic congruence between *Tulasnella* and Drakaeinae orchids (Welch's *t*-test; $t = 1.14$, $P = 0.27$).

To test whether "pseudoreplication" affect the cophylogenetic signal, all but one species in *Chiloglottis* and *Drakaea* were excluded. Cophylogenetic analyses with PACo showed that without "pseudoreplication", a significant cophylogenetic signal is still present; $m^2_{XY} = 31{,}616.4$, $P = 0.001$ (Figure S4).

Cophylogenetic analyses using an event-based method can reveal the most probable evolutionary events by imposing some cost on each evolutionary event. In line with the global-fit method, this result showed significant congruency between Drakaeinae and *Tulasnella* as the observed minimum total cost = 78, $P < 0.001$. This is significantly lower than optimal costs computed from randomly generated trees (mean 189.23 ± (SD) 8.62); cospeciations = 0, duplications = 2, host switches = 7, losses = 24 and failure to diverge = 38), Figure 5 & S5).

In contrast to the Drakaeinae orchids, cophylogenetic analyses of the *Cryptostylis-Tulasnella* relationships did not show significantly congruent phylogenies (Figure 6) with the PACo global value $m^2_{XY} = 0.01$ ($P = 0.61$). Although the interaction of *Tulasnella-C. subulata* and *C. ovata* indicate some support to a hypothesis of phylogenetic congruence compared with the rest of *Tulasnella-Cryptostylis* interactions (Welch's *t*-test; $t = -5.0$, $P = 0.0001$, Figure 7), the Procrustes residuals do not support the overall congruency between *Tulasnella* and Cryptostylidinae. The Procrustean superimposition plot shows no obvious residuals that support the congruency in *Tulasnella-Cryptostylis* interactions (Figure 8). The event-based method (JANE 4.0) also showed an incongruency as the observed minimum total cost = 43, $P = 0.36$ (Figure 9 & S6) is not significantly lower than optimal costs computed from randomly generated trees (mean 45.8 ± (SD) 4.46); cospeciations = 4, duplications = 9, host switches = 1, losses = 25 and failure to diverge = 7).

DISCUSSION

Are mycorrhizal fungi shared among Drakaeinae orchids?

Tulasnella from nine species of *Arthrochilus* are closely related and share *T. warcupii* among seven *Arthrochilus* species. *T. warcupii* is shared among *A. prolixus*, *A. rosulatus*, *A. oreophilus, A. stenophyllus, A. irritabilis, A. latipes* and *A. dockrillii,* which have a wide geographic distribution ranging from Kakadu National Park in the Northern Territory to Queensland and New South Wales. Despite overlap in geographic range of *A. huntianus* and *A. corinnae* with other *Arthrochilus,* they do not associate with *T. warcupii* but do associate with closely related *Tulasnella* from a separate clade (Figure 1). In addition, *Drakaea, Caleana* and *Paracaleana* share *T. secunda* (all nine *Drakaea* species, *C. major, P. minor, P. nigrita, P. disjuncta, P. lyonsii,* and *P. terminalis*). However, *P. nigrita* and *P. disjuncta* also associate with the seemingly widespread *T. warcupii*. Thus, *T. prima, T. secunda* and *T. warcupii* are shared among Drakaeinae.

Orchid specificity at the genus level

Previous studies showed that *Chiloglottis, Drakaea* and *Caleana* show a strict specificity with *Tulasnella* fungi. From Table 2, it is clear that *Chiloglottis* and *Drakaea* are highly specific with their fungal symbionts; which are two *Tulasnella* species for 18 *Chiloglottis* species and only one *Tulasnella* species for nine *Drakaea* species (Phillips et al., 2011; Roche et al., 2010; Ruibal et al., 2017). In this study, *Paracaleana* and *Arthrochilus* seem more generalized for their fungal partners with seven species of *Paracaleana* associating with three *Tulasnella* species and nine species of *Arthrochilus* associating with five *Tulasnella* species.

Do closely related orchids tend to associate with closely related fungi?

We found a significant cophylogenetic signal between Drakaeinae orchids and their *Tulasnella* associations using a global-fit method in PACo that indicates some degree of congruency between both phylogenies. The interactions between Drakaeinae orchids and *Tulasnella* fungi are measured by their contribution to the overall phylogenetic congruence, where thicker lines indicating a smaller residual distance or an interaction that has stronger support to the phylogenetic congruence. *Chiloglottis* contributes greater phylogenetic congruence (thickest lines in Figure 2) in Drakaeinae compared to the rest of the interactions (Figure 3). Furthermore, supported by a Procrustes superimposition plot (Figure 4), significant phylogenetic congruence at the whole Drakaeinae-*Tulasnella* network scale may largely be attributed to the interactions between *Chiloglottis* and their fungi.

Event-based analyses measuring the cost of coevolutionary events also show a significant congruency as the original total cost is significantly different compared to the distribution of costs in Drakaeinae-*Tulasnella* relationships. No cospeciation was observed in Drakaeinae-*Tulasnella* associations (total minimum event cost = 78; cospeciations = 0, duplications = 2, host switches = 7, losses = 24 and failure to diverge = 38) (Figure 5). Hence, significant congruency in Drakaeinae and their *Tulasnella* symbionts potentially occur because, according to the analyses, fungal lineages failed to diverge, but also due to a loss or extinction of a symbiont in a particular host, with host switches only contributing 7 to the total minimum event cost (Brooks, 1988b; de Vienne et al., 2007; Singh et al., 2017b). The failure to diverge in *Tulasnella* does not necessarily mean the fungi did not speciate, but rather that *Chiloglottis* rapidly diverged (Mant et al., 2002; Peakall et al., 2010). Therefore, the orchid taxa diverged much faster than the fungus.

Replication of taxa ("pseudoreplication") may increase Type I error rates (Hommola et al., 2009). However, orchid genera such as *Chiloglottis* and *Drakaea* associate with only 1-2 *Tulasnella* species, therefore it is not surprising that when only one species of *Chiloglottis* and *Drakaea* are included to test the effect of "pseudoreplication" (Figure S4), the cophylogenetic signal is still significant and the Drakaeinae-*Tulasnella* phylogenies keep maintaining some degree of congruency (Figure S8). However, OMF can grow independently of the orchids and the congruency between phylogenies do not necessarily show any coevolutionary process. It suggests that rather than coevolution, the process of phylogenetic niche conservatism may drive the patterns where niches are more conserved or similar than expected (Crisp & Cook, 2012; Pyron et al., 2015). Considering the apparently rapid diversification of species in *Chiloglottis* (Mant et al., 2005b; Mant et al., 2002) and *Drakaea*, these orchid genera speciate rapidly in response to pollinators, thereby conserving their fungal association. Recently diverged orchids tend to use different pollinator species, which suggests that pollinators but not the fungi drive orchid speciation (Waterman et al., 2011). Rapid diversification in orchids are recognized by a lack of deep branching pattern as in *Chiloglottis* and *Drakaea* (Figure S2); that also occurs in a genus of sexually-deceptive orchid *Ophrys* (Breitkopf et al., 2015) and *Dendrobium* (Niu et al., 2018; Xiang et al., 2013).

We did not detect a significant cophylogenetic signal between Cryptostylidinae and their *Tulasnella* symbionts, suggesting that orchids and OMF symbionts are not phylogenetically congruent, although they share evolutionary events. In the Cryptostylidinae-*Tulasnella* system, loss events are more frequent, suggesting the possibilities of the orchids to recruit a new fungal symbiont when their original fungal partner is not available in a new distant habitat (Xing et al., 2019), or when the initial fungal symbiont were killed by the extreme conditions (e.g. dry weather and fire) (McCormick et al., 2006). No congruency between Cryptostylidinae and *Tulasnella* might also suggests that the diversification in Cryptostylidinae is much slower compared to Drakaeinae due to pollinator sharing in all Australian *Cryptostylis* species (Coleman, 1928, 1929, 1930a, 1930b), whereas in Drakaeinae rapid diversification is likely driven by specialisation in their pollinators. Specialised pollination niches potentially increase the plant diversification (Phillips et al., 2020).

Significant cophylogenetic signal in Drakaeinae orchids and *Tulasnella* also support previous studies in *Orchis* (Jacquemyn et al., 2011b) and *Dendrobium* (Xing et al., 2017) and their *Tulasnella* fungal associations. Both orchid genera showed a phylogenetically conserved interaction, where closely related orchid hosts interact with a similar set of fungal OTUs. However, *Dendrobium* and *Orchis* have much lower mycorrhizal specificity compared to Drakaeinae orchids. *Dendrobium* consists of high level of diversity and high diversification rates (Givnish et al., 2015; Niu et al., 2018; Xiang et al., 2013).

CONCLUSIONS

We compared the cophylogenetic patterns of two orchid subtribes in Australia, Drakaeinae and Cryptostylidinae. Phylogenies of Drakaeinae orchids and *Tulasnella* fungi show a significant degree of congruency. Phylogenetic congruency in Drakaeinae-*Tulasnella* suggests that phylogenetic niche conservatism rather than coevolution contributes towards the congruency between phylogenies. Cryptostylidinae-*Tulasnella* phylogenies do not show significant congruency, thus the slower diversification in *Cryptostylis* compared to *Chiloglottis* and *Drakaea* may have lead to an absence of phylogenetic niche conservatism patterns. Although our study is the largest to date on Australian orchids addressing cophylogeny (for subtribe Drakaeinae), a broader taxonomic sampling with broader geographical distribution and species especially in Cryptostylidinae will be required as a more complete orchid phylogeny will increase the accuracy of cophylogenetic patterns, thus providing a more comprehensive understanding of orchid-fungi evolutionary relationships.

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Table 1. The number of orchid plants analysed and the number of *Tulasnella* isolates and clones from Australian orchids of the subtribe Drakaeinae.

No	Host Taxon	Locality	State/Country	Plant identification number	Number of plants	Number of Isolates	Number of Clones	*Tulasnella* Group (Cruz et al. 2014)
1	*Arthrochilus irritabilis* F.Muell.	Noosa National Park	Queensland	CL033	5	5	6	IV
2	*Arthrochilus huntianus* F.Muell.	Lake Eucumbene	New South Wales	CL045	1	0	8	IV
3	*Arthrochilus oreophilus* D.L.Jones.	Barron Gorge National Park	Queensland	CL112	5	7	1	IV
4	*Arthrochilus latipes* D.L.Jones.	Kakadu National Park	Northern Territory	CL130	8	5	6	IV
5	*Arthrochilus prolixus* D.L.Jones.	Bulahdelah	New South Wales	CL152	2	2	5	IV
6	*Arthrochilus corinnae* D.L.Jones.	Bloomfield	Queensland	CL153	5	4	5	IV
7	*Arthrochilus rosulatus* D.L.Jones.	Shiptons Flat	Queensland	CL154	5	9	6	IV
8	*Arthrochilus dockrillii* Lavarack.	Kuranda National Park	Queensland	CL155	5	5	7	IV
9	*Arthrochilus dockrillii* Lavarack.	Shiptons Flat	Queensland	CL156	5	3	8	IV
10	*Arthrochilus stenophyllus* D.L.Jones.	Cardwell in Melaleuca Flats	Queensland	CL157	2	5	3	IV
11	*Arthrochilus oreophilus* D.L.Jones.	Atherton	Queensland	CL158	5	4	5	IV
12	*Paracaleana hortiorum* Hopper & A.P.Br.	Capel	Western Australia	CL171	3	0	2	IV
13	*Paracaleana terminalis* Hopper & A.P.Br.	Northampton	Western Australia	CL068	3	8	—	IV
14	*Paracaleana disjuncta* D.L.Jones.	Kendenup	Western Australia	CL075	2	0	4	IV
15	*Paracaleana nigrita* (Lindl.) Blaxell.	Jane National Park	Western Australia	CL103	4	4	9	IV
16	*Caleana major* R.Br.	Nowra	New South Wales	CL108	3	—	2	IV
17	*Spiculaea ciliata* Lindl.	East Brookton	Western Australia	CL038	4	27	12	IV
						88	89	

—: not attempted; 0: failed

Table 2. Comparisons of *Tulasnella* diversity of genera of the orchid subtribes Drakaeinae and Cryptostylidinae.

Orchid genera	Number of orchid species	Number of *Tulasnella* species/OTUs
Chiloglottis	18	2 (*T. prima* and *T. sphagneti*)
Drakaea	9	1 (*T. secunda*)
Caleana	1	2 (*T. prima* and *T. secunda*)
Paracaleana	7	3 (*T. prima*, *T. warcupii* and *T. secunda*)
Arthrochilus	9	5 (*T. warcupii*, OTU A, OTU M, OTU N, OTU P)
Spiculaea	1	1 (OTU K)
Cryptostylis	8	15 (obtained from Chapter One and Nguyen et al. 2019)

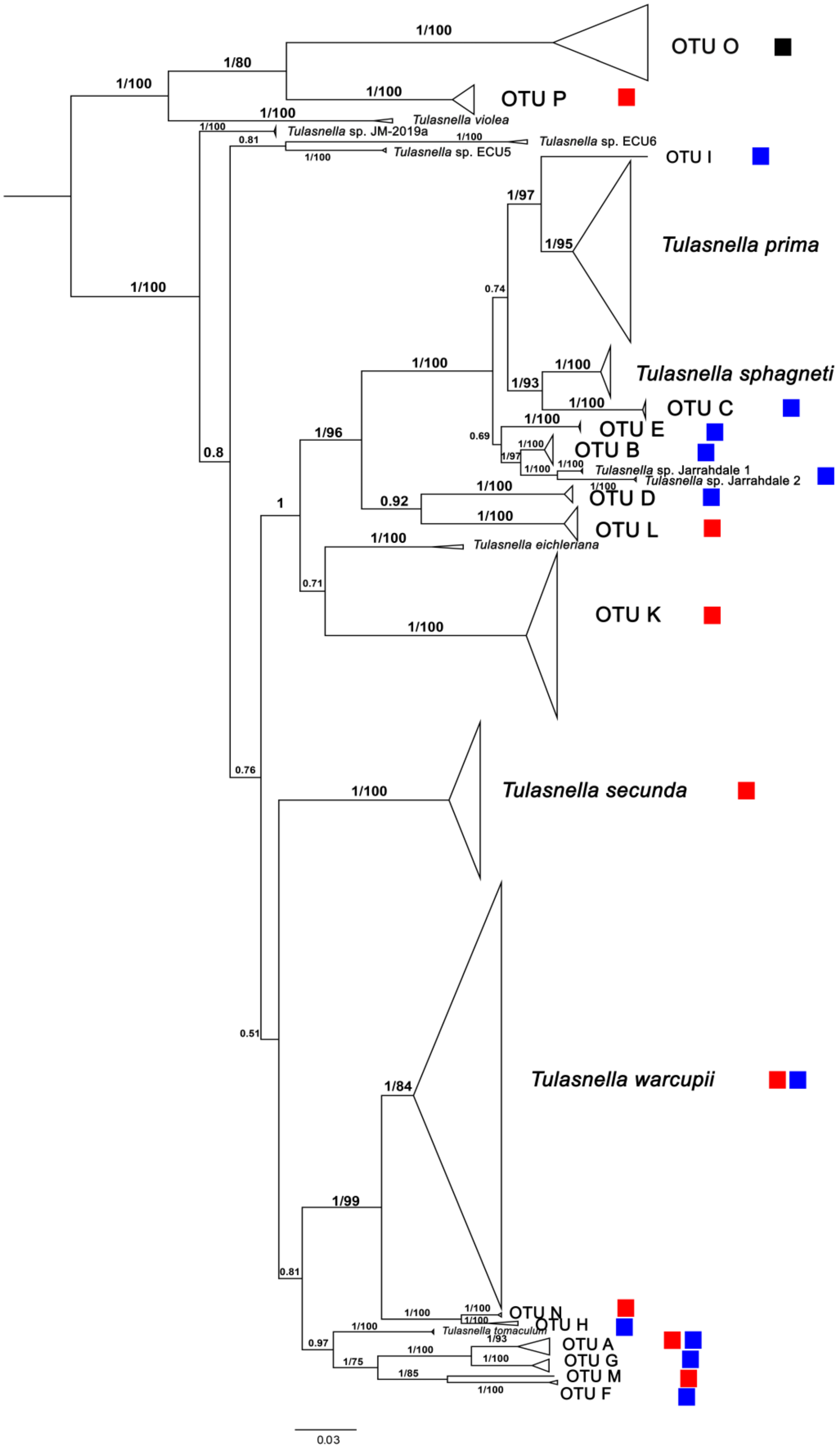
1/100
OTU O
1/100
1/80
1/100
OTU P
1/100
Tulasnella violea
1/100
Tulasnella sp. JM-2019a
0.81
1/100
Tulasnella sp. ECU6
1/100
Tulasnella sp. ECU5
OTU I
1/97
1/95
Tulasnella prima
1/100
0.74
1/100
Tulasnella sphagneti
1/93
1/100
OTU C
1/100
OTU E
0.69
1/100
OTU B
1/97
1/100
1/100
Tulasnella sp. Jarrahdale 1
Tulasnella sp. Jarrahdale 2
1/100
1/96
1/100
OTU D
0.92
1/100
OTU L
1
1/100
Tulasnella eichleriana
0.71
1/100
OTU K
0.8
0.76
1/100
Tulasnella secunda
0.51
1/84
Tulasnella warcupii
1/99
0.81
1/100
1/100
OTU N
1/100
OTU H
1/100
Tulasnella tomaculum
1/93
OTU A
0.97
1/100
1/100
OTU G
1/75
1/85
OTU M
1/100
OTU F
0.03

Figure 1. Midpoint rooted MrBayes phylogenetic tree for *Tulasnella* group IV associated with Drakaeinae (red box) and Cryptostylidinae (blue box) as well as Thelymitrinae (black box), obtained for the internal transcribed spacer (ITS). OTU: Operational Taxonomic Unit. The numbers above/under the branches are maximum likelihood bootstrap values/Bayesian posterior probabilities. Only Bootstrap values ≥ 70 are shown. Sequence details for each clade are shown in Table S1.

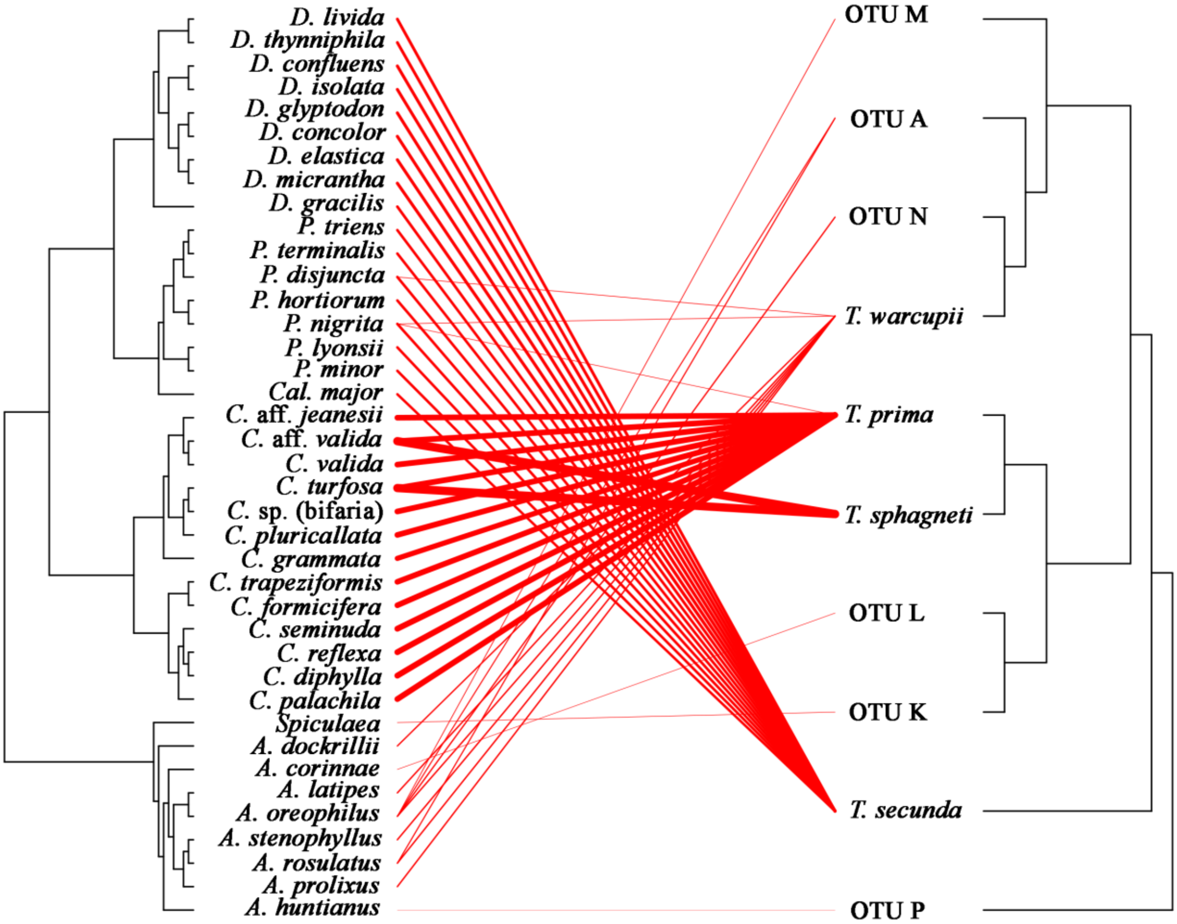

Figure 2. Interaction network and phylogenies of Drakaeinae orchids and *Tulasnella* fungi. The cophylogenetic analysis in PACo shows significant phylogenetic congruence (cophylogenetic signal $m^2_{XY} = 42{,}673.91$; $P < 0.001$) between them. The interactions are weighted by their contribution to the overall phylogenetic congruence. The thicker lines show smaller residual distance or interactions that show stronger support to the hypothesis of phylogenetic congruence. Orchid genera abbreviations – *D*: *Drakaea*; *P*: *Paracaleana*; *Cal*: *Caleana*; *C*: *Chiloglottis*; *A*: *Arthrochilus*. Fungal genus abbreviation – *T*: *Tulasnella*.

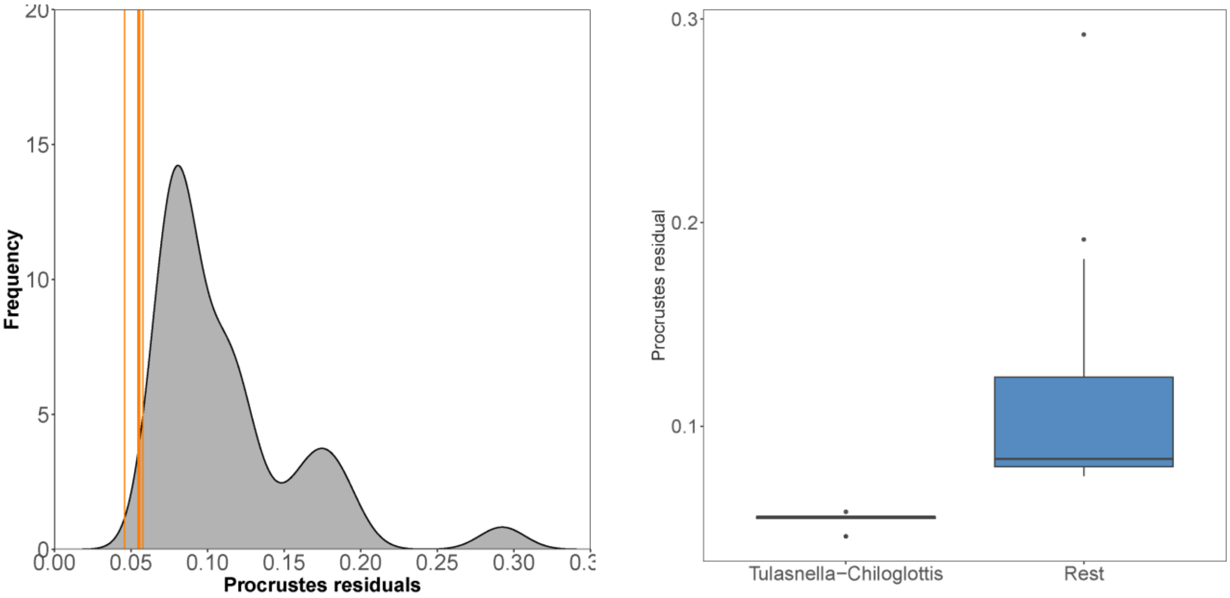

Figure 3. [Left]: The orange lines show the Procrustes residuals for the *Tulasnella*-*Chiloglottis* interactions that were strongly supported in Figure 2, and the distribution gives the values of the rest of the interactions in the Drakaeinae-*Tulasnella* network. [Right]: Comparison of the strongest interactions that are most phylogenetic congruent between *Tulasnella*-*Chiloglottis* compared to the rest of the network (Rest).

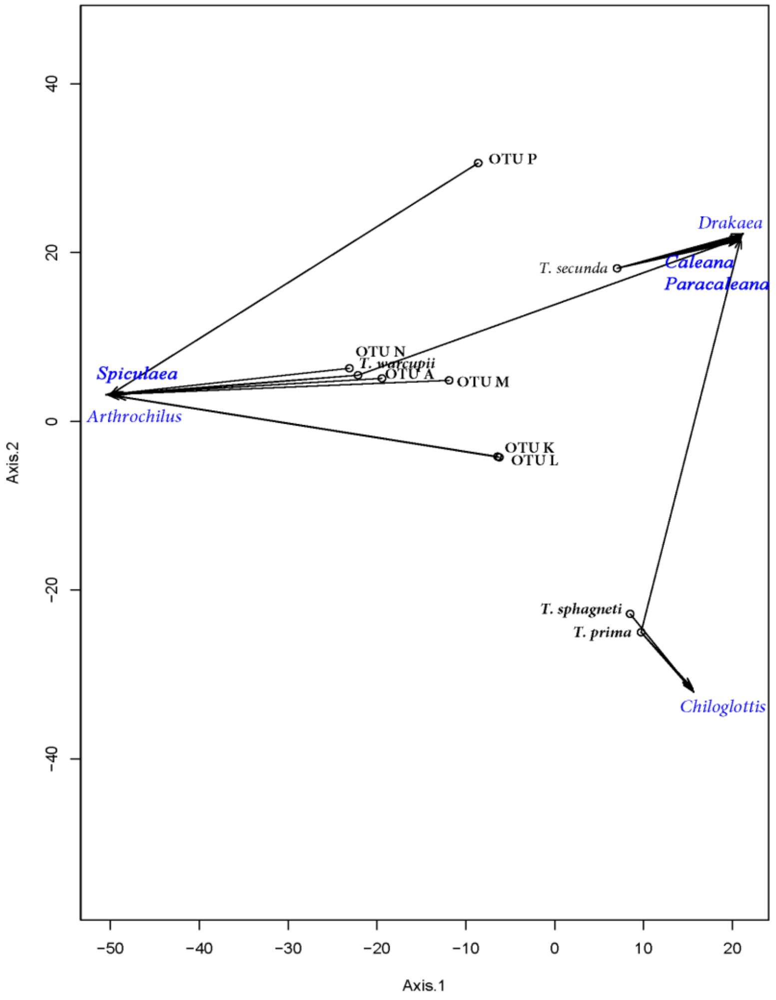

Figure 4. Procrustean superimposition plot of Drakaeinae orchids and their *Tulasnella* fungi. The starting points (dots) represent the configuration of *Tulasnella* symbionts and the arrowheads show the configuration of Drakaeinae hosts. Vector length represents the global-fit (residual sum of squares) which is inversely proportional for contribution to the congruency.

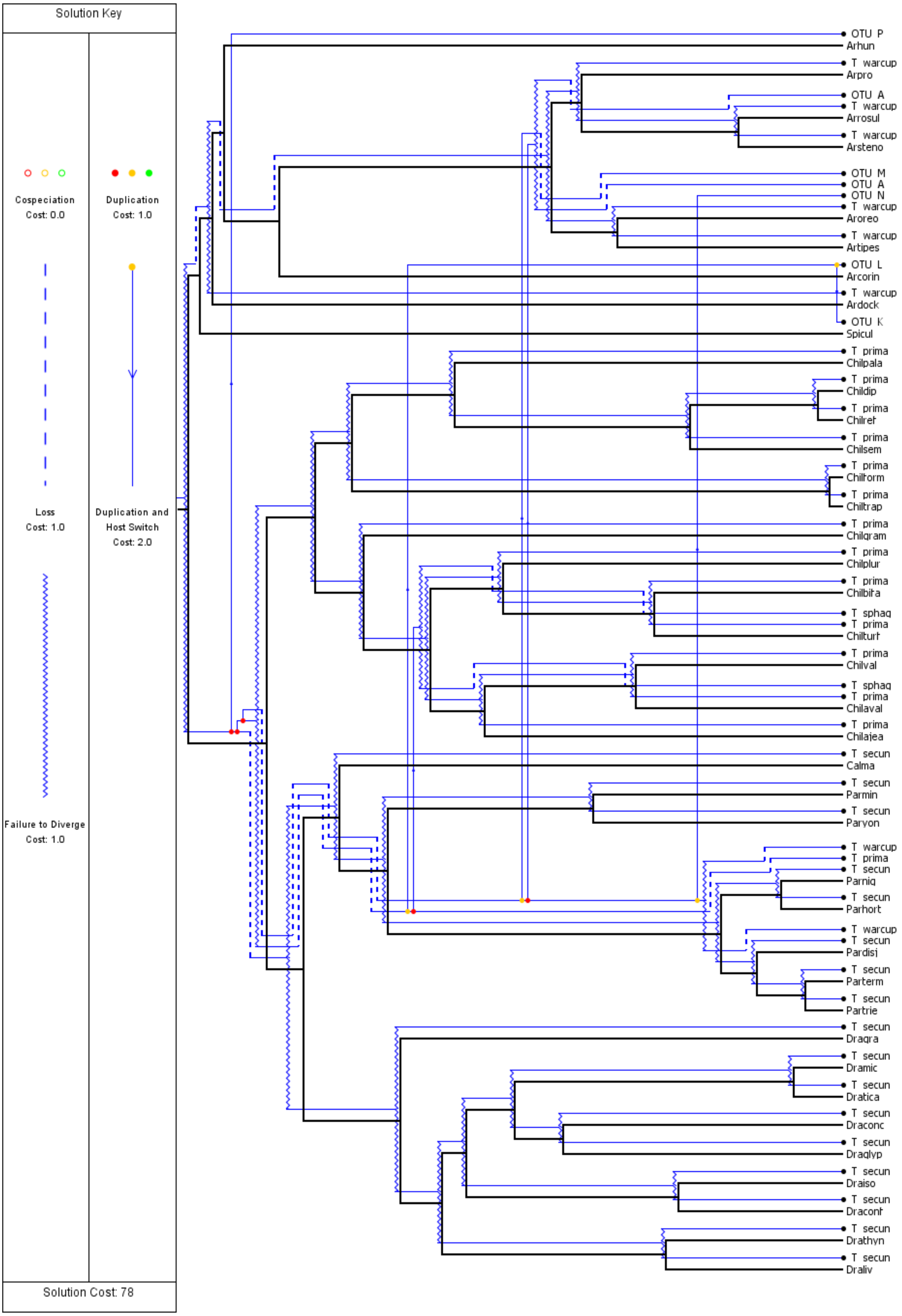
Solution Key
Cospeciation
Cost: 0.0
Duplication
Cost: 1.0
Loss
Cost: 1.0
Duplication and
Host Switch
Cost: 2.0
Failure to Diverge
Cost: 1.0
Solution Cost: 78
OTU P
Arhun
T warcup
Arpro
OTU A
T warcup
Arrosul
T warcup
Arsteno
OTU M
OTU A
OTU N
T warcup
Aroreo
T warcup
Artipes
OTU L
Arcorin
T warcup
Ardock
OTU K
Spicul
T prima
Chilpala
T prima
Childip
T prima
Chilret
T prima
Chilsem
T prima
Chilform
T prima
Chiltrap
T prima
Chilgram
T prima
Chilplur
T prima
Chilbita
T sphag
T prima
Chilturt
T prima
Chilval
T sphag
T prima
Chilaval
T prima
Chilajea
T secun
Calma
T secun
Parmin
T secun
Paryon
T warcup
T prima
T secun
Parniq
T secun
Parhort
T warcup
T secun
Pardisj
T secun
Parterm
T secun
Partrie
T secun
Dragra
T secun
Dramic
T secun
Dratica
T secun
Draconc
T secun
Draglyp
T secun
Draiso
T secun
Dracont
T secun
Drathyn
T secun
Draliv

Figure 5. Tanglegram showing the Drakaeinae (host) phylogenetic tree in black and the *Tulasnella* phylogenetic group IV in blue. The event-based method aims to find the most probable evolutionary events by imposing some cost on each event. Analysis was performed using JANE 4.0 with default cost settings based on Conow et al. (2010). observed minimum total cost = 78, P = 0 (cospeciations = 0, duplications = 2, host switches = 7, losses = 24 and failure to diverge = 38).

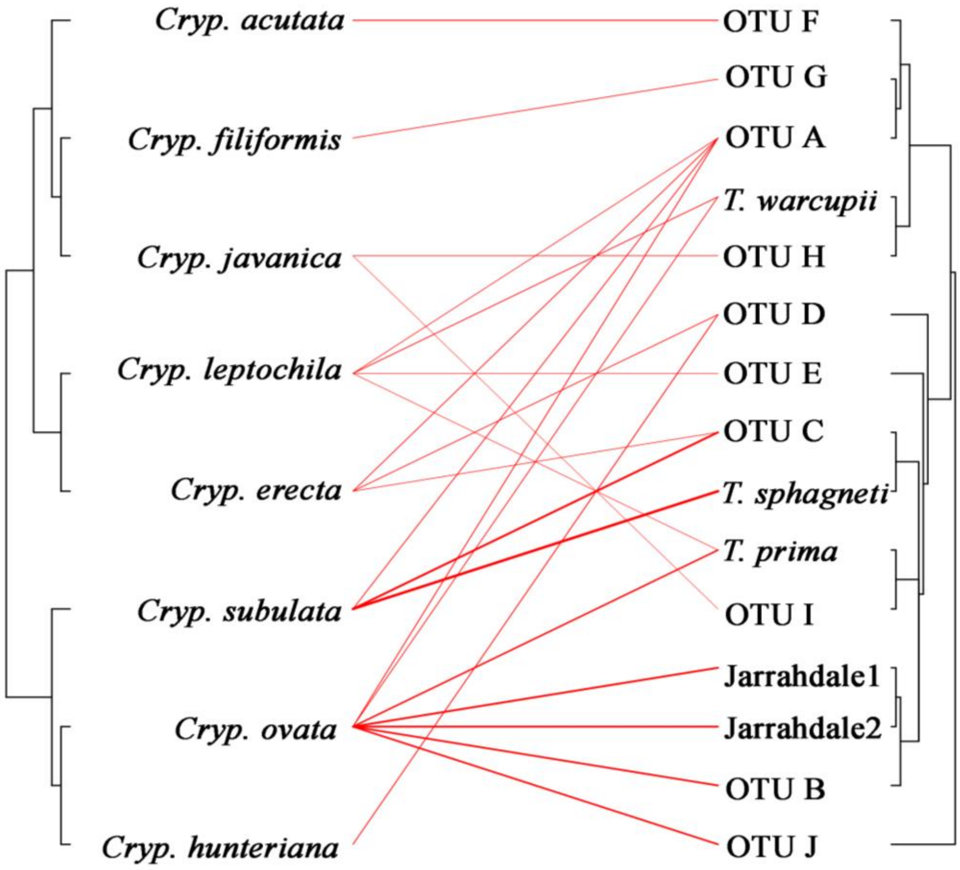

Figure 6. Interaction network and phylogenies of *Cryptostylis* orchids and *Tulasnella* fungi. The cophylogenetic analysis in PACo shows not significant phylogenetic congruence between them (cophylogenetic signal $m^2_{XY} = 0.01$; $P = 0.61$). The interactions are weighted (thicker lines = higher weight) by their contribution to the overall phylogenetic congruence.

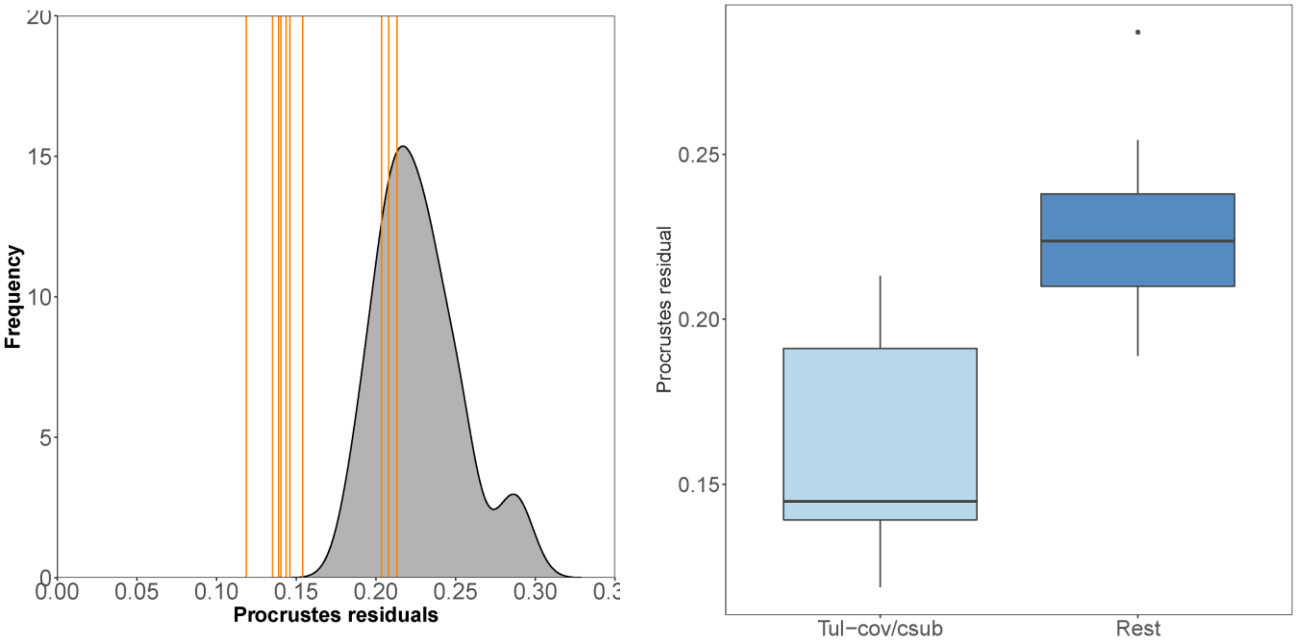

Figure 7. [Left]: The orange lines show the Procrustes residuals for the *Tulasnella*-*C. ovata/C. subulata* interactions that were strongly supported in Figure 6, and the distribution gives the values of the rest of the interactions in the *Cryptostylis*-*Tulasnella* network. [Right]: Comparison of the interactions between *Tulasnella*-*C. subulata* and *C. ovata* compared to the rest of the network (Rest).

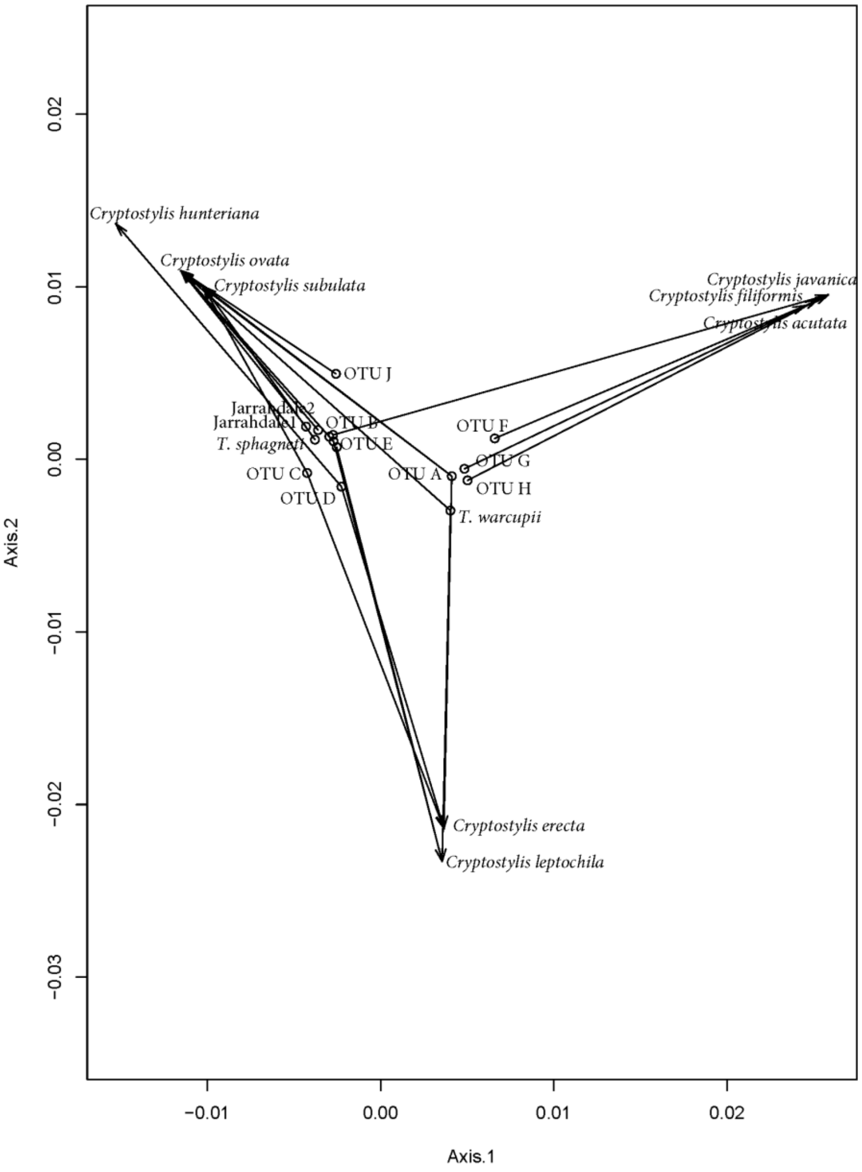

Figure 8. Procrustean superimposition plot of *Cryptostylis* orchids and their *Tulasnella* fungi. The starting points (dots) represent the configuration of *Tulasnella* symbionts and the arrowheads show the configuration of *Cryptostylis* hosts. Vector length represents the global-fit (residual sum of squares) which is inversely proportional for contribution to the congruency.

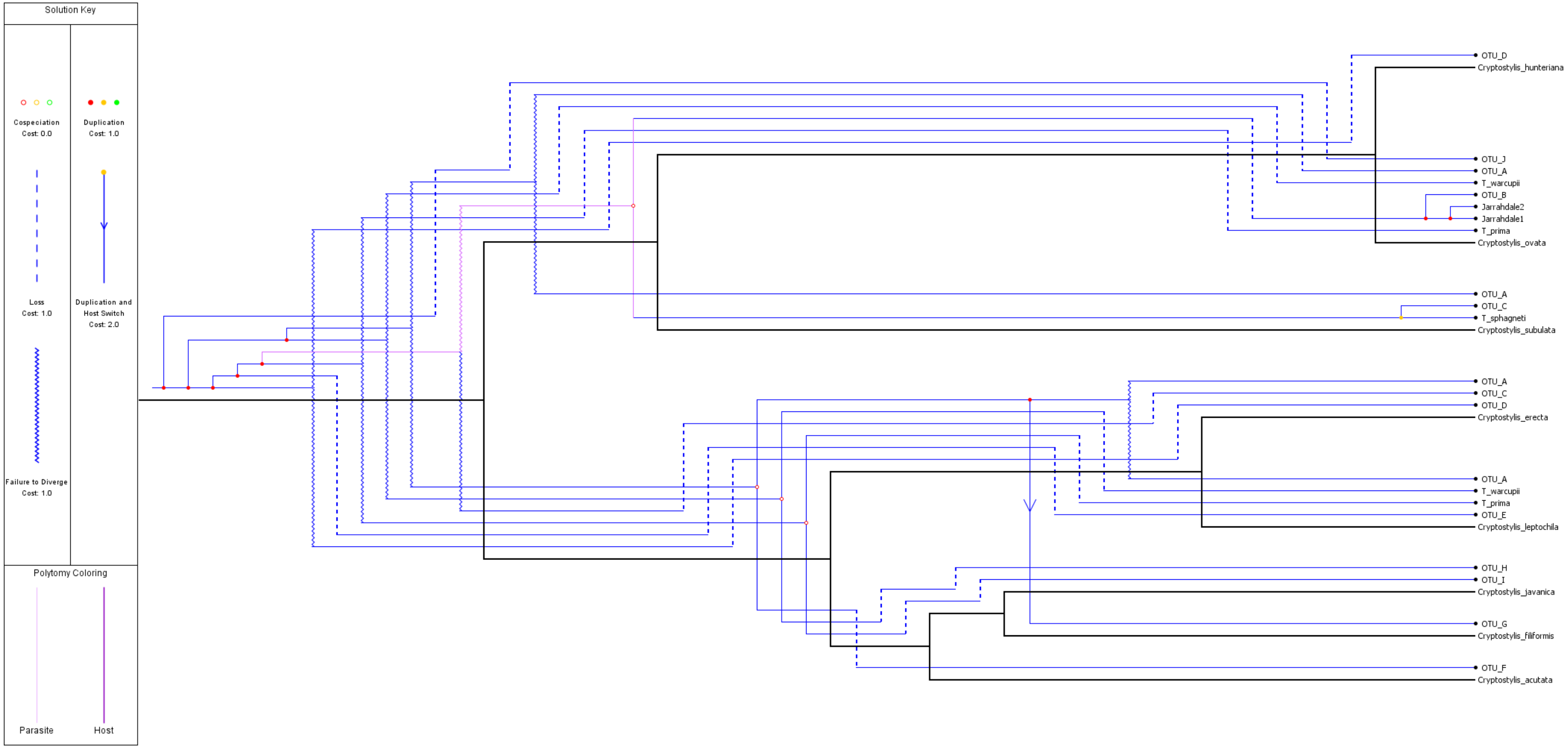

Figure 9. Tanglegram showing the Cryptostylidinae (host) phylogenetic tree in black and the *Tulasnella* phylogenetic tree in blue. The event-based method aims to find the most probable evolutionary events by imposing some cost on each event. Analysis was performed using JANE 4.0 with default cost settings based on Conow et al. (2010). The observed minimum total cost = 43, *P* = 0.36 (cospeciations = 4, duplications = 9, host switches = 1, losses = 25 and failure to diverge = 7).

SUPPLEMENTARY MATERIALS

Table S1. Sequences generated in this study as well as reference sequences for *Tulasnella* phylogenetic tree Group IV from GenBank. The order of the *Tulasnella* species/OTU is following the clade from top to bottom, as presented in Figure 1.

Tulasnella species/OTU	GenBank Number	Orchid host / substrates	Isolate / clone code	Study
OTU O		*Calochilus gracillimus*	CL134.1 clone1	Chapter Five
OTU O		*Calochilus gracillimus*	CL134.1 clone2	Chapter Five
OTU O		*Calochilus gracillimus*	CL134.1 clone3	Chapter Five
OTU O		*Calochilus gracillimus*	CL134.1 clone4	Chapter Five
OTU O		*Calochilus gracillimus*	CL134.2 clone2	Chapter Five
OTU O		*Calochilus gracillimus*	CL134.3 clone1	Chapter Five
OTU O		*Calochilus gracillimus*	CL134.3 clone2	Chapter Five
OTU O		*Calochilus gracillimus*	CL134.2 clone1	Chapter Five
OTU O		*Calochilus paludosus*	CL080 clone1	Chapter Five
OTU O		*Calochilus paludosus*	CL080 clone2	Chapter Five
OTU O		*Calochilus paludosus*	CL080 clone4	Chapter Five
OTU O		*Calochilus paludosus*	CL080 clone3	Chapter Five
OTU O		*Calochilus platychilus*	CL094.1 clone2	Chapter Five
OTU O		*Calochilus platychilus*	CL094.3 clone1	Chapter Five
OTU O		*Calochilus platychilus*	CL094.3 clone3	Chapter Five
OTU O		*Calochilus platychilus*	CL094.3 clone4	Chapter Five
OTU O		*Calochilus platychilus*	CL094.3 clone5	Chapter Five
OTU O		*Calochilus platychilus*	CL094.1 clone1	Chapter Five
OTU O		*Calochilus platychilus*	CL094.3 clone2	Chapter Five
OTU P		*Arthrochilus huntianus*	CL045 clone1	This study
OTU P		*Arthrochilus huntianus*	CL045 clone2	This study
OTU P		*Arthrochilus huntianus*	CL045 clone3	This study
OTU P		*Arthrochilus huntianus*	CL045 clone8	This study
OTU P		*Arthrochilus huntianus*	CL045 clone7	This study
OTU P		*Arthrochilus huntianus*	CL045 clone4	This study
OTU P		*Arthrochilus huntianus*	CL045 clone5	This study
OTU P		*Arthrochilus huntianus*	CL045 clone6	This study
Tulasnella violea	KC152437	Decayed wood	DC293	Cruz et al. 2014
Tulasnella violea	KC152415	Fallen branch	DC177	Cruz et al. 2014
Tulasnella sp. JM-2019a	MK626568	Rotten wood	DAOMC 252086	Mack & Seifert 2019*
Tulasnella sp. JM-2019a	MK626686	Rotten wood	DAOMC 2521988	Mack & Seifert 2019*
Tulasnella sp. JM-2019a	MK626687	Rotten wood	DAOMC 252083	Mack & Seifert 2019*
Tulasnella sp. ECU6	KC152401	Fallen branch	DC185	Cruz et al. 2014
Tulasnella sp. ECU6	KC152402	Fallen branch	DC262	Cruz et al. 2014
Tulasnella sp. ECU5	KC152397	Fallen branch	DC225	Cruz et al. 2014
Tulasnella sp. ECU5	KC152398	Fallen branch	DC225	Cruz et al. 2014
OTU I		*Cryptostylis javanica*	03_clone1	Chapter One
Tulasnella prima	HM196783	*Chiloglottis* aff. *jeanesii*		Roche et al. 2010
Tulasnella prima	HM196784	*Chiloglottis* aff. *jeanesii*		Roche et al. 2010
Tulasnella prima	HM196788	*Chiloglottis* aff. *jeanesii*		Roche et al. 2010
Tulasnella prima	HM196786	*Chiloglottis* aff. *jeanesii*		Roche et al. 2010
Tulasnella prima	HM196779	*Chiloglottis* aff. *jeanesii*		Roche et al. 2010
Tulasnella prima	HM196785	*Chiloglottis* aff. *jeanesii*		Roche et al. 2010
Tulasnella prima	HM196782	*Chiloglottis* aff. *jeanesii*		Roche et al. 2010
Tulasnella prima	HM196795	*Chiloglottis* aff. *jeanesii*		Roche et al. 2010
Tulasnella prima	HM196787	*Chiloglottis* aff. *jeanesii*		Roche et al. 2010

Tulasnella prima	HM196791	*Chiloglottis* aff. *jeanesii*		Roche et al. 2010
Tulasnella prima	HM196792	*Chiloglottis* aff. *jeanesii*		Roche et al. 2010
Tulasnella prima	KF476543	*Chiloglottis formicifera*		Roche et al. 2010
Tulasnella prima	KF476550	*Chiloglottis formicifera*	CLM306	Roche et al. 2010
Tulasnella prima	KF476551	*Chiloglottis formicifera*	CLM308	Roche et al. 2010
Tulasnella prima	HM196796	*Chiloglottis trapeziformis*		Roche et al. 2010
Tulasnella prima	HM196810	*Chiloglottis trapeziformis*		Roche et al. 2010
Tulasnella prima	HM196799	*Chiloglottis trapeziformis*		Roche et al. 2010
Tulasnella prima	HM196794	*Chiloglottis trapeziformis*		Roche et al. 2010
Tulasnella prima	HM196806	*Chiloglottis trapeziformis*		Roche et al. 2010
Tulasnella prima	HM196809	*Chiloglottis trapeziformis*		Roche et al. 2010
Tulasnella prima	HM196793	*Chiloglottis trapeziformis*		Roche et al. 2010
Tulasnella prima	HM196807	*Chiloglottis trapeziformis*		Roche et al. 2010
Tulasnella prima	HM196790	*Chiloglottis trapeziformis*		Roche et al. 2010
Tulasnella prima	HM196789	*Chiloglottis trapeziformis*		Roche et al. 2010
Tulasnella prima	HM196797	*Chiloglottis seminuda*		Roche et al. 2010
Tulasnella prima	HM196798	*Chiloglottis seminuda*		Roche et al. 2010
Tulasnella prima	HM196800	*Chiloglottis seminuda*		Roche et al. 2010
Tulasnella prima	MK430473	*Cryptostylis ovata*	CL13(6)	Nguyen et al. 2019
Tulasnella prima	MK430470	*Cryptostylis ovata*	CL12(5)	Nguyen et al. 2019
Tulasnella prima	MK430521	*Cryptostylis ovata*	CS43(17)	Nguyen et al. 2019
Tulasnella prima	HM196801	*Chiloglottis valida*		Roche et al. 2010
Tulasnella prima	HM196804	*Chiloglottis valida*		Roche et al. 2010
Tulasnella prima	KF476556	*Chiloglottis trilabra*		Roche et al. 2010
Tulasnella prima	HM196805	*Chiloglottis reflexa*		Roche et al. 2010
Tulasnella prima	HM196803	*Chiloglottis diphylla*		Roche et al. 2010
Tulasnella prima	KF476552	*Chiloglottis diphylla*		Roche et al. 2010
Tulasnella prima		*Cryptostylis leptochila*	CLM1933	Chapter One
Tulasnella prima		*Cryptostylis leptochila*	CLM1934	Chapter One
Tulasnella prima		*Cryptostylis leptochila*	CLM1936	Chapter One
Tulasnella prima		*Paracaleana nigrita*	CL103.3 clone1	This study
Tulasnella prima		*Paracaleana nigrita*	CL103.3 clone2	This study
Tulasnella prima		*Paracaleana nigrita*	CL103.3 clone3	This study
Tulasnella prima		*Paracaleana nigrita*	CL103.3 clone4	This study
Tulasnella prima		*Paracaleana nigrita*	CL103.3 clone5	This study
Tulsnella sphagneti		*Cryptostylis subulata*	CLM2131	Chapter One
Tulsnella sphagneti		*Cryptostylis subulata*	CLM2132	Chapter One
Tulsnella sphagneti		*Cryptostylis subulata*	CLM1952	Chapter One
Tulsnella sphagneti		*Chiloglottis turfosa*	12030	Roche et al. 2010
Tulsnella sphagneti		*Chiloglottis turfosa*	13102.1	Roche et al. 2010
Tulsnella sphagneti		*Chiloglottis turfosa*	13102.2	Roche et al. 2010
Tulsnella sphagneti		*Chiloglottis* sp.	13065.1	Roche et al. 2010
Tulsnella sphagneti		*Chiloglottis* sp.	13065.2	Roche et al. 2010
Tulsnella sphagneti	KY095117	*Chiloglottis* aff. *valida*	12033.1	Roche et al. 2010
Tulsnella sphagneti		*Chiloglottis* sp.	13058	Roche et al. 2010
Tulsnella sphagneti		*Chiloglottis* sp.	13139 1	Roche et al. 2010
Tulsnella sphagneti		*Chiloglottis* sp.	13143 1	Roche et al. 2010
OTU C		*Cryptostylis subulata*	CLM2020	Chapter One
OTU C		*Cryptostylis subulata*	CLM2017	Chapter One
OTU C		*Cryptostylis subulata*	CLM2019	Chapter One
OTU C		*Cryptostylis subulata*	CLM2031	Chapter One
OTU C		*Cryptostylis subulata*	CLM2032	Chapter One
OTU E		*Cryptostylis leptochila*	CLM2071	Chapter One
OTU E		*Cryptostylis leptochila*	CLM2072	Chapter One
OTU E		*Cryptostylis leptochila*	CLM2142	Chapter One
OTU B	MK430439	*Cryptostylis ovata*	CB14(1)	Nguyen et al. 2019
OTU B	MK430440	*Cryptostylis ovata*	CB14(3)	Nguyen et al. 2019

OTU B	MK430444	*Cryptostylis ovata*	CB43(6)	Nguyen et al. 2019
OTU B	MK430445	*Cryptostylis ovata*	CB43(9)	Nguyen et al. 2019
OTU B	MK430445	*Cryptostylis ovata*	CB43(9)	Nguyen et al. 2019
OTU B		*Cryptostylis ovata*	CLM1941	Chapter One
OTU B		*Cryptostylis ovata*	CLM1942	Chapter One
OTU B		*Cryptostylis ovata*	CLM1943	Chapter One
Tulasnella sp. Jarrahdale 1	MK430451	*Cryptostylis ovata*	CJ11(8)	Nguyen et al. 2019
Tulasnella sp. Jarrahdale 1	MK430452	*Cryptostylis ovata*	CJ13(3)	Nguyen et al. 2019
Tulasnella sp. Jarrahdale 2	MK430461	*Cryptostylis ovata*	CJ51(7)	Nguyen et al. 2019
Tulasnella sp. Jarrahdale 2	MK430468	*Cryptostylis ovata*	CJ51(17)	Nguyen et al. 2019
OTU D	JN015192	*Cryptostylis hunteriana*	BB0002_2_A	Howard & Clements 2011*
OTU D		*Cryptostylis hunteriana*	CLM2110	Chapter One
OTU D		*Cryptostylis hunteriana*	CLM2112	Chapter One
OTU D		*Cryptostylis hunteriana*	CLM2118	Chapter One
OTU L		*Arthrochilus corinnae*	CL153.2 clone1	This study
OTU L		*Arthrochilus corinnae*	CL153.2 clone2	This study
OTU L		*Arthrochilus corinnae*	CL153.2 clone3	This study
OTU L		*Arthrochilus corinnae*	CL153.4 clone1	This study
OTU L		*Arthrochilus corinnae*	CL153.4 clone2	This study
OTU L		*Arthrochilus corinnae*	CLM2245	This study
OTU L		*Arthrochilus corinnae*	CLM2246	This study
OTU L		*Arthrochilus corinnae*	CLM2247	This study
OTU L		*Arthrochilus corinnae*	CLM2248	This study
Tulasnella eichleriana	KC152389	Decayed wood	DC294	Cruz et al. 2014
Tulasnella eichleriana	AY373292	Unknown	KC852	Cruz et al. 2014
OTU K	JX138568	*Spiculaea ciliata*	MB-2012	Martos et al. 2012
OTU K		*Spiculaea ciliata*	EB9 clone1	This study
OTU K		*Spiculaea ciliata*	EB8 clone2	This study
OTU K		*Spiculaea ciliata*	EB8 clone1	This study
OTU K		*Spiculaea ciliata*	EB7 clone2	This study
OTU K		*Spiculaea ciliata*	EB7 clone1	This study
OTU K		*Spiculaea ciliata*	CL038.4 clone2	This study
OTU K		*Spiculaea ciliata*	CL038.4 clone1	This study
OTU K		*Spiculaea ciliata*	CL038.3 clone2	This study
OTU K		*Spiculaea ciliata*	CL038.3 clone1	This study
OTU K		*Spiculaea ciliata*	CLM1770	This study
OTU K		*Spiculaea ciliata*	CLM1773	This study
OTU K		*Spiculaea ciliata*	CLM1774	This study
OTU K		*Spiculaea ciliata*	CLM1775	This study
OTU K		*Spiculaea ciliata*	CLM1763	This study
OTU K		*Spiculaea ciliata*	CLM1765	This study
OTU K		*Spiculaea ciliata*	CLM1791	This study
OTU K		*Spiculaea ciliata*	CLM1792	This study
OTU K		*Spiculaea ciliata*	CLM1771	This study
OTU K		*Spiculaea ciliata*	CLM1776	This study
OTU K		*Spiculaea ciliata*	CLM1796	This study
OTU K		*Spiculaea ciliata*	CLM1767	This study
OTU K		*Spiculaea ciliata*	CLM1790	This study
OTU K		*Spiculaea ciliata*	CLM1797	This study
OTU K		*Spiculaea ciliata*	CLM1798	This study
OTU K		*Spiculaea ciliata*	CLM1820	This study
OTU K		*Spiculaea ciliata*	CLM1847	This study
OTU K		*Spiculaea ciliata*	CLM1848	This study
OTU K		*Spiculaea ciliata*	CLM1802	This study
OTU K		*Spiculaea ciliata*	CLM1804	This study
OTU K		*Spiculaea ciliata*	CLM1814	This study
OTU K		*Spiculaea ciliata*	CLM1827	This study

OTU K		*Spiculaea ciliata*	CLM1828	This study
OTU K		*Spiculaea ciliata*	CLM1829	This study
OTU K		*Spiculaea ciliata*	CLM1830	This study
OTU K		*Spiculaea ciliata*	CLM1831	This study
OTU K		*Spiculaea ciliata*	CLM1844	This study
Tulasnella secunda	KF476586	*Drakaea glyptodon*		Phillips et al. 2011
Tulasnella secunda	KF476583	*Drakaea glyptodon*		Phillips et al. 2011
Tulasnella secunda	KF476577	*Drakaea glyptodon*		Phillips et al. 2011
Tulasnella secunda	HQ386743	*Drakaea glyptodon*		Phillips et al. 2011
Tulasnella secunda	KF476590	*Drakaea livida*		Phillips et al. 2011
Tulasnella secunda	KF476576	*Drakaea livida*		Phillips et al. 2011
Tulasnella secunda	HQ386778	*Drakaea livida*		Phillips et al. 2011
Tulasnella secunda	KF476579	*Drakaea confluens*		Phillips et al. 2011
Tulasnella secunda	KF476592	*Drakaea confluens*		Phillips et al. 2011
Tulasnella secunda	KF476591	*Drakaea gracilis*		Phillips et al. 2011
Tulasnella secunda	KF476578	*Drakaea gracilis*		Phillips et al. 2011
Tulasnella secunda	KF476575	*Drakaea elastica*		Phillips et al. 2011
Tulasnella secunda	KF476593	*Drakaea elastica*		Phillips et al. 2011
Tulasnella secunda	KF476585	*Drakaea isolata*		Phillips et al. 2011
Tulasnella secunda	KF476588	*Drakaea concolor*		Phillips et al. 2011
Tulasnella secunda	KF476584	*Paracaleana hortiorum*		Linde et al. 2014
Tulasnella secunda		*Paracaleana hortiorum*	CL171.1 clone1	This study
Tulasnella secunda		*Paracaleana hortiorum*	CL171.2 clone1	This study
Tulasnella secunda		*Caleana major*	CL108 clone2	This study
Tulasnella secunda		*Caleana major*	CL108 clone3	This study
Tulasnella secunda	KF476574	*Paracaleana terminalis*		Linde et al. 2014
Tulasnella secunda		*Paracaleana terminalis*	CLM2212	This study
Tulasnella secunda		*Paracaleana terminalis*	CLM2214	This study
Tulasnella secunda		*Paracaleana terminalis*	CLM2217	This study
Tulasnella secunda		*Paracaleana terminalis*	CLM2218	This study
Tulasnella secunda		*Paracaleana terminalis*	CLM2219	This study
Tulasnella secunda		*Paracaleana terminalis*	CLM2215	This study
Tulasnella secunda		*Paracaleana terminalis*	CLM2216	This study
Tulasnella secunda		*Paracaleana terminalis*	CLM2213	This study
Tulasnella secunda	KF476580	*paracaleana triens*		Linde et al. 2014
Tulasnella secunda	KF476568	*Paracaleana minor*		Linde et al. 2014
Tulasnella secunda	KF476573	*Paracaleana lyonsii*		Linde et al. 2014
Tulasnella secunda		*Paracaleana nigrita*	CLM2224	This study
Tulasnella secunda		*Paracaleana nigrita*	CLM2225	This study
Tulasnella secunda		*Paracaleana nigrita*	CLM2226	This study
Tulasnella secunda		*Paracaleana nigrita*	CLM2228	This study
Tulasnella warcupii		*Arthrochilus irritabilis*	CL033.4 clone1	This study
Tulasnella warcupii		*Arthrochilus irritabilis*	CL033.4 clone2	This study
Tulasnella warcupii		*Arthrochilus irritabilis*	CL033.4 clone3	This study
Tulasnella warcupii		*Arthrochilus irritabilis*	CL033.4 clone4	This study
Tulasnella warcupii		*Arthrochilus irritabilis*	CL033.4 clone5	This study
Tulasnella warcupii		*Arthrochilus irritabilis*	CLM1674	This study
Tulasnella warcupii		*Arthrochilus irritabilis*	CLM1694	This study
Tulasnella warcupii		*Arthrochilus irritabilis*	CLM1696	This study
Tulasnella warcupii		*Arthrochilus irritabilis*	CLM1700	This study
Tulasnella warcupii		*Arthrochilus irritabilis*	CLM1701	This study
Tulasnella warcupii		*Arthrochilus irritabilis*	AI002-6	This study
Tulasnella warcupii		*Arthrochilus prolixus*	CL152.1 clone1	This study
Tulasnella warcupii		*Arthrochilus prolixus*	CL152.1 clone3	This study
Tulasnella warcupii		*Arthrochilus prolixus*	CL152.1 clone5	This study
Tulasnella warcupii		*Arthrochilus prolixus*	CL152.1 clone2	This study
Tulasnella warcupii		*Arthrochilus prolixus*	CL152.1 clone4	This study
Tulasnella warcupii		*Arthrochilus prolixus*	CLM2242	This study

Tulasnella warcupii		*Arthrochilus prolixus*	CLM2243	This study
Tulasnella warcupii		*Arthrochilus latipes*	CLM2236	This study
Tulasnella warcupii		*Arthrochilus latipes*	CLM2238	This study
Tulasnella warcupii		*Arthrochilus latipes*	CL130.3 clone1	This study
Tulasnella warcupii		*Arthrochilus latipes*	CL130.3 clone2	This study
Tulasnella warcupii		*Arthrochilus latipes*	CL130.2 clone1	This study
Tulasnella warcupii		*Arthrochilus latipes*	CL130.2 clone2	This study
Tulasnella warcupii		*Arthrochilus latipes*	CL130.2 clone3	This study
Tulasnella warcupii		*Arthrochilus latipes*	CL130.2 clone4	This study
Tulasnella warcupii		*Arthrochilus latipes*	CLM2239	This study
Tulasnella warcupii		*Arthrochilus latipes*	CLM2240	This study
Tulasnella warcupii		*Arthrochilus latipes*	CLM2237	This study
Tulasnella warcupii		*Paracaleana nigrita*	CL103.2 clone1	This study
Tulasnella warcupii		*Paracaleana nigrita*	CL103.2 clone2	This study
Tulasnella warcupii		*Paracaleana nigrita*	CL103.2 clone3	This study
Tulasnella warcupii		*Paracaleana nigrita*	CL103.4 clone4	This study
Tulasnella warcupii		*Arthrochilus rosulatus*	CL154.1 clone1	This study
Tulasnella warcupii		*Arthrochilus rosulatus*	CL154.1 clone2	This study
Tulasnella warcupii		*Arthrochilus rosulatus*	CL154.1 clone3	This study
Tulasnella warcupii		*Arthrochilus rosulatus*	CL154.2 clone1	This study
Tulasnella warcupii		*Arthrochilus rosulatus*	CL154.3 clone1	This study
Tulasnella warcupii		*Arthrochilus rosulatus*	CLM2249	This study
Tulasnella warcupii		*Arthrochilus rosulatus*	CLM2250	This study
Tulasnella warcupii		*Arthrochilus rosulatus*	CLM2251	This study
Tulasnella warcupii		*Arthrochilus rosulatus*	CLM2252	This study
Tulasnella warcupii		*Arthrochilus rosulatus*	CLM2253	This study
Tulasnella warcupii		*Arthrochilus rosulatus*	CLM2254	This study
Tulasnella warcupii		*Arthrochilus rosulatus*	CLM2255	This study
Tulasnella warcupii		*Arthrochilus rosulatus*	CLM2256	This study
Tulasnella warcupii		*Arthrochilus rosulatus*	CLM2257	This study
Tulasnella warcupii		*Arthrochilus rosulatus*	CLM2258	This study
Tulasnella warcupii		*Paracaleana disjuncta*	CL075.2 clone2	This study
Tulasnella warcupii		*Paracaleana disjuncta*	CL075.2 clone3	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CLM2259	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CLM2263	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CL155.3 clone1	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CL155.4 clone2	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CLM2260	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CLM2261	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CLM2262	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CL155.2 clone1	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CL155.2 clone2	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CL155.2 clone3	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CL155.4 clone1	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CL155.4 clone3	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CL156.1 clone3	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CL156.2 clone1	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CL156.2 clone2	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CL156.3 clone1	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CL156.3 clone2	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CL156.1 clone1	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CL156.1 clone2	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CL156.2 clone3	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CLM2264	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CLM2265	This study
Tulasnella warcupii		*Arthrochilus dockrillii*	CLM2266	This study
Tulasnella warcupii	KF476596	*Arthrochilus oreophilus*	CLM027	Linde et al. 2014
Tulasnella warcupii	KF476601	*Arthrochilus oreophilus*	CLM022	Linde et al. 2014

Tulasnella warcupii	KF476600	*Arthrochilus oreophilus*	CLM007	Linde et al. 2014
Tulasnella warcupii	KF476598	*Arthrochilus oreophilus*	CLM091	Linde et al. 2014
Tulasnella warcupii	KF476597	*Arthrochilus oreophilus*	CLM092	Linde et al. 2014
Tulasnella warcupii	KF476599	*Arthrochilus oreophilus*	CLM028	Linde et al. 2014
Tulasnella warcupii		*Arthrochilus oreophilus*	CLM2230	This study
Tulasnella warcupii		*Arthrochilus oreophilus*	CLM2231	This study
Tulasnella warcupii		*Arthrochilus oreophilus*	CLM2232	This study
Tulasnella warcupii		*Arthrochilus oreophilus*	CLM2233	This study
Tulasnella warcupii		*Arthrochilus oreophilus*	CLM2234	This study
Tulasnella warcupii		*Arthrochilus oreophilus*	CLM2235	This study
Tulasnella warcupii		*Arthrochilus oreophilus*	CLM2277	This study
Tulasnella warcupii		*Arthrochilus oreophilus*	CLM2268	This study
Tulasnella warcupii		*Arthrochilus oreophilus*	CLM2275	This study
Tulasnella warcupii		*Arthrochilus oreophilus*	CLM2276	This study
Tulasnella warcupii		*Arthrochilus oreophilus*	CLM2229	This study
Tulasnella warcupii		*Arthrochilus oreophilus*	CL158.2 clone1	This study
Tulasnella warcupii		*Arthrochilus oreophilus*	CL158.3 clone1	This study
Tulasnella warcupii		*Arthrochilus oreophilus*	CL158.1 clone1	This study
Tulasnella warcupii		*Arthrochilus oreophilus*	CL158.1 clone2	This study
Tulasnella warcupii		*Arthrochilus oreophilus*	CL158.2 clone2	This study
Tulasnella warcupii		*Arthrochilus stenophyllus*	CLM2269	This study
Tulasnella warcupii		*Arthrochilus stenophyllus*	CLM2271	This study
Tulasnella warcupii		*Arthrochilus stenophyllus*	CLM2272	This study
Tulasnella warcupii		*Arthrochilus stenophyllus*	CLM2273	This study
Tulasnella warcupii		*Arthrochilus stenophyllus*	CLM2274	This study
Tulasnella warcupii		*Arthrochilus stenophyllus*	CL157.2 clone1	This study
Tulasnella warcupii		*Arthrochilus stenophyllus*	CL157.2 clone2	This study
Tulasnella warcupii		*Arthrochilus stenophyllus*	CL157.2 clone3	This study
OTU N	KF476594	*Arthrochilus oreophilus*	CLM084	Linde et al. 2014
OTU N	KF476595	*Arthrochilus oreophilus*	CLM085	Linde et al. 2014
OTU H		*Cryptostylis javanica*	01_clone1	Chapter One
OTU H		*Cryptostylis javanica*	01_clone3	Chapter One
Tulasnella tomaculum	AY373296	Unknown	KC429	McCormick et al. 2004
Tulasnella tomaculum	KC152380	Unknown	K(M)123675	Cruz et al. 2014
OTU A	KF476602	*Arthrochilus oreophilus*	CLM031	Linde et al. 2014
OTU A		*Arthrochilus rosulatus*	CL154.2 clone3	This study
OTU A		*Cryptostylis leptochila*	CLM1670	Chapter One
OTU A		*Cryptostylis erecta*	CLM1944	Chapter One
OTU A		*Cryptostylis erecta*	CLM1945	Chapter One
OTU G		*Cryptostylis filiformis*	Sabah1_clone2	Chapter One
OTU G		*Cryptostylis filiformis*	Sabah1_clone3	Chapter One
OTU G		*Cryptostylis filiformis*	GK06_clone1	Chapter One
OTU G		*Cryptostylis filiformis*	GK06_clone2	Chapter One
OTU M		*Arthrochilus oreophilus*	CL112.2 clone1	This study
OTU F		*Cryptostylis acutata*	Sabah9_clone1	Chapter One
OTU F		*Cryptostylis acutata*	Sabah9_clone2	Chapter One

Table S2. Drakaeinae-*Tulasnella* association matrix

	OTU_P	T_secun	OTU_K	OTU_L	T_sphag	T_prima	T_warcup	OTU_N	OTU_A	OTU_M
Arhun	1	0	0	0	0	0	0	0	0	0
Arpro	0	0	0	0	0	0	1	0	0	0
Arrosul	0	0	0	0	0	0	1	0	1	0
Arsteno	0	0	0	0	0	0	1	0	0	0
Aroreo	0	0	0	0	0	0	1	1	1	1
Artipes	0	0	0	0	0	0	1	0	0	0
Arcorin	0	0	0	1	0	0	0	0	0	0
Ardock	0	0	0	0	0	0	1	0	0	0
Spicul	0	0	1	0	0	0	0	0	0	0
Chilpala	0	0	0	0	0	1	0	0	0	0
Childip	0	0	0	0	0	1	0	0	0	0
Chilref	0	0	0	0	0	1	0	0	0	0
Chilsem	0	0	0	0	0	1	0	0	0	0
Chilform	0	0	0	0	0	1	0	0	0	0
Chiltrap	0	0	0	0	0	1	0	0	0	0
Chilgram	0	0	0	0	0	1	0	0	0	0
Chilplur	0	0	0	0	0	1	0	0	0	0
Chilbifa	0	0	0	0	0	1	0	0	0	0
Chilturf	0	0	0	0	1	1	0	0	0	0
Chilval	0	0	0	0	0	1	0	0	0	0
Chilaval	0	0	0	0	1	1	0	0	0	0
Chilajea	0	0	0	0	0	1	0	0	0	0
Calma	0	1	0	0	0	0	0	0	0	0
Parmin	0	1	0	0	0	0	0	0	0	0
Paryon	0	1	0	0	0	0	0	0	0	0
Parnig	0	1	0	0	0	1	1	0	0	0
Parhort	0	1	0	0	0	0	0	0	0	0
Pardisj	0	1	0	0	0	0	1	0	0	0
Parterm	0	1	0	0	0	0	0	0	0	0
Partrie	0	1	0	0	0	0	0	0	0	0
Dragra	0	1	0	0	0	0	0	0	0	0
Dramic	0	1	0	0	0	0	0	0	0	0
Dratica	0	1	0	0	0	0	0	0	0	0
Draconc	0	1	0	0	0	0	0	0	0	0
Draglyp	0	1	0	0	0	0	0	0	0	0
Draiso	0	1	0	0	0	0	0	0	0	0
Draconf	0	1	0	0	0	0	0	0	0	0
Drathyn	0	1	0	0	0	0	0	0	0	0
Draliv	0	1	0	0	0	0	0	0	0	0

Notes:

Drakaeinae orchids: Arhun: *Arthrochilus huntianus*; Arpro: *Arthrochilus prolixus*; Arrosul: *Arthrochilus rosulatus*; Arsteno: *Arthrochilus stenophyllus*; Aroreo: *Arthrochilus oreophilus*; Artipes: *Arthrochilus latipes*; Arcorin: *Arthrochilus corinnae*; Ardock: *Arthrochilus dockrillii*; Spicul: *Spiculaea ciliata*; Chilpala: *Chiloglottis palachila*; Childip: *Chiloglottis diphylla*; Chilref: *Chiloglottis reflexa*; Chilsem: *Chiloglottis seminuda*; Chilform: *Chiloglottis formicifera*; Chiltrap: *Chiloglottis trapeziformis*; Chilgram: *Chiloglottis grammata*; Chilplur: *Chiloglottis pluricallata*; Chilbifa: *Chiloglottis* sp. (bifaria); Chilturf: *Chiloglottis turfosa*; Chilval: *Chiloglottis valida*; Chilaval: *Chiloglottis* aff. *valida*; Chilajea: *Chiloglottis* aff. *jeanesii*; Calma: *Caleana major*; Parmin: *Paracaleana minor*; Paryon: *Paracaleana lyonsii*; Parnig: *Paracaleana nigrita*; Parhort: *Paracaleana hortiorum*; Pardisj: *Paracaleana disjuncta*; Parterm: *Paracaleana terminalis*; Partrie: *Paracaleana triens*; Dragra: *Drakaea gracilis*; Dramic: *Drakaea micrantha*; Dratica: *Drakaea elastica*; Draconc: *Drakaea concolor*; Draglyp: *Drakaea glyptodon*; Draiso: *Drakaea isolata*; Draconf: *Drakaea confluens*; Drathyn: *Drakaea thynniphila*; Draliv: *Drakaea livida*.

***Tulasnella* symbionts:** T_secun: *Tulasnella secunda*; T_warcup: *Tulasnella warcupii*; T_prima: *Tulasnella prima*; T_sphag: *Tulasnella sphagneti*.

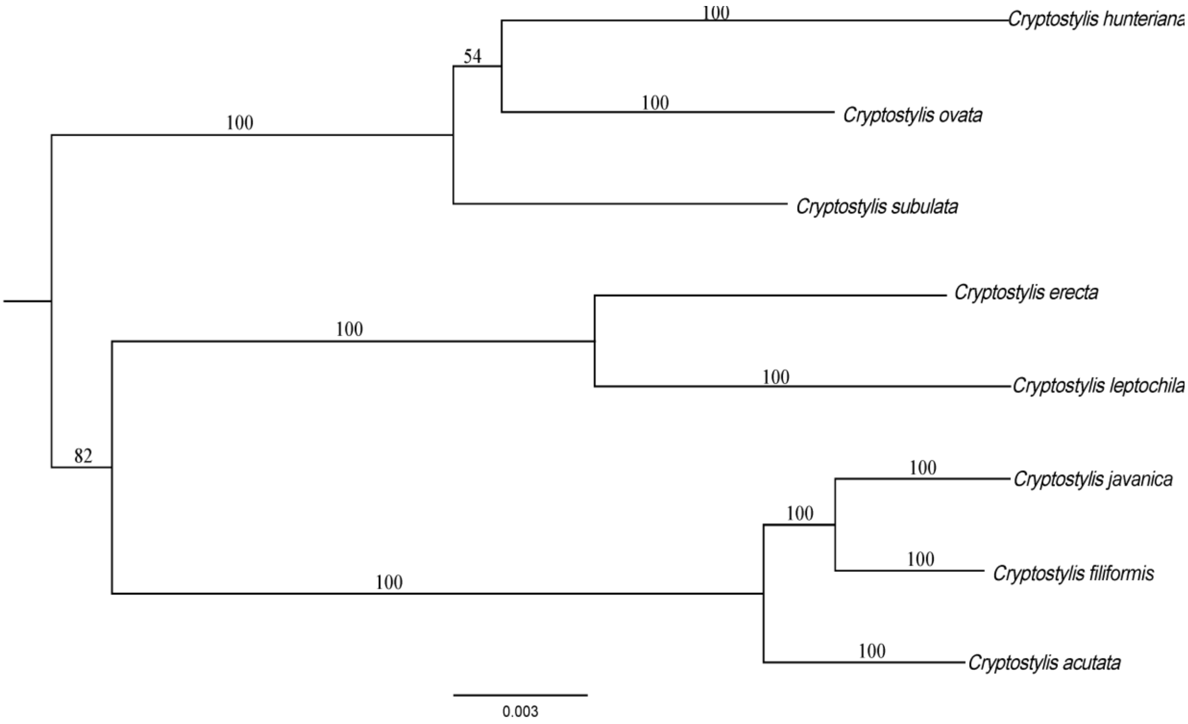

Figure S1. Midpoint-rooted *Cryptostylis* orchids phylogeny obtained from 211 single-copy orthologous genes (186,827 bp) identified in Deng et al. (2015), following the methodology of Peakall et. al., (2020 *in prep*). The numbers above the branches are percentage bootstrap support.

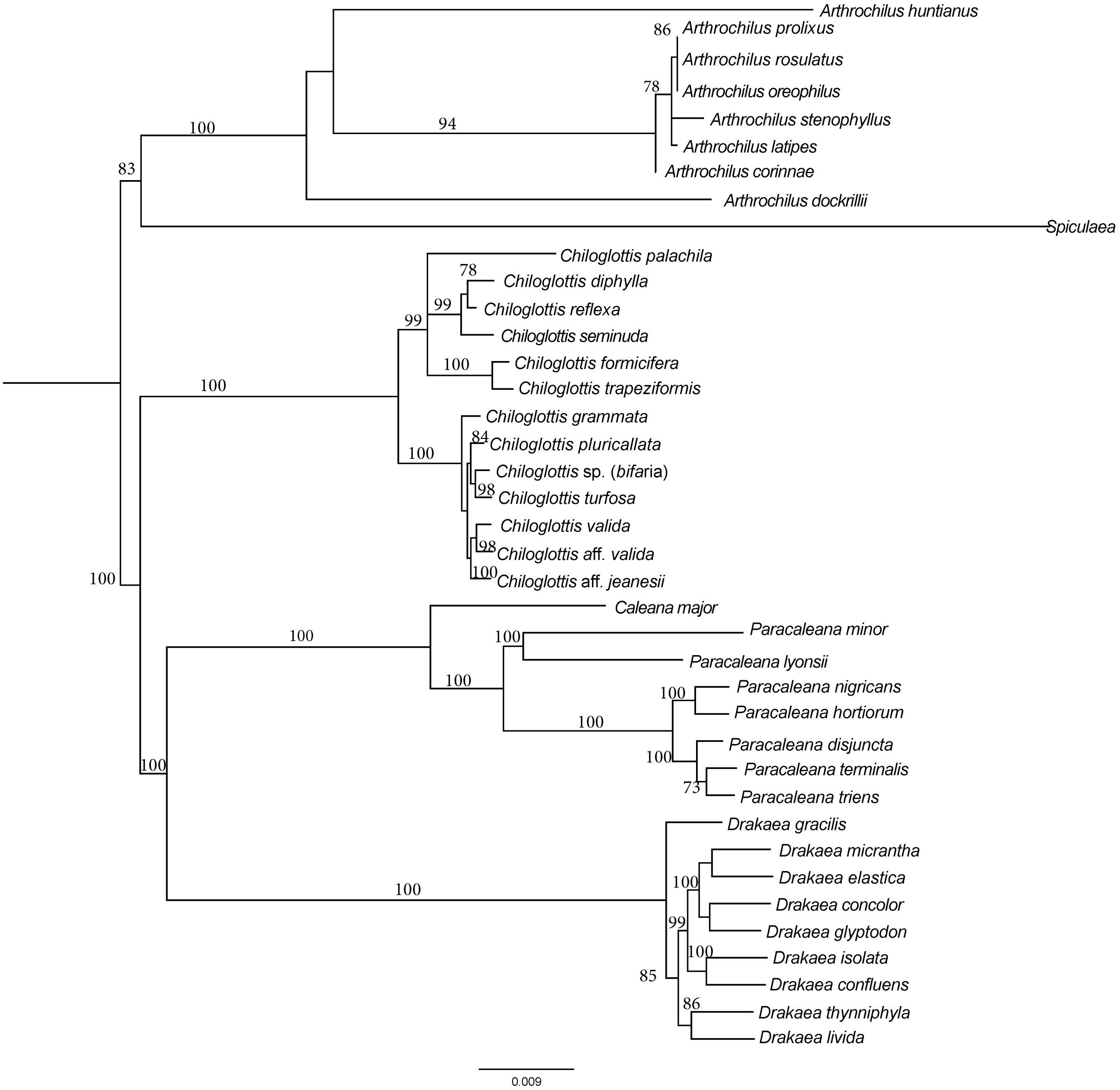

Figure S2. Drakaeinae orchid phylogeny obtained from 200 single-copy orthologous genes (196,180 bp in total) developed for the Drakaeinae orchids, was used following the methodology of Peakall et. al., (2020 *in prep*). The numbers above the branches are Bootstrap support. Only Bootstrap values ≥ 70 are shown. Tree is rooted with *Pyrorchis* (pruned in this study).

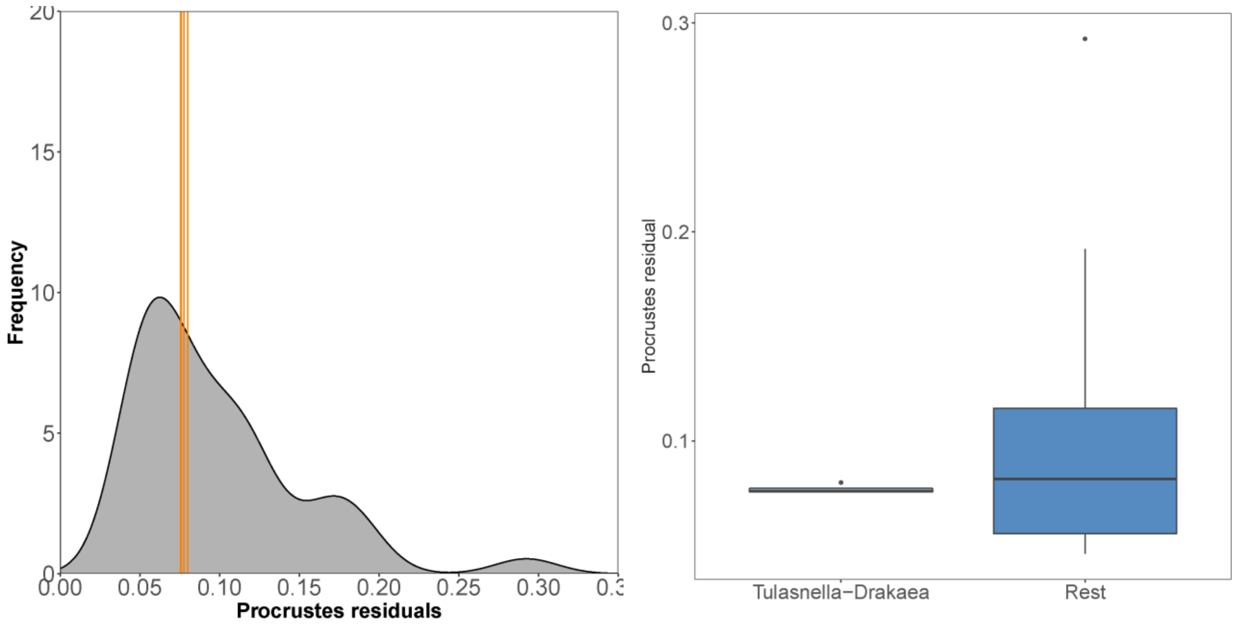

Figure S3. [Left]: The orange lines show interactions between *Tulasnella* and *Drakaea*, and the distribution gives the values of the rest of the interactions in the Drakaeinae-*Tulasnella* network. [Right]: Comparison of the *Tulasnella*-*Drakaea* compared to the rest of the network (Rest).

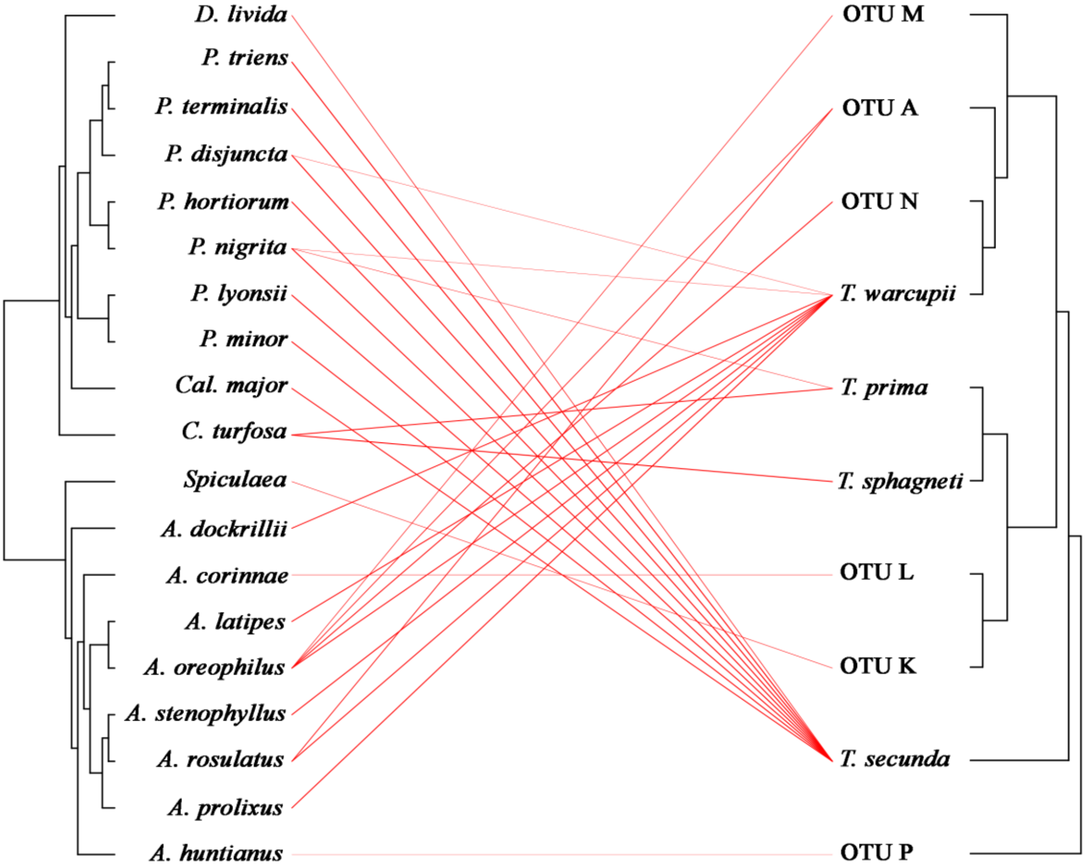

Figure S4. Interaction network and phylogenies of Drakaeinae orchids and *Tulasnella* fungi, excluding all but one species of *Drakaea* and *Chiloglottis* to test whether “pseudoreplication” of taxa within a genus affect cophylogenetic signal. The cophylogenetic analysis in PACo still shows significant phylogenetic congruence (cophylogenetic signal $m^2_{xy} = 31{,}616$; $P < 0.001$). The interactions are weighted by their contribution to the overall phylogenetic congruence. Orchid genera abbreviations – *D*: *Drakaea*; *P*: *Paracaleana*; *Cal*: *Caleana*; *C*: *Chiloglottis*; *A*: *Arthrochilus*. Fungal genus abbreviation – *T*: *Tulasnella*.

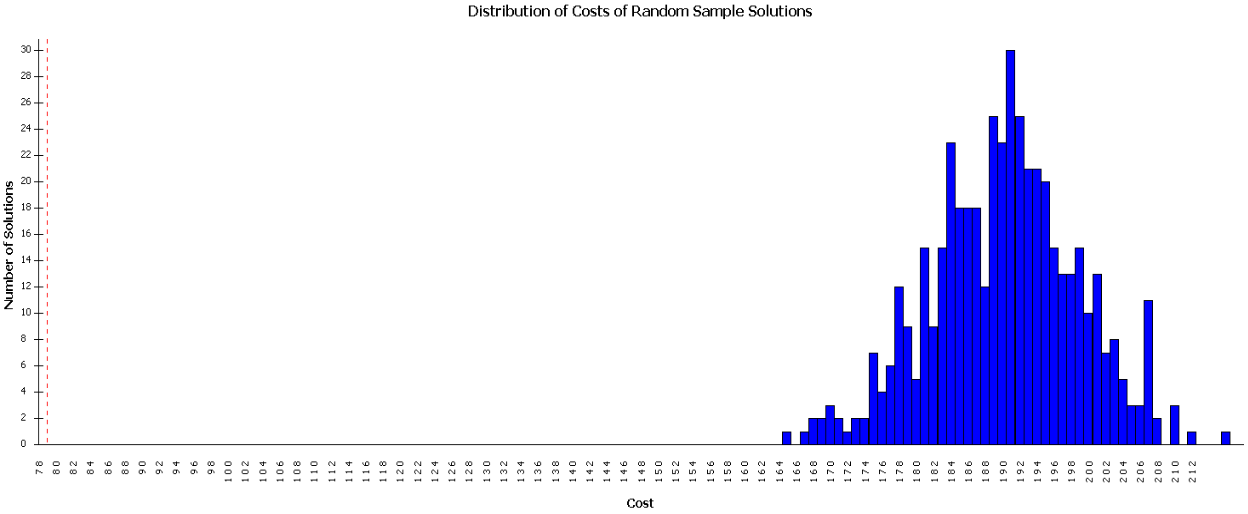

Figure S5. The total cost of event (78 – red dotted line) is significantly different compared to the distribution of costs in Drakaeinae-*Tulasnella*.

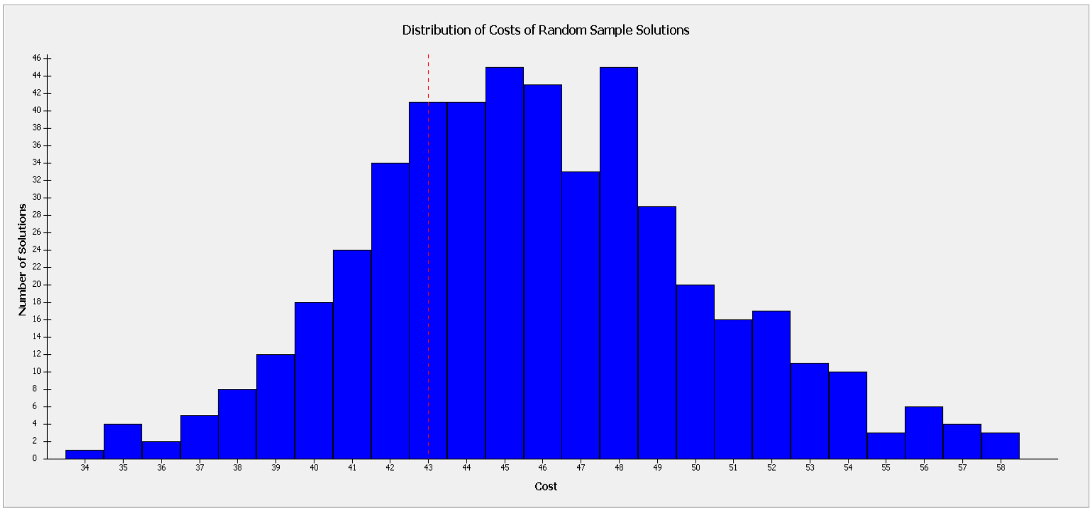

Figure S6. The total cost of event (43) is not significantly different compared to the distribution of costs in *Cryptostylis-Tulasnella.*

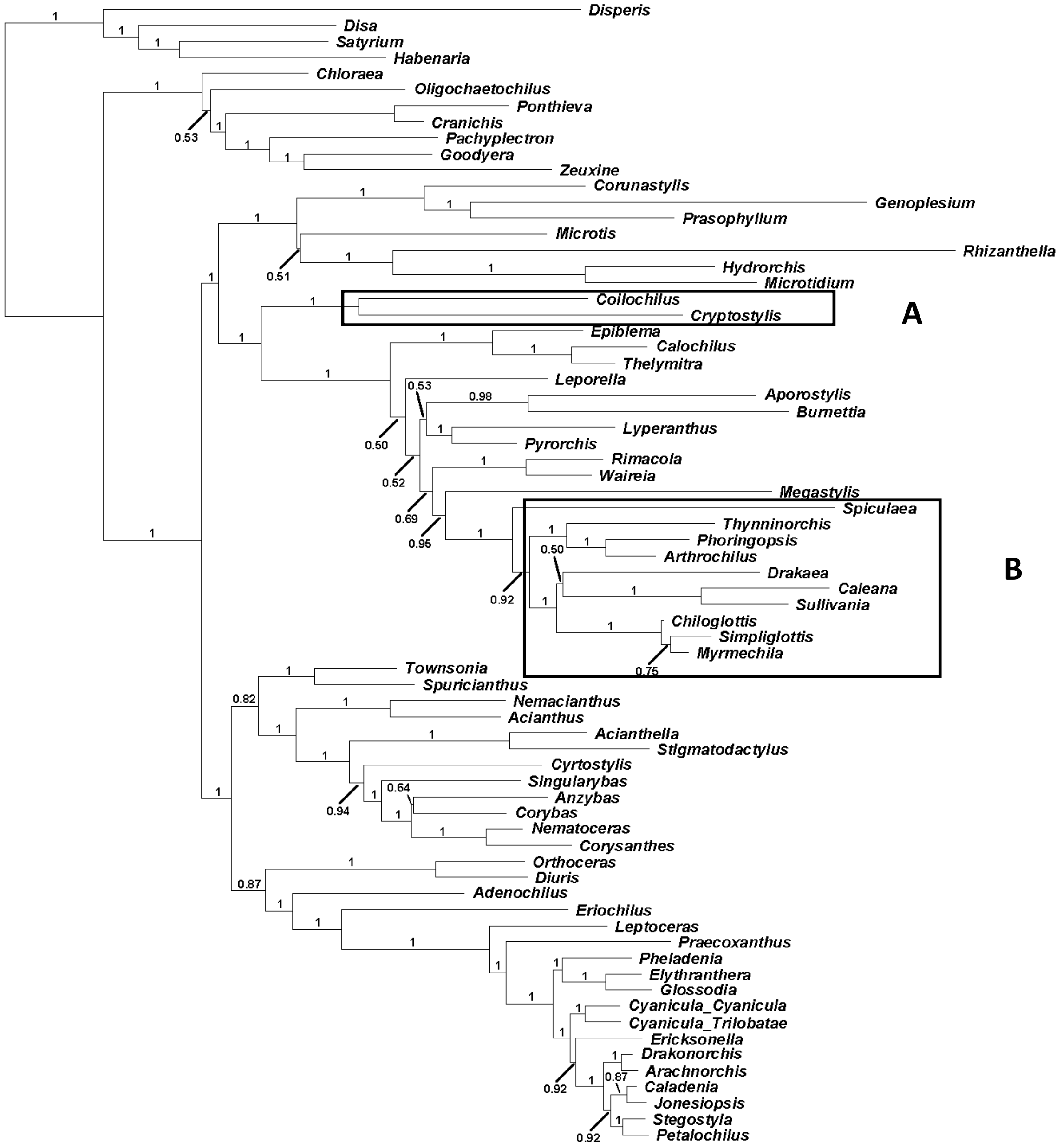

Figure S7. Bayesian probability Diurideae phylogenetic tree for the combined molecular data set (ITS nrDNA, *trnL-trnF*, *matK* and *rbcL*) of orchid tribe Diurideae (Weston et al., 2014). Cryptostylidinae (A) and Drakaeinae (B) are distantly related orchid subtribes.

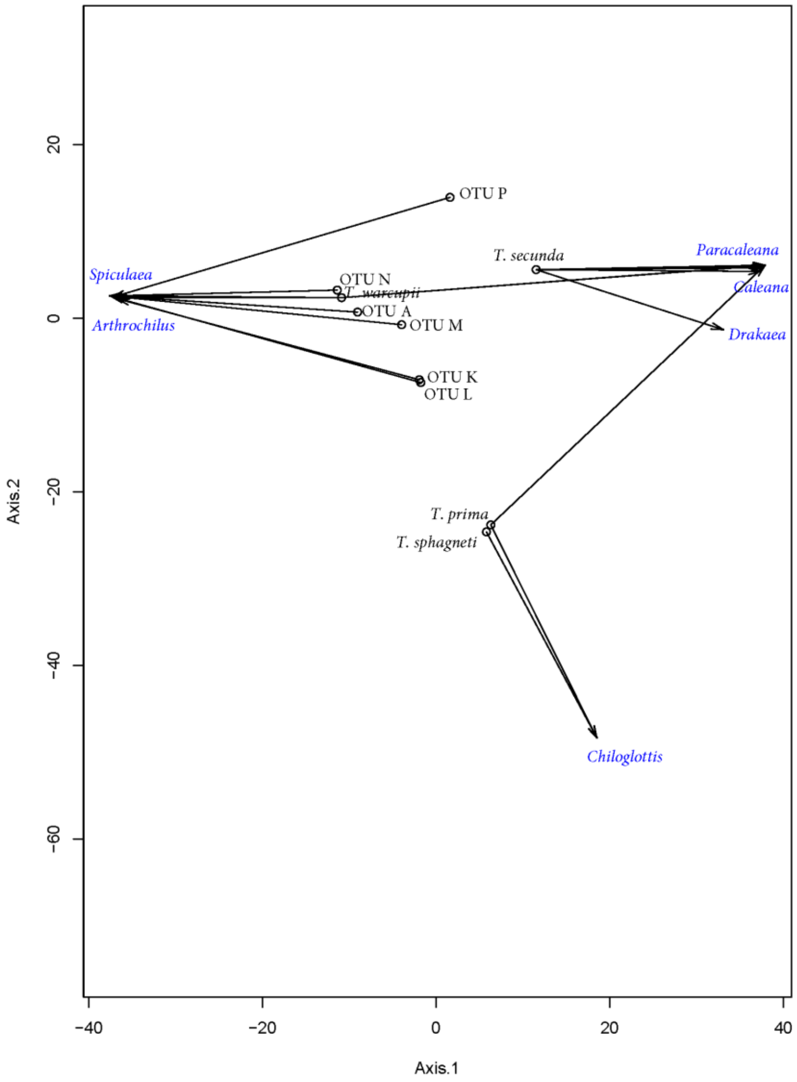

Figure S8. Procrustean superimposition plot of Drakaeinae orchids and their *Tulasnella* fungi, excluding all but one species of *Drakaea* and *Chiloglottis* to test whether “pseudoreplication” of taxa within a genus affect cophylogenetic signal. The starting points (dots) represent the configuration of *Tulasnella* symbionts and the arrowheads show the configuration of Drakaeinae hosts.

CHAPTER THREE

New species of *Tulasnella* associated with Australian terrestrial orchids in the Cryptostylidinae and Drakaeinae

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New species of *Tulasnella* associated with Australian terrestrial orchids in the Cryptostylidinae and Drakaeinae

ABSTRACT

Many orchids have an obligate relationship with *Tulasnella* mycorrhizal fungi for seed germination and support into adulthood. Despite the importance of *Tulasnella* as mycorrhizal partners, many species remain undescribed. Here we use multiple sequence locus phylogenetic analyses to delimit and describe six new *Tulasnella* species associated with Australian terrestrial orchids from the subtribes Cryptostylidinae and Drakaeinae. Five of the new species, *Tulasnella australiensis, T. occidentalis, T. punctata, T. densa*, and *T. concentrica*, all associate with *Cryptostylis* (Cryptostylidinae), whereas *T. rosea* associates with *Spiculaea ciliata* (Drakaeinae). Isolates representing *T. australiensis* were previously also reported in association with *Arthrochilus* (Drakaeinae). All newly described *Tulasnella* species were delimited by phylogenetic analyses of four loci (nuc rDNA ITS1-5.8S-ITS2 (ITS), *C14436* (ATP synthase), *C4102* (glutamate synthase) and mt 16S rDNA (mtLSU)). The pairwise sequence divergence between species for the ITS region ranged from 5.6% to 25.2%, and the maximum sequence divergence within the newly described species ranged from 1.64–4.97%. There was a gap in the distribution of within and between species pairwise divergences in the region of 4–6%, with only one within-species value of 4.97% (for two *T. australiensis* isolates) and one between-species value of 5.6% (involving an isolate of *T. occidentalis*) falling within this region. Based on fluorescence staining, all six new *Tulasnella* species are binucleate and have septate cylindrical hyphae. There was some subtle variation in culture morphology, but colony diameter as measured on 3MN+vitamin media after six wk growth did not differ among species. However, *T. australiensis* grew significantly ($P < 0.02$) slower than others on ½ FIM and ¼ PDA media. Formal description of these *Tulasnella* species contributes significantly to documentation of *Tulasnella* diversity and provides names and delimitations to underpin further research on the fungi and their relationships with orchids.

Keywords: Orchid mycorrhizal fungi, binucleate, colony diameter, multilocus phylogenetic analyses, morphology, **6 new taxa.**

INTRODUCTION

Tulasnella J. Schröt. (Cantharellales, Tulasnellaceae Juel) is an important orchid mycorrhizal genus of fungi (OMF) and widespread around the world. Approximately 100 species names have been proposed in the genus, most of which are currently accepted (http://www.speciesfungorum.org/). *Tulasnella* species occur worldwide as free-living saprotrophs in decayed wood (Roberts 1994; Cruz et al. 2011), as mycobionts in orchids (Warcup 1981; Rasmussen and Rasmussen 2009) and liverworts (Kottke et al. 2008), and as ectomycorrhizal associates of the non-photosynthetic liverwort *Cryptothallus mirabilis* (Bidartondo et al. 2003). The asexual (anamorph) form was previously named *Epulorhiza* R.T. Moore (Moore 1987; Oberwinkler et al. 2017). However, based on the concept "one fungus, one name", the separate anamorph nomenclature was discontinued (Hawksworth 2011). Studies by Moncalvo et al. (2006) and Gónzalez et al. (2016) placed Tulasnellaceae in the order Cantharellales based on phylogenetic analyses.

Delimitation of *Tulasnella* on morphology is difficult because there are few morphologically distinct characters among species, and induction of sexual spores is challenging (Roberts 1994; Suarez et al. 2006; Cruz et al. 2011; Cruz et al. 2014). Two or more species can be considered as cryptic species if they are morphologically indistinguishable (Bickford et al. 2007), and such species occur in *Tulasnella*. Therefore, molecular characterization using multiple sequence loci from mitochondrial and/or nuclear DNA is necessary, as used to delimit cryptic species in various groups of fungi (Leavitt et al. 2011; Linde et al. 2014; Stefani et al. 2014; Wang et al. 2014; Whitehead et al. 2017). Once species are delimited on multi-gene data, a barcode locus can be selected. For Basidiomycota, nuc rDNA ITS1-5.8S-ITS2 (ITS) is generally considered the best performing locus for resolving species delimitation compared to other loci (Schoch et al. 2012), and this region has been shown to be suitable for distinguishing *Tulasnella* species (Linde et al. 2017).

Diurideae is a tribe of terrestrial orchids that occur in the southern hemisphere, namely Australia, New Zealand, and New Caledonia (Kores et al. 2001). At least four of eight subtribes in this group – Cryptostylidinae (*Cryptostylis*) (Nguyen et al. 2019), Diuridinae (*Diuris*) (Warcup 1971; Smith et al. 2010), Drakaeinae (*Chiloglottis, Drakaea, Arthrochilus*) (Roche et al. 2010; Phillips et al. 2011; Linde et al. 2014), and Thelymitrinae (*Thelymitra*) – associate with *Tulasnella* (Reiter et al. 2018). Recently, Linde et al. (2017) used multiple DNA loci to distinguish cryptic species in a species delimitation study, in which four new *Tulasnella* species were described from Diurideae: *T. sphagneti* Linde & T.W. May and *T. prima* Linde & T.W. May from *Chiloglottis, T. secunda* Linde & T.W. May from *Drakaea* and *Caleana*, and *T. warcupii* Linde & T.W. May from *Arthrochilus oreophilus*. In a previous study of *Tulasnella* isolated from Australian orchids, eight loci were used to distinguish three species, and of these loci, ITS was the most polymorphic and provided similar phylogenetic resolution as the full set of loci (Linde et al. 2014). A further five *Tulasnella* species have since been described using ITS and the D1/D2 region of nuc rDNA 28S (28S), four of which are associated with orchids (Rachanarin et al. 2018; Fujimori et al. 2019) and one with oak trees (Solís et al. 2017).

The aim of this study is to identify and describe phylogenetic species among the *Tulasnella* symbionts associated with several Australian orchids (*Cryptostylis, Arthrochilus*, and *Spiculaea*) by assessing the concordance of clades present in phylogenetic trees based on multiple loci. Formal descriptions of six new species are provided along with details of culture morphology, microscopic characteristics, and nuclear characterization.

MATERIALS AND METHODS

Orchid collections

Root samples of five Australian *Cryptostylis* (*Cryptostylis ovata, C. erecta, C. leptochila, C. subulata*, and *C. hunteriana*) and *Spiculaea ciliata* were collected in the period 2016–2018, mostly during their flowering time to enable identification of sympatric orchid species. All root samples were wrapped in damp paper towels and kept at 5 C until processing. *Cryptostylis ovata* plants were obtained from Boyanup, Johnston Rd, Muja Conservation Park, Granite Peaks, and Rockingham, all in Western Australia (WA); *C. subulata* plants were obtained from Ulladulla, Frenchs Forest, Erowal Bay, and Karuah, and Nowra, all in New South Wales (NSW); samples of *C. erecta* were obtained from Fitzroy Falls, Nowra, Frenchs Forest, Erowal Bay, Carls mountain Rd, all in NSW; *C. hunteriana* were obtained from Bulahdelah, Meroo National Park, and Erowal Bay, all from NSW; *C. leptochila* were obtained from Cabbage Tree Creek, Cape Conran, and Bunyip State Park in Victoria (VIC), as well as Carls Mountain Rd, Bundanoon, and Fitzroy Falls in NSW. Additionally, samples of *S. ciliata* were obtained from WA populations; Boulder Rock, East Brookton, and Canning Mills (SUPPLEMENTARY TABLE 1).

Fungal isolation

Half-strength Fungal Isolation Media (½ FIM) (Clements and Ellyard 1979) supplemented with 50 mg/mL streptomycin sulfate was used to isolate *Tulasnella* fungi from orchid samples. Root pieces were washed thoroughly under running water, dissected to localise the pelotons, and rinsed three times with sterile water before plating onto ½ FIM + streptomycin 50 mg/mL agar plates. All plates were incubated at 22 C in the dark. Hyphal tips from germinated pelotons were transferred onto new plates of ½ FIM + streptomycin sulfate (50 mg/mL) to obtain single and pure cultures. Stock cultures were kept on ½ FIM agar slants and also stored as agar blocks in sterile miliQ water. Voucher specimens of the fungi were deposited as living cultures (stored metabolically inactive) at the Royal Botanic Gardens Victoria Fungal Culture Collection (RBGV-FCC) and Victorian Plant Pathology Herbarium (VPRI), whereas dried material was deposited at the National Herbarium of Victoria (MEL).

DNA extraction, PCR amplification, and sequencing

For fungal DNA extractions, all isolates (total 190 isolates) were grown in liquid ½ FIM medium, with conditions as above. In cases where isolates did not grow in the liquid medium, fungal mycelium was obtained directly from ½ FIM agar plates. To extract the mycelium growing on agar, it was treated with QG buffer (Qiagen, Hilden, Germany) and washed thoroughly with sterile water to remove remaining buffer. Harvested mycelium was lyophilized prior to fungal genomic DNA isolation. Fungal genomic DNA was extracted from lyophilized mycelium using a DNAeasy Plant Mini Kit following the manufacturer’s protocol (Qiagen, Hilden, Germany).

ITS was amplified and sequenced using primers ITS5 and ITS4 (White et al. 1990; Gardes and Bruns 1993) following Linde et al. (2014). A portion of a second nuclear locus, *C4102*, which encodes glutamate synthase (Linde et al. 2014; Ruibal et al. 2013), as well as two mitochondrial loci, *C14436* (ATP synthase) (Linde et al. 2014; Ruibal et al. 2013) and mt 16S rDNA (mitochondrial large subunit/mtLSU) (White et al. 1990; Bruns et al. 1998) were amplified using primers and PCR conditions as described in Ruibal et al. (2013) (TABLE 1). We attempted amplification of ITS for all fungal samples, whereas at least four isolates per species were used to amplify the two mt loci (*C14436* and mtLSU) and the nuclear locus *C4102*. To observe possible unculturable *Tulasnella*, we also extracted the peloton-rich root genomic DNA of several orchid hosts using DNeasy Plant Mini Kit. The peloton-rich DNA was amplified with the ITS *Tulasnella* specific primer combination of ITS5 and ITS4Tul (Taylor and McCormick 2008) and PCR products were cloned with the pCR4-TOPO cloning vector (ThermoFisher Scientific, Waltham, Massachusetts) and transformed using competent *Escherichia coli* DH10B. Results of direct isolation from roots are provided only when they extend the host range of species considered herein.

PCR products were purified using Exonuclease I (NEB, Ipswich, Massachusetts) and (Calf intestinal) Alkaline Phosphatase (NEB, Ipswich, Massachusetts) and incubated at 37 C for 30 min followed by 95 C for 10 min. Cycle sequencing reactions consisted of 4.5 μL cleaned PCR product, 1 μL BigDye Terminator v3.1 (Applied Biosystems, Foster City, California), 2 μL 5X BDT buffer, 3 mmol sequencing primer (forward or reverse), and water to final volume 15 μL. Sequencing was undertaken on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, California). All sequences were edited and checked manually using SEQUENCHER 5.4.1 (Gene Codes Corp., Ann Arbor, Michigan). Multiple sequence alignments were constructed using GENEIOUS 10.2.6 (Kearse et al. 2012) and manually checked.

Phylogenetic analysis

Closely related *Tulasnella* sequences identified in a previous study (Cruz et al. 2014), as well as *Tulasnella* obtained from Cryptostylidinae (Nguyen et al. 2019) and Drakaeinae (Linde et al. 2017) were downloaded from NCBI GenBank (SUPPLEMENTARY TABLE 2). Initial phylogenetic analyses of ITS revealed that all fungi belonged to *Tulasnella* Group IV based on Cruz et al. (2014). Therefore, *Tulasnella* Group IV representatives were also added to the ITS dataset. In total, 97 sequences from GenBank (51 ITS, 15 *C14436*, 14 *C4102*, and 17 mtLSU) and 280 sequences from this study (190 ITS, 30 each for *C14436*, *C4102*, and mtLSU) were analyzed. Due to the unavailability of non-ITS sequences of *T. sphagneti* (Linde et al. 2017), we sequenced isolates of *T. sphagneti* (CLM541, CLM587), as well as an additional four *T. sphagneti* isolates obtained from *Cryptostylis* in this study.

Phylogenetic trees were inferred by Bayesian inference (BI) using MRBAYES 3.2.6 (Ronquist and Huelsenbeck 2003). Two parallel runs of four chains each were run for 1.2 million generations and trees sampled every 400 generations after a 10% burn-in. To verify that the burn-in was sufficient, likelihood profiles were examined. Convergence of runs was confirmed when the average standard deviation was <0.012 and effective sample sizes >200. The best-fit substitution model was selected by ModelFinder **(**Kalyaanamoorthy et al. 2017) in IQ-TREE 2.0 according to the Bayesian Information Criterion (Nguyen et al. 2014) for each locus partitioned into coding and intronic regions. The concatenated alignment was partitioned by each locus and their codon positions. Support for nodes was assessed with

Bayesian posterior probabilities (BPP), with BPP>0.95 considered significant. To check BI tree support and topology, a RAxML maximum likelihood (ML) analysis (Stamatakis et al. 2008) was conducted in addition to 1000 bootstrap searches. Phylogenetic trees were inferred for each locus separately, as well as for a concatenated dataset. Congruency among loci was assessed visually based on tree topology. Phylogenetic trees were visualized using FIGTREE 1.4.3. For the ITS, *Tulasnella violea* (Quél.) Bourdot & Galzin was selected as the outgroup based on results presented in Cruz et al. (2014). Trees from other loci and concatenated loci were midpoint rooted due to the unavailability of *T. violea* sequences. Pairwise sequence divergence of the ITS sequences within and among lineages were estimated with a distance matrix in GENEIOUS 10.2.6 (Kearse et al. 2012).

Morphological and culture characteristics

Morphology of cultures and microcharacters were examined for three isolates of each species as identified in the phylogenetic analyses as follows:

Culture morphology

Isolates were grown on four different media; ½ FIM (Clements and Ellyard 1979), 3MN (Wright et al. 2010) + A-Z vitamin (Centrum "Balanced Formula", Wyeth Consumer Healthcare, Baulkham Hills, Australia), E-Medium (Caldwell et al. 1991), and ¼ PDA (Potato Dextrose Agar, Alpha Biosciences, Baltimore, Maryland), all supplemented with streptomycin sulfate 50 mg/mL. Characteristics of the cultures that were recorded include: shape of colony margin, colony color, presence/absence of aerial hyphae, colony texture, growth uniformity, presence/absence of concentric zones, hyphal size and types, and other distinctive features. Morphology was assessed after approximately four wk incubation at 22 C in the dark.

To induce basidiospore formation, we transferred mycelium blocks of the holotype (as representative) from high nutrient media (¼ PDA) to Water Agar (WA) (Stalpers and Andersen 1996). Moniloid cells were induced by transferring the mycelium to Potato Agar (1000 mL of filtrate of 200 g of boiled potato and 15 g agar) as suggested in Fujimori et al. (2019). All cultures were incubated at 22 C in the dark and examined after four wk.

Growth assessment

We used four different media as described above (½ FIM, 3MN, E-Medium, and ¼ PDA, all supplemented with streptomycin sulfate 50 mg/mL) to assess whether *Tulasnella* species differ in growth rate. Prior to transfer to the four designated media, stock isolates were grown on ½ FIM agar + streptomycin (50mg/mL) for approximately two wk. These were transferred to the center of a petri dish of each of the four growth media as a small agar block (5 mm^2). Three replicate plates per isolate per medium were used. All isolates were incubated as above for four wk before colony diameters were measured with calipers. To account for uneven growth of colonies, two perpendicular measurements for colony diameter were made per plate to obtain the average colony diameter per plate. Statistical analysis was performed using a linear-mix model ("LMERTEST" package) in R (R Core Team 2019) and a one-way ANOVA was used to determine significance differences in colony growth between *Tulasnella* species within each medium.

Microscopic characteristics and fluorescence staining of fungal nuclei

At least two isolates per phylogenetic species were examined for their microscopic characteristics and number of nuclei per hypha following Yang et al. (1991). However, here we used Hoechst dye 33342 (ThermoFisher Scientific, Waltham, Massachusetts) instead of 33258. Stock solution of Hoechst 33342 was prepared according to manufacturer's protocol (10 mg/mL or 16.23 mM) with 0.1 M KH_2PO_4 and 0.1 M NaOH pH 7.8, as this pH allowed the best visualisation of nuclei and septa of *Rhizoctonia* (Yang et al. 1991; Fang et al. 2013). Isolates of *T. prima* (CLM2088) and *T. warcupii* (CLM2235) were included for comparison. For nuclear staining, glass slides were coated with PDA. For each isolate a small agar block of mycelium (1 mm^2) was placed on the middle of a slide. A damp filter paper was laid under the slide to maintain humidity. Due to the slow growth of most of the isolates studied here, slides were incubated at 22–23 C for up to two wk in the dark, then air-dried in a laminar flow cabinet for 15 min. A drop of Hoechst 33342 dye working solution was added to the dried hyphae and covered with a cover glass.

Examination of nuclei in hyphal cells was conducted using a Leica fluorescence microscope DM5500B at 40X and 100X magnification. The number of nuclei was counted for at least 20 hyphal cells per isolate. Photographs were taken using an Automated Upright Microscope Leica DM5500B Camera (Leica Microsystems, Wetzlar, Germany). In some cases the mycelium of isolates of *T. occidentalis* (CLM1938, CLM1942) and *T. densa* (CLM2112, CLM2117)) did not grow on glass slides, in which case, we cultured the isolates on liquid cultures of ½ FIM and/or 3MN + A-Z for two wk. A small amount of mycelium was gently pipetted (using 1 mL wide-bore tips) to prevent rupturing the mycelium, then mounted carefully on glass slides. The slides were air-dried and processed as described above for nuclear staining.

RESULTS

Based on the phylogenetic analyses, six new species are described: *Tulasnella australiensis* sp. nov. (64 isolates), *T. occidentalis* sp. nov. (29 isolates), *T. punctata* sp. nov. (28 isolates), *T. densa* sp. nov. (16 isolates), *T. concentrica* sp. nov. (26 isolates), and *T. rosea* sp. nov. (27 isolates) (SUPPLEMENTARY TABLE 1). Direct cloning from roots provided an example of an otherwise undetected host-fungus relationship for *Cryptostylis ovata* and *T. australiensis* (GenBank accession MT320989).

In the ITS phylogeny (FIG. 1), the new species *T. rosea* clustered at the base of the tree sister to all remaining members of the ingroup. Two known species, *T. prima* and *T. sphagneti*, isolated from Australian orchids, clustered with the clades of new isolates with *T. punctata* sister to *T. sphagneti* and *T. prima* sister to *T. concentrica*. Sequences from two undescribed *Tulasnella* species from Western Australia (*Tulasnella* sp. Jarrahdale 1 and Jarrahdale 2 of Nguyen et al. (2019)) formed a clade that was sister to *T. occidentalis*. Sequences from the new species *T. densa* were sister to the clade containing *T. prima*, *T. concentrica, T. occidentalis, T. sphagneti,* and *T. punctata.* Sequences from the previously described species *T. warcupii* clustered with two sequences of an undescribed *Tulasnella* isolated from Australian *Arthrochilus* orchids from Linde et al. (2017) (KF476594 and KF476595). The clade containing the *T. warcupii* sequences was sister to a large clade that contained numerous sequences of the novel species *T. australiensis*. The *T. australiensis* + *T. warcupii* clade was sister to a clade composed of *T. tomaculum* from the U.S. (McCormick et al. 2004) and U.K. (Cruz et al. 2014) and *T. secunda*. The clade containing *T. australiensis*, *T. secunda*, and *T. warcupii* was sister to a larger clade including three clades, two of which contain sequences from undescribed species from Ecuador (ECU5 and ECU6 of Cruz et al. (2014)) and a third clade containing sequences from rotten wood in Canada (JM 2019a DAOMC of Mack and Seifert (2019)).

Several other existing isolates from current and previous *Tulasnella* studies clustered within our newly described species, as evident in a detailed phylogeny (SUPPLEMENTARY FIG. 1). For example, Sommer et al. (2012) identified *Tulasnella* MB-2012 (GenBank accession number JX138568) from *S. ciliata* — this clustered with *T. rosea*. Sommer et al. (2012) also included an unassigned *Tulasnella* sequence (JN015192) from an unspecified Australian terrestrial orchid — this clustered with *T. densa*. Previously Linde et al. (2017) reported a *Tulasnella* symbiont from *Arthrochilus* (KF476602) — this clustered with *T. australiensis*. Furthermore, Nguyen et al. (2019) reported several *Tulasnella* isolates from *C. ovata* — these clustered with either *T. prima* or *T. occidentalis*.

The six new clades that were apparent in the ITS tree were also recovered in the phylogenies based on the other three loci (*C14436*, *C4102*, and mtLSU) when analyzed separately (SUPPLEMENTARY FIGS. 2–4) and concatenated (FIG. 2). All new species were supported by high BPP (1) and bootstrap values (>96) for all loci except mtLSU where the clades comprising isolates of *T. punctata* (0.63, 83) and *T. concentrica* (0.93, 66) were present but with lower support (TABLE 2). Phylogenetic trees of *C14436* (SUPPLEMENTARY FIG. 2) and *C4102* loci (SUPPLEMENTARY FIG. 3) were largely congruent with the overall structure of the ITS phylogenetic tree (FIG. 1). However, in the tree based on the *C4102* locus there was poor support for some relationships (e.g. the clade formed by the *T. prima* and *T. concentrica*) (SUPPLEMENTARY FIG. 3). In addition, the mtLSU locus showed a large polytomy comprising isolates of *T. sphagneti* along with individual clades of *T. prima*, *T. densa*, *T. concentrica*, *T. occidentalis* and *T. punctata* (SUPPLEMENTARY FIG. 4). Phylogenetic trees based on the concatenated 4-locus dataset (ITS, *C14436*, *C4102*, and mtLSU) (mid-point rooted) (FIG. 2) had similar topologies and high support values for most clades in comparison to the tree based on ITS (rooted to *T. violea*) (FIG. 1). However, the concatenated phylogenetic tree showed *T. warcupii* sister to *T. secunda* — which were then sister to *T. australiensis*, whereas the ITS tree showed *T. australiensis* and *T. warcupii* as sister groups — most closely related to *T. secunda*. Using ITS, the percentage sequence divergence threshold (i.e. “barcode gap”) that separated *Tulasnella* species in this study was in the region of 4 to 6% (FIG. 3) with maximum within species divergence ranging from 1.64 to 4.97% (TABLE 3).

Colony morphology on the four media showed slight differences between the six newly described *Tulasnella* species (FIG. 4), as discussed below, and was consistent across isolates of the same species on the most diagnostic medium, 3MN (SUPPLEMENTARY FIG. 5). Average colony diameter on the four media for all six species was 40–60 mm (after 4 wk). *Tulasnella concentrica* and *T. rosea* showed the largest colony diameters (up to 80 mm) on all media except on 3MN, where all species grew comparatively slowly (< 40 mm). Five of the six species showed similar growth on all media after four wk (SUPPLEMENTARY TABLE 3, SUPPLEMENTARY FIG. 6), the exception is *T. australiensis* which had significantly ($P < 0.05$) smaller colony diameters on ½ FIM and ¼ PDA. Attempts to induce basidiospore formation failed and moniliod cells were not observed. All six new *Tulasnella* species examined had binucleate hyphal cells based on fluorescence staining (FIGS. 5–10).

TAXONOMY

Tulasnella australiensis Arifin, T.W. May & Linde, sp. nov.

MycoBank: MB 834516

Typification: AUSTRALIA. NEW SOUTH WALES: isolated from *Cryptostylis erecta*, collected from Forest Road, Nowra, 3 Feb 2017, *A. Arifin CLM1945* (**holotype** RBGV-FCC 190913, preserved in a metabolically inactive state; isotypes: VPRI 43022, MEL 2417116, preserved in a metabolically inactive state).

Etymology: *australiensis* (Latin), referring to occurrence in Australia.

Culture morphology: On ¼ PDA, 33–47 mm diam, off-white above and below, with very thin, appressed surface mycelium, with no growth on inoculum block and most mycelium submerged; no concentric zonation; near margin growth becoming finely dendroid and sparser; margin undulate. Characters on other media the same as ¼ PDA except for ½ FIM. On ½ FIM, cultures with slower growth compared to other media, diameter less than 20 mm, and with 2–3 concentric zones that gradually decrease in the density of mycelium from the center to the edge; no aerial mycelium; growth more irregular at the margin; margin more even and more sparse compared to growth on other media. On 3MN and E-medium similar to ¼ PDA.

Hyphae septate, 3–7 µm diam, length 25–40 µm between septa, cylindrical, thin-walled; terminal elements cylindrical; branched, with branching mostly at right angles, with constriction at base of branch hypha; lacking clamp connections; binucleate (FIG. 5).

Ecology and distribution: Orchidaceae hosts *Cryptostylis erecta, C. subulata, C. leptochila* - New South Wales and Victoria, *C. ovata* - Western Australia (no culturable isolates – directly sequenced from roots), *Arthrochilus oreophilus* - Queensland; Australia. Oct–Mar.

Other specimens examined: AUSTRALIA. NEW SOUTH WALES: isolated from *Cryptostylis erecta*, Forest Road, Nowra, 30 Jan 2018, *A. Arifin CLM2095* (RBGV-FCC 190910, VPRI 43535); isolated from *Cryptostylis erecta*, Erowal Bay, 1 Sep 2018, *A. Arifin CLM2179* (RBGV-FCC 190912, VPRI 43537).

Notes: Overall, *T. australiensis* grew comparatively slowly on three of the media tested but had similar growth rates to other species on 3MN. On ½ FIM the growth did not exceed 20 mm in four wk. The most distinctive feature of *T. australiensis* compared to others is the undulate outline of the colony margin especially on ¼ PDA.

Tulasnella occidentalis Arifin, T.W. May & Linde, sp. nov.
MycoBank: MB 834526

Typification: AUSTRALIA. WESTERN AUSTRALIA: isolated from *Cryptostylis ovata*, collected from Boyanup, 18 Dec 2016, *A. Arifin CLM1938* (**holotype** RBGV-FCC 190916, preserved in a metabolically inactive state; isotypes: VPRI 43538, MEL 2470321, preserved in a metabolically inactive state).

Etymology: *occidentalis* (Latin), referring to the distribution in western Australia.

Culture morphology: Cultures on ¼ PDA, 55–70 mm diam, off-white above and below, with thin layer of appressed growth, most hyphae submerged; growth regular without dendroid appearance; with concentric rings (five or more ring zones) formed by slightly dense mycelium; without minute spirals; margin with sparse growth, even. On ½ FIM and E-medium, cultures show 1–2 concentric zones. On ½ FIM agar, no appressed mycelium is visible and cultures showed sparser growth compared to ¼ PDA and 3MN. On 3MN, aerial hyphae are more obvious compared to other media and there are 2–3 concentric zones.

Hyphae septate, 2–6 µm diam, length 30–50 µm between septa, cylindrical, thin-walled; terminal elements cylindrical; branched, branches mostly at right angles, with constriction at base of branch hypha; lacking clamp connections; binucleate (FIG. 6).

Ecology and distribution: Orchidaceae host *Cryptostylis ovata,* Western Australia, Australia. Nov–Feb.

Other specimens examined: AUSTRALIA. WESTERN AUSTRALIA: isolated from *Cryptostylis ovata*, Rockingham, 10 Jul 2018, *A. Arifin CLM2136* (RBGV-FCC 190915, VPRI 43536, MEL 2470322); isolated from *Cryptostylis ovata*, Rockingham, 10 Jul 2018, *A. Arifin CLM2137* (RBGV-FCC 190914, VPRI 43539, MEL 2470323).

Notes: *T. occidentalis* had a similar growth rate on the four media tested. On 3MN, cultures of this species showed the growth is strongly zoned. Other species on 3MN have more sparse growth towards the margin. Nine sequences labelled as the Bertram clade from a previous study (Nguyen et al. 2019) clustered within *T. occidentalis*.

Tulasnella punctata Arifin, T.W. May & Linde, sp. nov.
MycoBank: MB 834529

Typification: AUSTRALIA. NEW SOUTH WALES: isolated from *Cryptostylis subulata*, collected from Frenchs Forest, 14 Jun 2017, *A. Arifin CLM2017* (**holotype** RBGV-FCC 190918, preserved in a metabolically inactive state; isotypes: VPRI 43540, MEL 2469724, preserved in a metabolically inactive state).

Etymology: Referring to the morphology of small denser spots in colonies on agar plates.

Culture morphology: On ¼ PDA, 38–67 mm diam, off white above and below, with very thin appressed surface mycelium, with no growth on inoculum block and most mycelium submerged, sparser near margin, with 5–6 randomly occurring small denser spots (1–5 mm diam); with 10 or more concentric zones; occasionally with minute spirals; margin even. On ½ FIM and E-medium, similar to ¼ PDA except with at least two concentric zones and growth very sparse, without denser spots. On 3MN, cultures with the most dense spots; margin undulate.

Hyphae septate, 3–5 µm diam, length 40–50(–60) µm between septa, cylindrical, rarely with fusoid cells, thin-walled; terminal elements cylindrical, rarely clavate; branched, branches mostly at right angles, with constriction at base of branch hypha; lacking clamp connections; binucleate (FIG. 7).

Ecology and distribution: Orchidaceae host *Cryptostylis subulata*, New South Wales, Australia. Oct–Mar.

Other specimens examined: AUSTRALIA. NEW SOUTH WALES: isolated from *Cryptostylis subulata*, Erowal Bay, 1 Sep 2018, *A. Arifin CLM2176* (RBGV-FCC 190919, VPRI 43541, MEL 2469725); isolated from *Cryptostylis subulata*, Frenchs Forest, 14 Jun 2017, *A. Arifin CLM2018* (RBGV-FCC 190920, VPRI 43516, MEL 2469726).

Notes: Growth of *T. punctata* was relatively similar on the four media tested. On the most diagnostic medium 3MN, *T. punctata* showed more obvious denser spots. Other species do not have this distinctive morphological characteristic.

Tulasnella densa Arifin, T.W. May & Linde, sp. nov.

MycoBank: MB 834530

Typification: AUSTRALIA. NEW SOUTH WALES: isolated from *Cryptostylis hunteriana*, collected from Meroo National Park, 4 Dec 2017, *A. Arifin CLM2117* (**holotype** RBGV-FCC 190923, preserved in a metabolically inactive state. Isotypes: VPRI 43511, preserved in a metabolically inactive state, MEL 2469735).

Etymology: *densa* (Latin), referring to the most dense mycelium growth on agar plates among newly described *Tulasnella* species in this study.

Culture morphology: on ¼ PDA, 73–79 mm diam, off-white in colour on both sides, with thin layer of appressed hyphae but most hyphae submerged; growth regular and radial without dendroid pattern; no concentric zones; minute spirals present on surface; margin even, with slightly denser growth. On E-medium and 3MN, cultures showed denser hyphae with undulate margin, otherwise similar to ¼ PDA. On ½ FIM, mycelium is mostly submerged with very thin appressed growth; growth generally sparse in a radial pattern, dendroid towards the margin; concentric rings with more than five zones present; minute spirals absent; margin even.

Hyphae septate, width 4–6 µm diam, length 20–45 µm between septa, cylindrical, thin-walled; terminal elements often clavate; with granules in almost all cell bodies; rarely with small peg-like protuberances; branched, branches mostly at right angles, with constriction at base of branch hypha; lacking clamp connections; binucleate (FIG. 8).

Ecology and distribution: Orchidaceae host *Cryptostylis hunteriana*, New South Wales, Australia. Dec–Feb.

Other specimens examined: AUSTRALIA. NEW SOUTH WALES: isolated from *Cryptostylis hunteriana*, Meroo National Park, 4 Dec 2017, *A. Arifin CLM2112* (RBGV-FCC 190922, VPRI 43512, MEL 2469734); isolated from *Cryptostylis hunteriana*, Meroo National Park, 4 Dec 2017, *A. Arifin CLM2118* (RBGV-FCC 190921, VPRI 43514, MEL 2469733).

Notes: Growth on E-medium and ½ FIM was similar to other species, but growth on ¼ PDA was comparatively fast and that on 3MN comparatively slow. Generally, among the novel species *T. densa* showed the most dense hyphal growth on all media tested except on ½ FIM. One previously reported isolate (*Tulasnella* BB0002_2_A, GenBank accession number JN015192; Howard & Clements 2011, unpublished) belongs to *T. densa*.

Tulasnella concentrica Arifin, T.W. May & Linde, sp. nov.

MycoBank: MB 834531

Typification: AUSTRALIA. NEW SOUTH WALES: isolated from *Cryptostylis leptochila*, collected from Morton National Park, 13 Oct 2017, *A. Arifin CLM2071* (**holotype** RBGV-FCC 190925, preserved in a metabolically inactive state. Isotypes: VPRI 43504, preserved in a metabolically inactive state, MEL 2469728).

Etymology: *concentrica* (Latin), referring to the multiple concentric zones of cultures on all culture media.

Culture morphology: On ¼ PDA, 71–79 mm diam, off-white in colour on both sides, with thin layer of appressed growth but most hyphae submerged; growth regular and radial, without dendroid pattern; concentric rings present, with more than 10 zones; minute spirals absent; margin even; mycelium moderately dense. On E-medium, similar to ¼ PDA. On ½ FIM similar to ¼ PDA but growth sparser; concentric rings with five or more zones present. On 3MN, colonies are the slowest growing among all media (35–41 mm diam.), with dense hyphal growth in the center surrounded by sparser mycelium.

Hyphae septate, width (2.5–)3–4 µm diam, length between septa 30–40 µm, cylindrical, thin-walled; not clavate at terminal ends; branched, with branches usually at right angles; with constriction at base of branch, rarely with swollen element arising from the branch; lacking clamp connections; binucleate (FIG. 9).

Ecology and distribution: Orchidaceae host *Cryptostylis leptochila,* New South Wales and Victoria, Australia. Nov–Apr.

Other specimens examined: AUSTRALIA. NEW SOUTH WALES: isolated from *Cryptostylis leptochila,* Morton National Park, 13 Oct 2017, *A. Arifin CLM2072* (RBGV-FCC 190926, VPRI 43502, MEL 2469729); isolated from *Cryptostylis leptochila*, Morton National Park, 13 Oct 2017, *A. Arifin CLM2142* (RBGV-FCC 190927, VPRI 43503, MEL 2469730).

Notes: Compared to the other species, *T. concentrica* had faster growth on all media, except for 3MN, where growth was similar to other species. The most distinctive feature of the culture morphology of *T. concentrica* is the presence of a strong pattern of concentric zones on all media tested. The growth seemed erratic on 3MN but the concentric zones are still noticeable.

Tulasnella rosea Arifin, T.W. May & Linde, sp. nov.

MycoBank: MB 834532

Typification: AUSTRALIA. WESTERN AUSTRALIA: isolated from *Spiculaea ciliata*, collected from Boulder Rock, 17 Aug 2016, *C. Linde CLM1773* (**holotype** RBGV-FCC190929, preserved in a metabolically inactive state. Isotypes: VPRI 43508, preserved in a metabolically inactive state, MEL 2470318).

Etymology: *rosea* (Latin), referring to the rose pink color on agar cultures.

Culture morphology: On ¼ PDA, 72–79 mm diam, underside and upperside pink in colour, almost no aerial mycelium including around mycelium block, most mycelium submerged, with radially dendroid growth pattern; no concentric zonation; minute spirals absent; margin even and with growth sparser near the margin;. On ½ FIM and E-medium similar to ¼ PDA but colour less pink and mycelium sparser. On 3MN growth slower (26–46 mm diameter) and denser compared to the other three media.

Hyphae septate, 4–6 µm diam, length between septa 30–40 µm long, cylindrical, sometimes with swollen element (to 8 µm in diam), thin-walled; terminal elements mainly cylindrical, sometimes clavate; branched, with branches often at right angles, with constriction at base of branch; lacking clamp connections; binucleate (FIG. 10).

Ecology and distribution: Orchidaceae host *Spiculaea ciliata*, Western Australia, Australia. Sep–Oct.

Other specimens examined: AUSTRALIA. WESTERN AUSTRALIA: isolated from *Spiculaea ciliata*, Boulder Rock, 17 Aug 2016, *C. Linde CLM1771* (RBGV-FCC 190928, VPRI 43507, MEL 2470317); isolated from *Spiculaea ciliata*, Boulder Rock, 17 Aug 2016, *C. Linde CLM1773* (RBGV-FCC 190929, VPRI 43508, MEL 2470318); isolated from *Spiculaea ciliata* collected from Boulder Rock, 17 Aug 2016, *C. Linde CLM1774* (RBGV-FCC 190930, VPRI 43506, MEL 2470319).

Notes: *Tulasnella rosea* showed a similar growth pattern to *T. concentrica* but with comparatively low growth on 3MN. On the most diagnostic medium 3MN, growth seemed repressed compared to other media. On all tested media cultures were distinctly pink but the colour is more obvious on ¼ PDA and when cultures are older (more than 6 wk). A sequence labelled *Tulasnella* MB-2012 (GenBank accession number JX138568) from Martos et al. (2012) clustered with *T. rosea.*

DISCUSSION

This study is the first to formally describe species of *Tulasnella* associated with Australian *Cryptostylis* and *Spiculaea* orchids, using both morphological and molecular characterization. *Tulasnella* is a common mycorrhizal symbiont in Drakaeinae orchids, including *Arthrochilus*, *Caleana* (Ruibal et al. 2013; Linde et al. 2014), *Chiloglottis* (Roche et al. 2010), *Drakaea* (Linde et al. 2014; Linde et al. 2017) and *Spiculaea* (Sommer et al. 2012). A recent study showed that *Cryptostylis ovata* (Diurideae: Cryptostylidinae) also associates with an undescribed species of *Tulasnella* (Nguyen et al. 2019). Despite orchids studied here belonging to two subtribes Drakaeinae and Cryptostylidinae (Kores et al. 2001; Clements et al. 2015), their mycorrhizal associations are with closely-related *Tulasnella* species, all belonging to *Tulasnella* phylogenetic Group IV (Cruz et al. 2014).

Use of several unlinked loci for species delimitation is recommended for fungal species difficult to distinguish morphologically (Taylor et al. 2000). Here we used multilocus (two nuclear and two mitochondrial loci) phylogenetic analyses and found that species delimited for individual loci and the combined four locus matrix were largely congruent (FIGS. 1, 2) similar to Linde et al. (2014) using the same loci. All six newly described *Tulasnella* species were delimited with high support values for species-level clades across three out of the four individual loci. The exception was mtLSU, where species-level clades were only moderately supported for three of the six novel species. In addition, one of the previously described species (*T. sphagneti*) did not form a distinct clade in the mtLSU analysis, but the individual sequences were part of a polytomy. However, for these exceptions, there was no contradiction of the placement of sequences within their respective phylogenetic species (SUPPLEMENTARY FIG. 4). Lower phylogenetic resolution using mtLSU was not surprising, as Linde et al. (2014, 2017) found a similar trend elsewhere in *Tulasnella*.

ITS is the most commonly used sequence locus in Dikarya and generally provides robust species-level resolution (Schoch et al. 2012). In this study, phylogenetic analysis of an ITS-only dataset provided well-supported clades and confirmed that this locus can be used to distinguish species of *Tulasnella* at a threshold of 4–6%, similar to the ~3.3–5.7% threshold previously suggested for other species in the genus (Linde et al. 2017) and the 4–6% threshold for *Serendipita* P. Roberts in the Sebacinales (Whitehead et al. 2017). Overall, divergence thresholds useful for operational recognition of *Tulasnella* species are higher than the universal 3% proposed by Nilsson et al. (2008).

Sequences of the four new species described by Linde et al. (2017) from Drakaeinae (FIG. 1, SUPPLEMENTARY FIG. 1) were quite distinct from the new taxa described here. Furthermore, Warcup and Talbot (1967, 1971, 1980) described three species of *Tulasnella* from Australian orchids. However, the hosts for the original material of these species are not from Drakaeinae: *Tulasnella asymmetrica* Warcup & P.H.B Talbot was described from *Thelymitra* (Thelymitrinae), *T. cruciata* Warcup & P.H.B Talbot from *Acianthus* (Acianthinae), and *T. irregularis* Warcup & P.H.B Talbot from *Dendrobium* (Warcup and Talbot 1967, 1971, 1980). It will be important to typify these three species, and associate the types with reference sequences, especially when investigating *Tulasnella* associated with other tribes of Australian orchids. However, given the host-specificity of the phylogenetically-defined *Tulasnella* species from Australian orchids in this and previous studies (Linde et al. 2017), these three names clearly do not apply to any of the novel species we have introduced here. Warcup and colleagues did subsequently record two of their novel *Tulasnella* species from members of the Diurideae (see Table 3 of Linde et al. 2017), with *T. asymmetrica* reported from *Chiloglottis* and *Cryptostylis* and *T. cruciata* from *Chiloglottis*, based on features of the sexual spores. Surviving cultures identified by Warcup as *T. asymmetrica* were all isolated from *Thelymitra*, and those that have been sequenced do not fall within the clade containing novel species from the Diurideae. Furthermore, isolates of *T. asymmetrica* from Warcup (1981) were separated into two clades; *T. asymmetrica* and *'T. asymmetrica'* based on phylogenetic divergence (Cruz et al. 2014). However, both *T. asymmetrica* clades fell into *Tulasnella* phylogenetic Group III based on Cruz et al. (2014), and do not match any new isolates in this study that are of *Tulasnella* phylogenetic Group IV. Therefore, there are no surviving cultures for Warcup isolates from orchid hosts in the Diurideae, and based on current knowledge of phylogenetic species and their host associations in *Tulasnella*, the identification of *Cryptostylis* isolates as *T. asymmetrica* by Warcup should be regarded as misidentifications.

We assessed morphological characters to find diagnostic features for the six new *Tulasnella* species described here. However, basidiospores were not observed despite one month growing on water agar. In addition, moniloid cells were not detected while growing the isolates on potato agar, which was effective to induce moniloid cells in some species of *Tulasnella* (Fujimori et al. 2019). Nevertheless, some morphological features such as dense hyphae (*T. densa*), hyphal dense spots in cultures (*T. punctata*), multiple concentric zones (*T. concentrica*), and pink coloured hyphae (*T. rosea*) could be used to aid in species identification (FIG. 4, SUPPLEMENTARY FIG. 5). However, it is necessary to delimit *Tulasnella* based on molecular characterization, and the morphological features alone are not sufficient to delimit species in the absence of basidiospores (Cruz et al. 2014) or fruiting bodies (Suarez et al. 2006; Cruz et al. 2011). Thus, morphological characteristics described here have little taxonomical value.

All six *Tulasnella* species described here (FIGS. 5–10), previously described *T. prima* and *T. warcupii* (SUPPLEMENTARY FIG. 7), as well as all *Tulasnella* studied to date are binucleate (Andersen 1996; Pereira et al. 2003; Almeida et al. 2007; Rachanarin et al. 2018; Fujimori et al. 2019). Therefore, *Tulasnella* appears to be almost always binucleate, with the only known exception being *Tulasnella tubericola* K. Solís et al., which showed bi- or trinucleate hyphae (Solís et al. 2017). By contrast, *Ceratobasidium* D. P. Rogers (Cantharellales, Ceratobasidiaceae G. W. Martin) (Sharon et al. 2008) and *Serendipita* (Andersen 1996), both orchid mycorrhizal fungi, vary from having uninucleate, binucleate, or even multinucleate hyphal cells.

Interestingly, *Arthrochilus* and *Cryptostylis* orchids are considered distantly-related genera (Miller and Clements 2014), yet they share phylogenetically related *Tulasnella* symbionts. An undescribed *Tulasnella* isolate (CLM031) previously identified from *A. oreophilus* (GenBank accession number KF476602) (Linde et al. 2017) has been identified as *T. australiensis* in this study, which was otherwise isolated from *Cryptostylis erecta*, *C. subulata*, and *C. leptochila*. *Tulasnella australiensis* is sister to a clade comprised of *T. warcupii* (with sequence divergence 15.1–19.5 %) and another undescribed *Tulasnella* species from *Arthrochilus* (GenBank accession numbers KF476594 and KF476595). Considering the distribution and host associations of *T. australiensis* in this study, this species is the only broadly distributed *Tulasnella* with multiple orchid hosts, and occurs through Queensland (*A. oreophilus*, see Linde et al. 2014), eastern Australia (*C. erecta*, *C. subulata*, *and C. leptochila*), and western Australia (*C. ovata*, direct sequencing from roots only, see SUPPLEMENTARY FIG. 1).

In contrast to *T. australiensis*, which associates with several orchid species and has a wide distribution, the other newly described *Tulasnella* species in this study show a restricted host association. For example, *T. occidentalis* was only found in Western Australia from *C. ovata* (also see Nguyen et al. 2019, GenBank numbers MK430439, MK430440, MK430444, and MK430445). It is evident that there are two further un-named *Tulasnella* isolated from *C. ovata* (Nguyen et al. (2019), currently designated *Tulasnella* sp. Jarrahdale 1 (GenBank accession number MK430451 and MK430452) and Jarrahdale 2 (GenBank accession number MK430461 and MK430468), thus adding to the *Tulasnella* species diversity associated with *Cryptostylis* (SUPPLEMENTARY FIG. 1).

Three *Tulasnella* species in this study were restricted to eastern Australian *Cryptostylis* hosts. *Tulasnella concentrica* was shown to associate with *C. leptochila* only. A *Tulasnella* sequence from an unspecified Australian terrestrial orchid (GenBank accession number JN015192) clustered together with all *T. densa* isolates from *C. hunteriana* in this study and likely was from *C. hunteriana*. *Tulasnella punctata* mostly associated with *C. subulata*, with the one exception of an isolate obtained from *C. erecta*. In addition, sequences of all isolates from *Spiculaea*, including one from a previous study (Sommer et al. 2012) grouped into a single clade, *T. rosea*, which is phylogenetically separated from all other *Tulasnella* species from *Cryptostylis*. These highly specific relationships do frequently occur in Australian orchids. For example, the orchid genus *Drakaea* is considered to have a highly specific fungal interaction, with nine *Drakaea* species associating with only *T. secunda* (Phillips et al. 2011; Linde et al. 2017). Furthermore, *Chiloglottis* orchids also show a high degree of fungal specificity, with approximately 18 *Chiloglottis* species associating with only *T. sphagneti* and *T. prima* (Roche et al. 2010; Linde et al. 2017).

In summary, six formally-described *Tulasnella* species from Australian orchids extend the diversity of *Tulasnella* fungi in Australia. Phylogenetic-based analyses provide strong evidence to delimit cryptic species in *Tulasnella* and other fungi that are challenging to distinguish based on culture morphology alone. Morphological characteristics are consistent with the delimitation of new species and show some distinctive features for individual species. By delimiting and naming these *Tulasnella* species, we provide biologically meaningful and identifiable units to be used in further research on the fungi and their relationships with orchids.

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TABLE 1. Primers and PCR conditions used in this study.

Locus or gene region (primers)	Forward	Reverse	PCR condition	References
ITS (ITS5, ITS4)	5′-TCCTCCGCTTATTGATATGC-3′	5′-GGAAGTAAAAGTCGTAACAAGG-3′	95 C for 10 min; 35 cycles of 95 C for 15 s, 52 C for 30 s, and 72 C for 1 min; 72 C for 7 min; and 11 C on hold	White et al. 1990
ATP Mitochondrial (C14436F, C14436R)	5′-ATGGACGGTACYGADGGTCTYG-3′	5′-CACGGAAGTAYTCNGCRATGG-3′	94 C for 3 min; 12 cycles (touchdown) of 94 C for 30 s, 66 C for 40 s (-3 C/second cycle), 72 C for 1 min; 30 cycles of 94 C for 30 s, 48 C for 40 s, 72 C for 1 min; 72 C for 20 min; and 11 C on hold	Ruibal et al. 2013
Glutamate synthase (C4102F, C4102R)	5′-ATCAARTAYGCTGGCCTKCCTTGG-3′	5′-CGRCCGCCWGTCATGTACTCGCA-3′		Ruibal et al. 2013
Mitochondrial large subunit (MLIN3, ML6)	5′-CACAGGTTCGTAGGTAG-3′	5′-CAGTAGAAGCTGCATAGGGTC-3′	95 C for 2 min; 35 cycles of 95 C for 1 min, 55 C for 1 min, and 72 C for 1 min; 72 C for 10 min; and 11 C on hold	Bruns et al. 1998

TABLE 2. Posterior probability / bootstrap support values for clades of six new *Tulasnella* species across four loci when analysed separately and concatenated.

	ITS	**C14436**	**C4102**	**mtLSU**	**Concatenated loci**
Tulasnella australiensis	1/100	1/100	1/100	1/97	1/100
Tulasnella occidentalis	1/99	1/100	1/100	0.90/91	1/100
Tulasnella punctata	1/100	1/100	1/100	0.63/83	1/100
Tulasnella densa	1/100	1/100	1/100	1/100	1/100
Tulasnella concentrica	1/98	1/99	1/97	0.93/66	1/100
Tulasnella rosea	1/100	1/100	1/98	1/100	1/100

TABLE 3. Maximum percentage pairwise sequence divergence within species and range (minimum-maximum) of percentage pairwise sequence divergence between species, for the ITS region of *Tulasnella* species.

	Max. within species	***T. sphagneti***	***T. warcupii***	***T. prima***	***T. australiensis* sp. nov**	***T. occidentalis* sp. nov**	***T. punctata* sp. nov**	***T. densa* sp. nov**	***T. concentrica* sp. nov**
T. sphagneti									
T. warcupii		19.3 – 20.2							
T. prima		9.3 – 10.9	20.0 – 21.2						
***T. australiensis* sp. nov**	4.97	19.7 – 23.6	15.1 – 19.5	18.7 – 23.3					
***T. occidentalis* sp. nov**	1.73	8.2 – 9.8	19.8 – 21.7	6.7 – 8.9	19.0 – 22.7				
***T. punctata* sp. nov**	1.64	8.9 – 9.9	8.9 – 22.2	11.5 – 13.6	20.9 – 25.2	9.7 – 11.5			
***T. densa* sp. nov**	1.69	16.8 – 18.4	19.2 – 20.6	16.7 – 20.1	18.4 – 22.1	16.5 – 18.2	18.7 – 20.8		
***T. concentrica* sp. nov**	3.24	9.8 – 11.3	19.8 – 21.8	6.5 – 8.9	18.3 – 22.2	5.6 – 7.5	11.6 – 13.3	17.3 – 19.4	
***T. rosea* sp. nov**	3.08	22.0 – 22.9	20.2 – 22.7	16.9 – 23.7	18.3 – 22.6	16.0 – 23.1	22.6 – 24.5	17.7 – 19.6	21.5 – 23.8

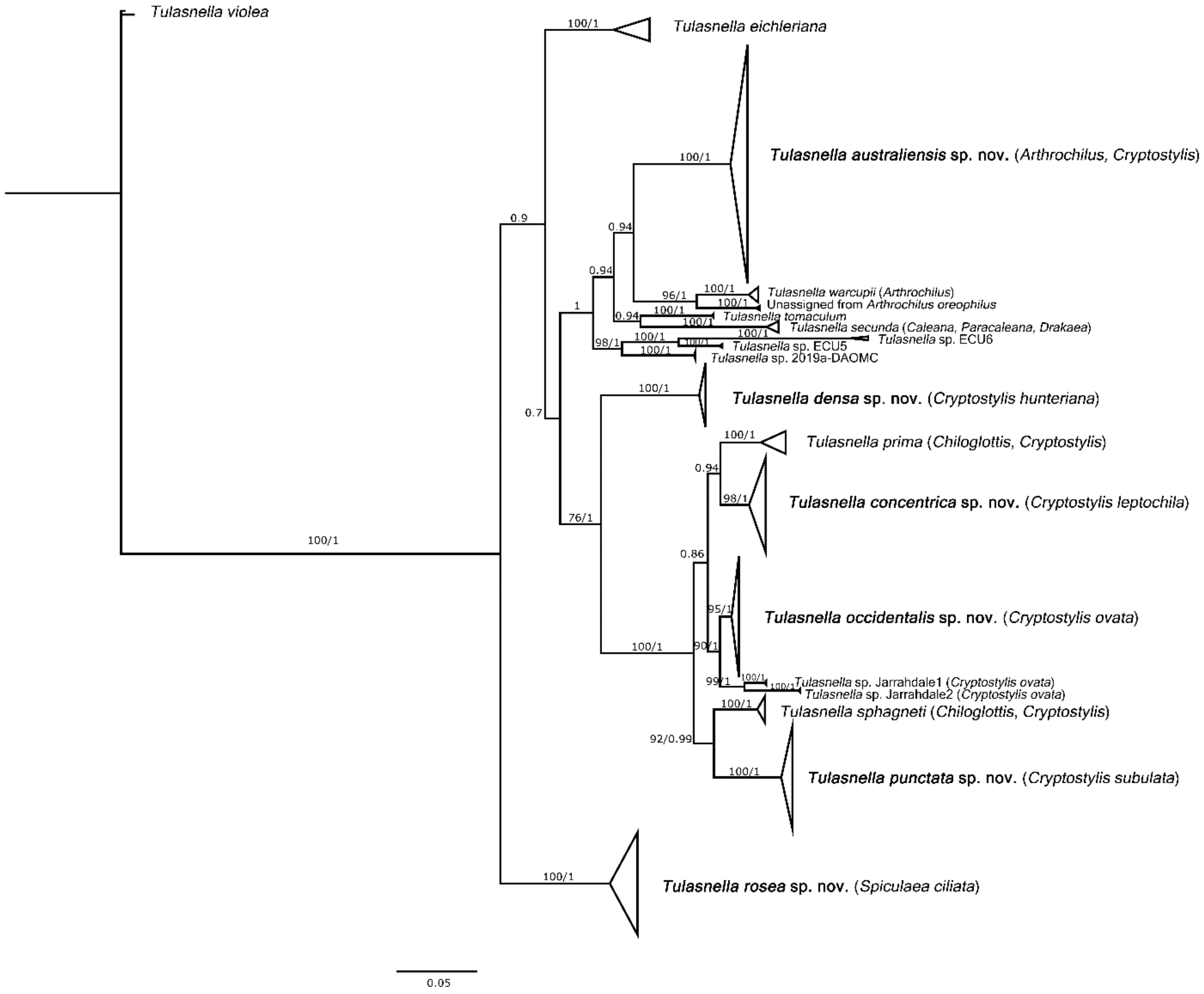


FIGURE 1. BI tree for *Tulasnella* using the ITS region. Numbers on the branches are bootstrap (>70%) / BPP (>0.70) support values.

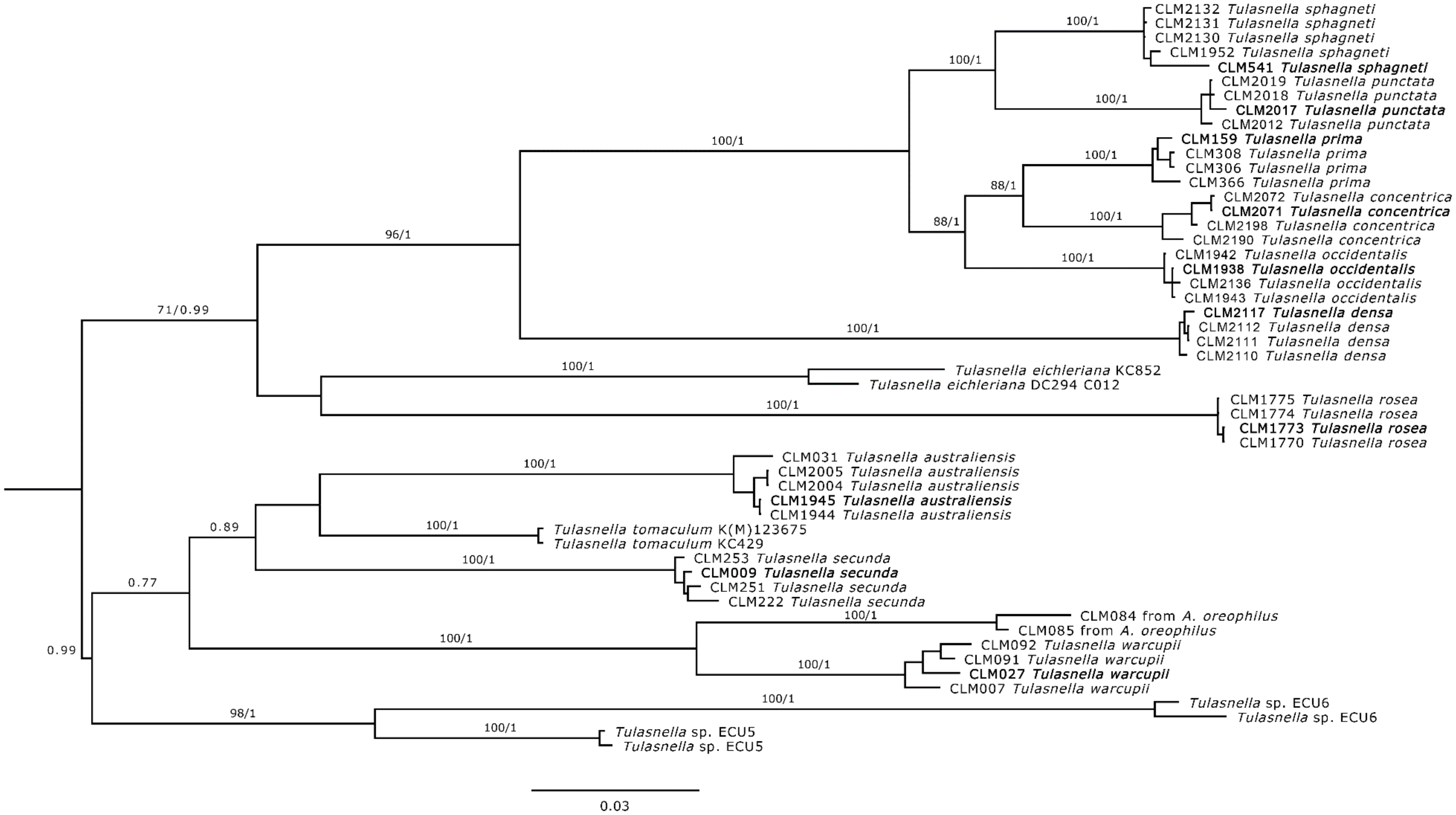


FIGURE 2. BI tree for *Tulasnella* using four concatenated loci: ITS, *C14436*, *C4102*, and mtLSU region. Numbers on the branches are bootstrap (≥70%) / BPP (≥0.70) support values. Sequences derived from type cultures shown in bold.

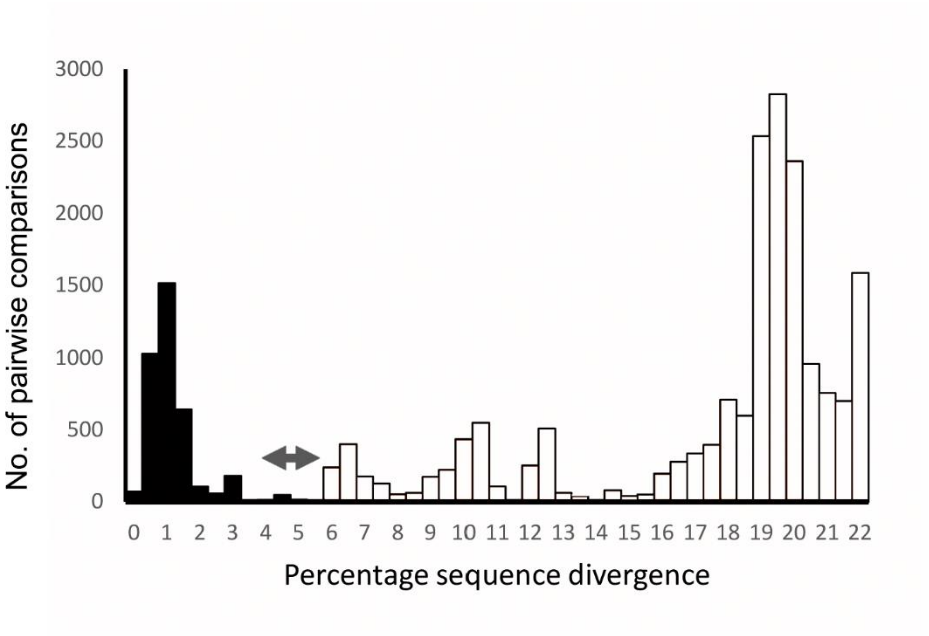


FIGURE 3. Percentage sequence divergence within and between isolates of six species of Australian *Tulasnella* from orchids. The arrow shows the 4–6% ITS sequence divergence gap. Intraspecific values are shown in black.

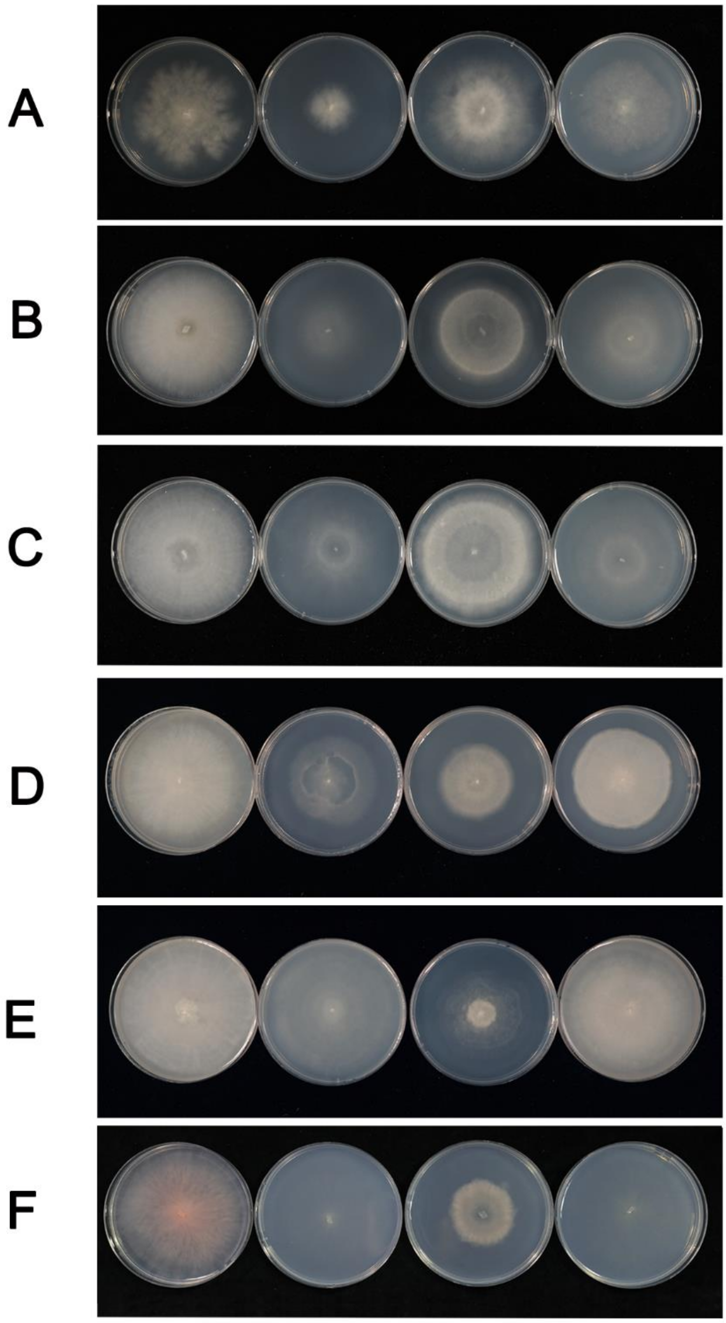


FIGURE 4. *Tulasnella* cultures on (from left to right) ¼ PDA, ½ FIM, 3MN+vitamin, and E-medium; all supplemented with streptomycin sulfate 50 mg/mL. A. *Tulasnella australiensis* (CLM1945); B. *Tulasnella occidentalis* (CLM1938); C. *Tulasnella punctata* (CLM2017); D. *Tulasnella densa* (CLM2118); E. *Tulasnella concentrica* (CLM2071); F. *Tulasnella rosea* (CLM1773).

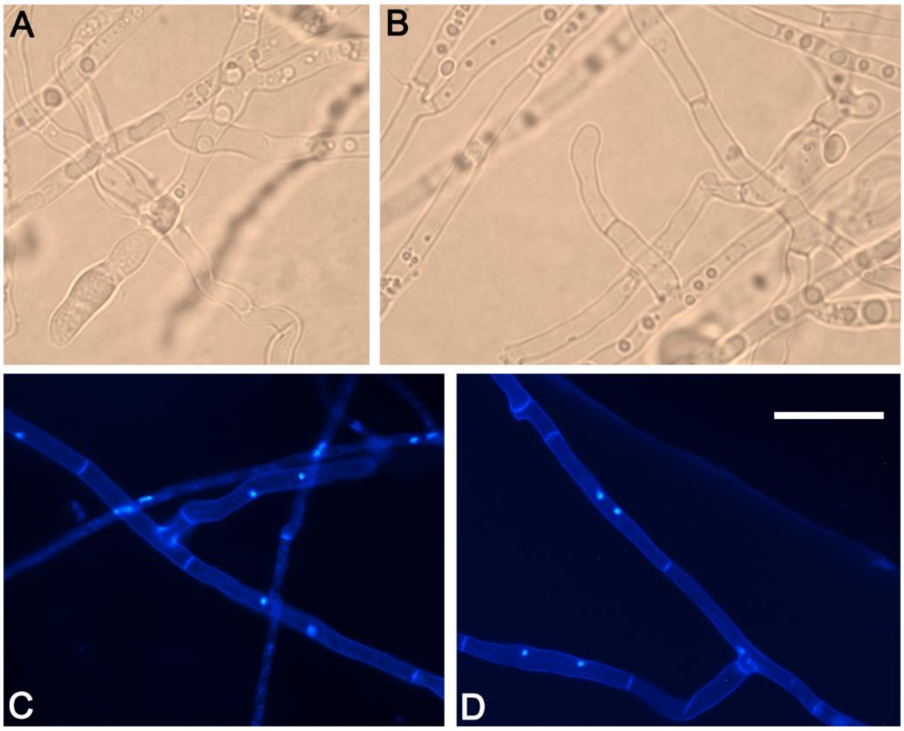


FIGURE 5. Micromorphological features of *Tulasnella australiensis* showing septate and cylindrical hyphae, with cylindrical terminal elements (A–B); branches mostly at right angles with constrictions, and binucleate (C–D). Bar = 20 µm.

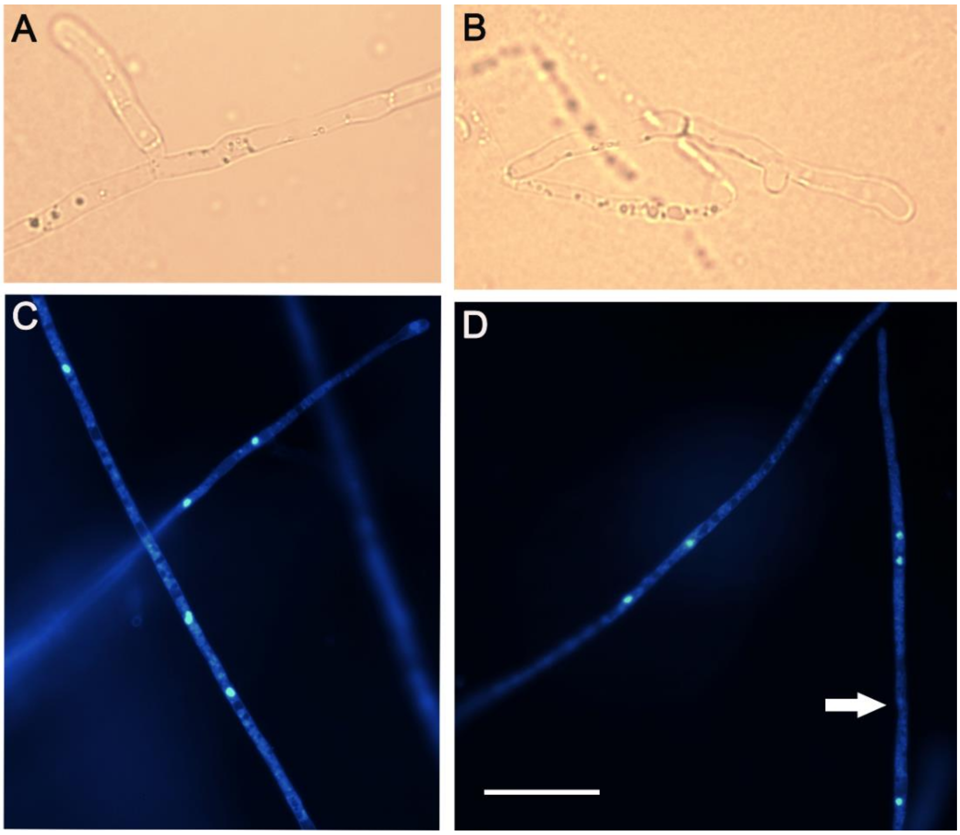


FIGURE 6. Micromorphological features of *Tulasnella occidentalis* showing septated and cylindrical hyphae, with cylindrical terminal elements, branches mostly at right angles with constrictions (A–B), and runner hyphae which are binucleate (C–D, septum shown by white arrow). Bar = 20 µm.

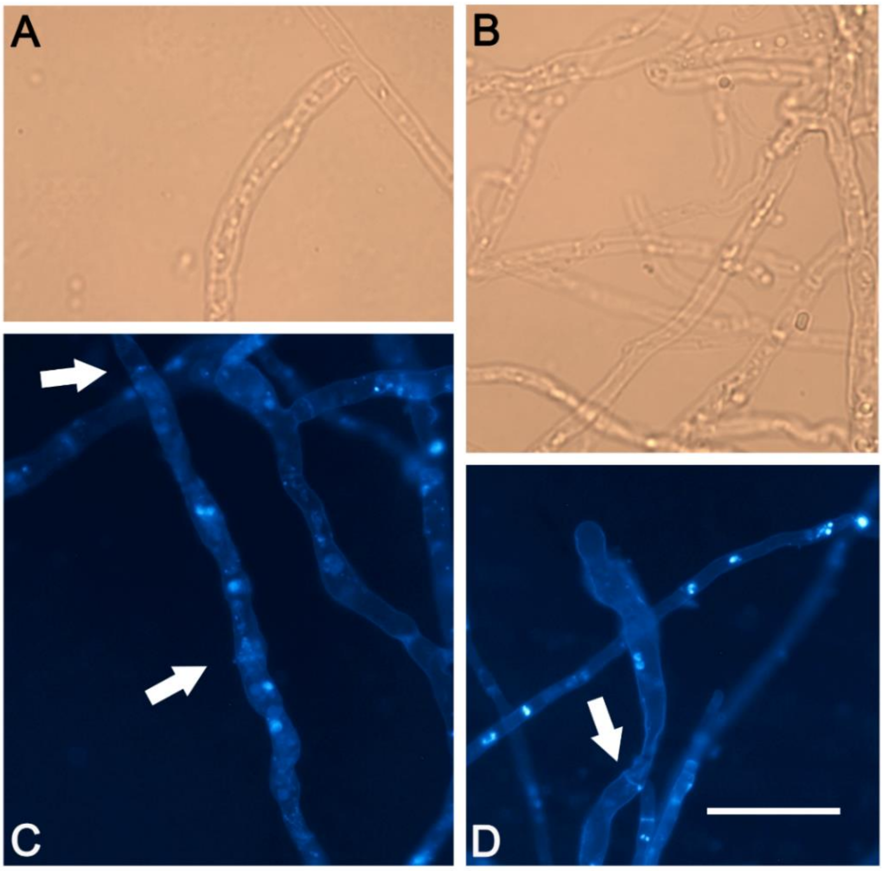


FIGURE 7. Micromorphological features of *Tulasnella punctata* showing branches mostly at right angles with constrictions, septate and cylindrical hyphae (A–B), with cylindrical terminal elements (D); and binucleate (C–D). Septa shown by white arrows. Bar = 20 µm.

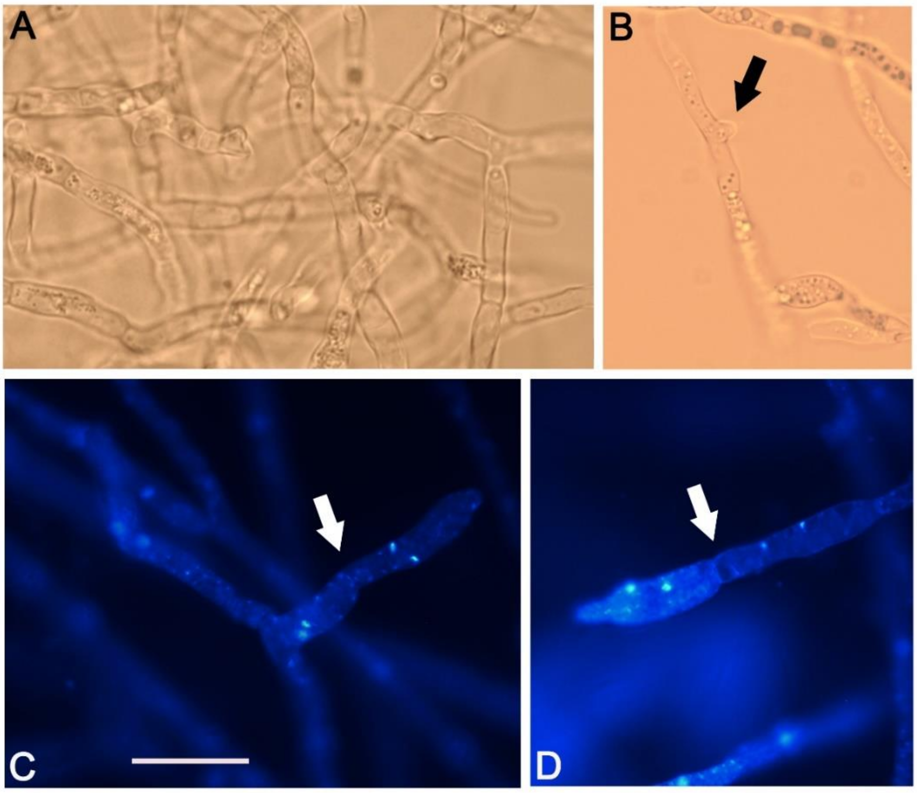


FIGURE 8. Micromorphological features of *Tulasnella densa* showing branches mostly at right angles, cylindrical hyphae with constrictions (A), septate (C–D, white arrows), with cylindrical terminal elements (C–D); and binucleate (C–D). Also rarely seen peg-like protuberance (B, black arrow). Bar = 20 µm.

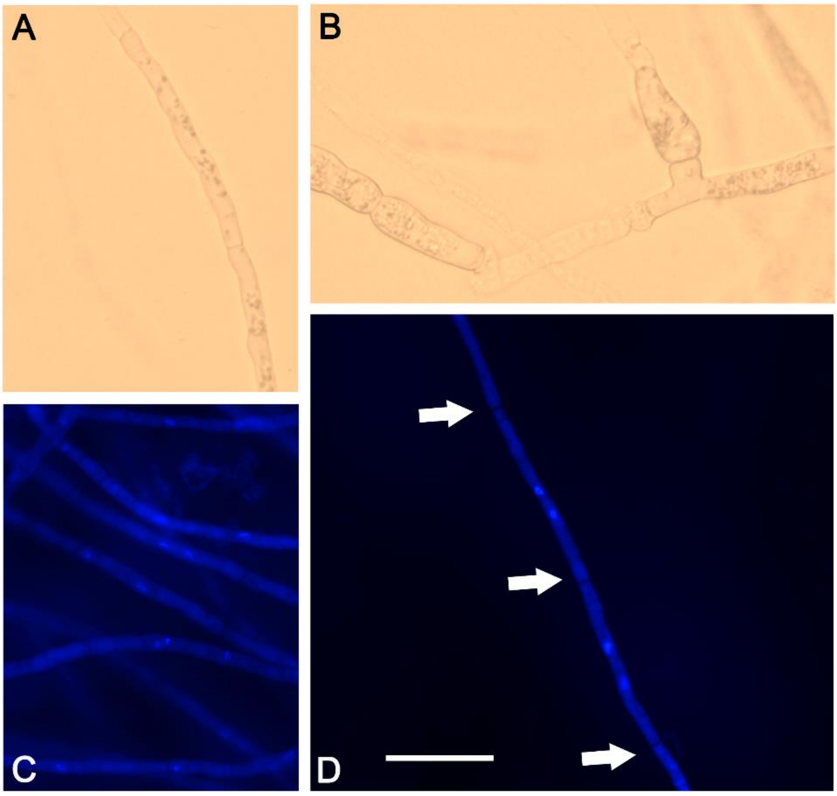


FIGURE 9. Micromorphological features of *Tulasnella concentrica* showing branches mostly at right angles, cylindrical hyphae with constrictions (B), septate (C–D, white arrows), and binucleate (C–D). Bar = 20 µm.

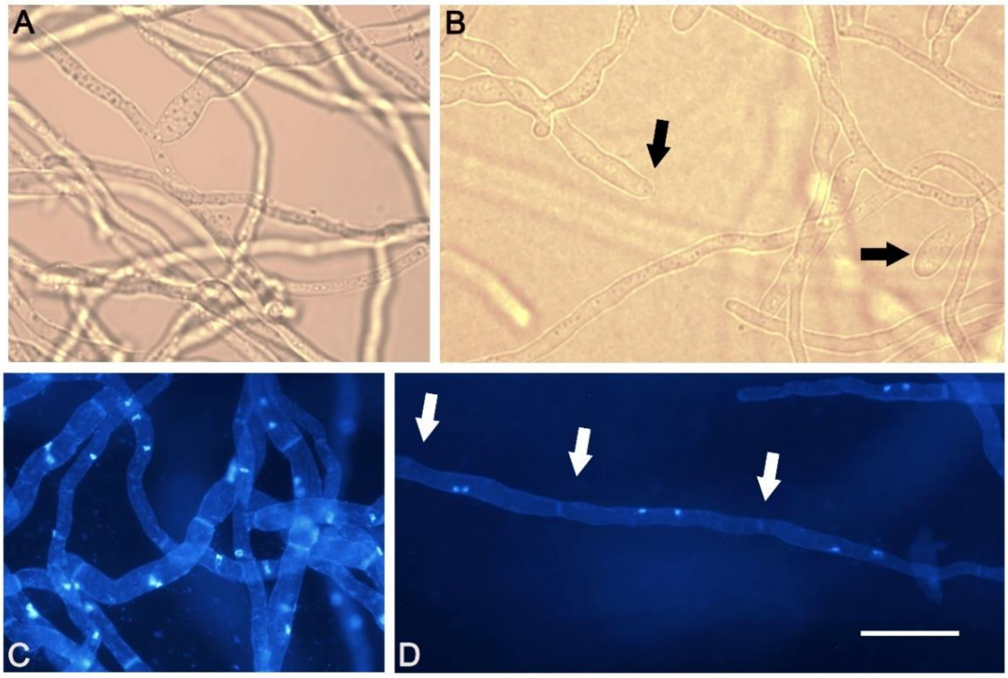


FIGURE 10. Micromorphological features of *Tulasnella rosea* showing branches mostly at right angles and cylindrical hyphae with constrictions (A, B), septate (C–D, white arrows), terminal elements cylindrical, sometimes clavate (B, black arrows), and binucleate (C–D). Bar = 20 µm.

SUPPLEMENTARY MATERIALS

SUPPLEMENTARY TABLE 1. *Tulasnella* isolates from which ITS sequences were newly generated in this study.

Tulasnella species	Isolate	GenBank ITS accession	Orchid Host	Origin	Habitat
Tulasnella australiensis sp. nov	CLM1647	MT003766	*Cryptostylis erecta*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1648	MT003765	*Cryptostylis erecta*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1649	MT003764	*Cryptostylis erecta*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1650	MT003763	*Cryptostylis erecta*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1651	MT003762	*Cryptostylis erecta*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1652	MT003761	*Cryptostylis erecta*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1656	MT003760	*Cryptostylis erecta*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1657	MT003759	*Cryptostylis erecta*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1660	MT003758	*Cryptostylis erecta*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1661	MT003757	*Cryptostylis erecta*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1662	MT003756	*Cryptostylis erecta*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1663	MT003755	*Cryptostylis erecta*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1664	MT003754	*Cryptostylis erecta*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1667	MT003753	*Cryptostylis erecta*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1668	MT003752	*Cryptostylis erecta*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1670	MT003751	*Cryptostylis leptochila*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1685	MT003750	*Cryptostylis leptochila*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1692	MT003749	*Cryptostylis leptochila*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1693	MT003748	*Cryptostylis leptochila*	Fitzroy Falls, NSW	*Eucalyptus* forest with shrubby understorey
	CLM1944	MT003731	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina

Tulasnella australiensis sp. nov	CLM1945(H)	MT003730	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM1946	MT003729	*Cryptostylis subulata*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM1947	MT003728	*Cryptostylis subulata*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM1953	MT003738	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM1954	MT003737	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM1955	MT003734	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM1956	MT003733	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM1957	MT003736	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM1958	MT003732	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM2004	MT003715	*Cryptostylis erecta*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2005	MT003714	*Cryptostylis erecta*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2006	MT003713	*Cryptostylis erecta*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2007	MT003712	*Cryptostylis erecta*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2008	MT003711	*Cryptostylis erecta*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2009	MT003710	*Cryptostylis erecta*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2010	MT003709	*Cryptostylis erecta*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2011	MT003708	*Cryptostylis erecta*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2013	MT003727	*Cryptostylis erecta*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2014	MT003726	*Cryptostylis erecta*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2073	MT003747	*Cryptostylis leptochila*	Bundanoon, NSW	Eucalypt woodland with shrubby understorey
	CLM2092	MT003746	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM2093	MT003743	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM2094	MT003742	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM2095	MT003735	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina

Tulasnella australiensis sp. nov	CLM2096	MT003767	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM2097	MT003745	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM2098	MT003744	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM2100	MT003741	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM2101	MT003740	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM2102	MT003739	*Cryptostylis erecta*	Nowra, NSW	Spotted gum forest with Casuarina
	CLM2119	MT003725	*Cryptostylis leptochila*	Fitzroy Falls, NSW	Eucalyptus forest with shrubby understorey
	CLM2120	MT003724	*Cryptostylis leptochila*	Fitzroy Falls, NSW	Eucalyptus forest with shrubby understorey
	CLM2121	MT003723	*Cryptostylis leptochila*	Fitzroy Falls, NSW	Eucalyptus forest with shrubby understorey
	CLM2178	MT003722	*Cryptostylis erecta*	Erowal Bay, NSW	Eucalypt and Casuarina woodland
	CLM2179	MT003721	*Cryptostylis erecta*	Erowal Bay, NSW	Eucalypt and Casuarina woodland
	CLM2180	MT003720	*Cryptostylis erecta*	Erowal Bay, NSW	Eucalypt and Casuarina woodland
	CLM2181	MT003719	*Cryptostylis erecta*	Erowal Bay, NSW	Eucalypt and Casuarina woodland
	CLM2182	MT003718	*Cryptostylis erecta*	Erowal Bay, NSW	Eucalypt and Casuarina woodland
	CLM2183	MT003717	*Cryptostylis erecta*	Erowal Bay, NSW	Eucalypt and Casuarina woodland
	CLM2184	MT003716	*Cryptostylis erecta*	Erowal Bay, NSW	Eucalypt and Casuarina woodland
	CLM2185	MT003707	*Cryptostylis erecta*	Carls mountain Rd, NSW	Eucalypt woodland with shrubby understorey
	CLM2186	MT003706	*Cryptostylis erecta*	Carls mountain Rd, NSW	Eucalypt woodland with shrubby understorey
	CLM2187	MT003705	*Cryptostylis erecta*	Carls mountain Rd, NSW	Eucalypt woodland with shrubby understorey
Tulasnella occidentalis sp. nov	CLM1938(H)	MT008096	*Cryptostylis ovata*	Booyanup, WA	Jarrah woodland with Xanthorrea
	CLM1939	MT008095	*Cryptostylis ovata*	Booyanup, WA	Jarrah woodland with Xanthorrea
	CLM1940	MT008094	*Cryptostylis ovata*	Booyanup, WA	Jarrah woodland with Xanthorrea

Tulasnella occidentalis sp. nov	CLM1941	MT008093	*Cryptostylis ovata*	Booyanup, WA	Jarrah woodland with Xanthorrea
	CLM1942	MT008092	*Cryptostylis ovata*	Booyanup, WA	Jarrah woodland with Xanthorrea
	CLM1943	MT008091	*Cryptostylis ovata*	Booyanup, WA	Jarrah woodland with Xanthorrea
	CLM2074	MT008090	*Cryptostylis ovata*	Johnston Rd, WA	Eucalypt woodland
	CLM2075	MT008089	*Cryptostylis ovata*	Johnston Rd, WA	Eucalypt woodland
	CLM2076	MT008088	*Cryptostylis ovata*	Johnston Rd, WA	Eucalypt woodland
	CLM2077	MT008087	*Cryptostylis ovata*	Johnston Rd, WA	Eucalypt woodland
	CLM2078	MT008086	*Cryptostylis ovata*	Johnston Rd, WA	Eucalypt woodland
	CLM2079	MT008085	*Cryptostylis ovata*	Johnston Rd, WA	Eucalypt woodland
	CLM2080	MT008084	*Cryptostylis ovata*	Johnston Rd, WA	Eucalypt woodland
	CLM2081	MT008083	*Cryptostylis ovata*	Muja Conservation Park, WA	Open Casuarina and Banksia woodland
	CLM2082	MT008082	*Cryptostylis ovata*	Muja Conservation Park, WA	Open Casuarina and Banksia woodland
	CLM2083	MT008081	*Cryptostylis ovata*	Muja Conservation Park, WA	Open Casuarina and Banksia woodland
	CLM2084	MT008080	*Cryptostylis ovata*	Muja Conservation Park, WA	Open Casuarina and Banksia woodland
	CLM2085	MT008079	*Cryptostylis ovata*	Muja Conservation Park, WA	Open Casuarina and Banksia woodland
	CLM2103	MT008077	*Cryptostylis ovata*	Granite Peaks, WA	*Eucalyptus* open forest
	CLM2104	MT008078	*Cryptostylis ovata*	Granite Peaks, WA	*Eucalyptus* open forest
	CLM2105	MT008076	*Cryptostylis ovata*	Granite Peaks, WA	*Eucalyptus* open forest
	CLM2106	MT008075	*Cryptostylis ovata*	Granite Peaks, WA	*Eucalyptus* open forest
	CLM2127	MT008069	*Cryptostylis ovata*	Johnston Rd, WA	Eucalypt woodland

Tulasnella occidentalis sp. nov	CLM2128	MT008068	*Cryptostylis ovata*	Muja Conservation Park, WA	Open Casuarina and Banksia woodland
	CLM2136	MT008074	*Cryptostylis ovata*	Rockingham, WA	Jarrah woodland
	CLM2137	MT008073	*Cryptostylis ovata*	Rockingham, WA	Jarrah woodland
	CLM2138	MT008072	*Cryptostylis ovata*	Rockingham, WA	Jarrah woodland
	CLM2139	MT008071	*Cryptostylis ovata*	Rockingham, WA	Jarrah woodland
	CLM2140	MT008070	*Cryptostylis ovata*	Rockingham, WA	Jarrah woodland
Tulasnella punctata sp. nov	CLM1901	MT008104	*Cryptostylis subulata*	Ulladulla, NSW	Open Casuarina woodland heath
	CLM2012	MT008124	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2016	MT008123	*Cryptostylis erecta*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2017(H)	MT008122	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2018	MT008121	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2019	MT008120	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2020	MT008119	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2021	MT008118	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2022	MT008117	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2023	MT008116	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2024	MT008115	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2025	MT008114	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2026	MT008113	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2027	MT008112	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2028	MT008111	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2029	MT008110	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2030	MT008109	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2031	MT008108	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey

Tulasnella punctata sp. no	CLM2032	MT008107	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2033	MT008106	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2034	MT008105	*Cryptostylis subulata*	Frenchs Forest, NSW	Eucalypt woodland with shrubby midstorey
	CLM2175	MT008102	*Cryptostylis subulata*	Erowal Bay, NSW	Eucalypt and Casuarina woodland
	CLM2176	MT008101	*Cryptostylis subulata*	Erowal Bay, NSW	Eucalypt and Casuarina woodland
	CLM2177	MT008100	*Cryptostylis subulata*	Erowal Bay, NSW	Eucalypt and Casuarina woodland
	CLM2123	MT008103	*Cryptostylis subulata*	Karuah, NSW	Open Eucalypt woodland
	CLM2124	MT008099	*Cryptostylis subulata*	Karuah, NSW	Open Eucalypt woodland
	CLM2125	MT008098	*Cryptostylis subulata*	Karuah, NSW	Open Eucalypt woodland
	CLM2126	MT008097	*Cryptostylis subulata*	Karuah, NSW	Open Eucalypt woodland
Tulasnella densa sp. nov	CLM2107	MT036529	*Cryptostylis hunteriana*	Bulahdelah, NSW	Eucalypt woodland with shrubby understorey
	CLM2108	MT036528	*Cryptostylis hunteriana*	Bulahdelah, NSW	Eucalypt woodland with shrubby understorey
	CLM2109	MT036527	*Cryptostylis hunteriana*	Bulahdelah, NSW	Eucalypt woodland with shrubby understorey
	CLM2110	MT036526	*Cryptostylis hunteriana*	Bulahdelah, NSW	Eucalypt woodland with shrubby understorey
	CLM2111	MT036525	*Cryptostylis hunteriana*	Meroo National Park, WA	Eucalypt woodland with shrubby understorey
	CLM2112	MT036524	*Cryptostylis hunteriana*	Meroo National Park, WA	Eucalypt woodland with shrubby understorey
	CLM2114	MT036523	*Cryptostylis hunteriana*	Meroo National Park, WA	Eucalypt woodland with shrubby understorey

Tulasnella densa sp. nov	CLM2115	MT036522	*Cryptostylis hunteriana*	Meroo National Park, WA	Eucalypt woodland with shrubby understorey
	CLM2116	MT036521	*Cryptostylis hunteriana*	Meroo National Park, WA	Eucalypt woodland with shrubby understorey
	CLM2117(H)	MT036520	*Cryptostylis hunteriana*	Meroo National Park, WA	Eucalypt woodland with shrubby understorey
	CLM2118	MT036519	*Cryptostylis hunteriana*	Meroo National Park, WA	Eucalypt woodland with shrubby understorey
	CLM2129	MT036514	*Cryptostylis hunteriana*	Meroo National Park, WA	Eucalypt woodland with shrubby understorey
	CLM2161	MT036518	*Cryptostylis hunteriana*	Erowal Bay, NSW	Eucalypt and Casuarina woodland
	CLM2162	MT036517	*Cryptostylis hunteriana*	Erowal Bay, NSW	Eucalypt and Casuarina woodland
	CLM2163	MT036516	*Cryptostylis hunteriana*	Erowal Bay, NSW	Eucalypt and Casuarina woodland
	CLM2164	MT036515	*Cryptostylis hunteriana*	Erowal Bay, NSW	Eucalypt and Casuarina woodland
Tulasnella concentrica sp. nov	CLM2071(H)	MT036547	*Cryptostylis leptochila*	Bundanoon, NSW	Eucalypt woodland with shrubby understorey
	CLM2072	MT036546	*Cryptostylis leptochila*	Bundanoon, NSW	Eucalypt woodland with shrubby understorey
	CLM2142	MT036545	*Cryptostylis leptochila*	Bundanoon, NSW	Eucalypt woodland with shrubby understorey
	CLM2160	MT036544	*Cryptostylis leptochila*	Penrose Nature Reserve, NSW	Eucalypt woodland with *Lomandra*
	CLM2165	MT036552	*Cryptostylis leptochila*	Carls mountain Rd, NSW	Eucalypt woodland with shrubby understorey
	CLM2166	MT036551	*Cryptostylis leptochila*	Carls mountain Rd, NSW	Eucalypt woodland with shrubby understorey

Tulasnella concentrica sp. nov	CLM2167	MT036550	*Cryptostylis leptochila*	Carls mountain Rd, NSW	Eucalypt woodland with shrubby understorey
	CLM2168	MT036549	*Cryptostylis leptochila*	Carls mountain Rd, NSW	Eucalypt woodland with shrubby understorey
	CLM2169	MT036548	*Cryptostylis leptochila*	Carls mountain Rd, NSW	Eucalypt woodland with shrubby understorey
	CLM2170	MT036555	*Cryptostylis leptochila*	Carls mountain Rd, NSW	Eucalypt woodland with shrubby understorey
	CLM2171	MT036554	*Cryptostylis leptochila*	Carls mountain Rd, NSW	Eucalypt woodland with shrubby understorey
	CLM2172	MT036553	*Cryptostylis leptochila*	Carls mountain Rd, NSW	Eucalypt woodland with shrubby understorey
	CLM2173	MT036543	*Cryptostylis leptochila*	Carls mountain Rd, NSW	Eucalypt woodland with shrubby understorey
	CLM2174	MT036542	*Cryptostylis leptochila*	Carls mountain Rd, NSW	Eucalypt woodland with shrubby understorey
	CLM2188	MT036541	*Cryptostylis leptochila*	Cabbage tree Ck, Vic	*Eucalyptus* open forest with shrubby understorey
	CLM2189	MT036531	*Cryptostylis leptochila*	Cabbage tree Ck, Vic	*Eucalyptus* open forest with shrubby understorey
	CLM2190	MT036530	*Cryptostylis leptochila*	Cabbage tree Ck, Vic	*Eucalyptus* open forest with shrubby understorey
	CLM2191	MT036540	*Cryptostylis leptochila*	Cabbage tree Ck, Vic	*Eucalyptus* open forest with shrubby understorey

Tulasnella concentrica sp. nov	CLM2192	MT036539	*Cryptostylis leptochila*	Cabbage tree Ck, Vic	*Eucalyptus* open forest with shrubby understorey
	CLM2193	MT036538	*Cryptostylis leptochila*	Cabbage tree Ck, Vic	*Eucalyptus* open forest with shrubby understorey
	CLM2194	MT036537	*Cryptostylis leptochila*	Cape Conran, Vic	*Eucalyptus* open forest with shrubby understorey
	CLM2195	MT036536	*Cryptostylis leptochila*	Cape Conran, Vic	*Eucalyptus* open forest with shrubby understorey
	CLM2196	MT036535	*Cryptostylis leptochila*	Bunyip State Park, Vic	*Eucalyptus* open forest with shrubby understorey
	CLM2197	MT036534	*Cryptostylis leptochila*	Bunyip State Park, Vic	*Eucalyptus* open forest with shrubby understorey
	CLM2198	MT036533	*Cryptostylis leptochila*	Bunyip State Park, Vic	*Eucalyptus* open forest with shrubby understorey
	CLM2199	MT036532	*Cryptostylis leptochila*	Bunyip State Park, Vic	*Eucalyptus* open forest with shrubby understorey
Tulasnella rosea sp. nov	CLM1763	MN947565	*Spiculaea ciliata*	Boulder Rock, WA	Apron of granite rock among moss and *Borya* sp.
	CLM1765	MN947566	*Spiculaea ciliata*	Boulder Rock, WA	Apron of granite rock among moss and *Borya* sp.
	CLM1767	MN947567	*Spiculaea ciliata*	Boulder Rock, WA	Apron of granite rock among moss and *Borya* sp.
	CLM1770	MN947568	*Spiculaea ciliata*	Boulder Rock, WA	Apron of granite rock among moss and *Borya* sp.

Tulasnella rosea sp. nov	CLM1771	MN947572	*Spiculaea ciliata*	Boulder Rock, WA	Apron of granite rock among moss and *Borya* sp.
	CLM1773(H)	MN947569	*Spiculaea ciliata*	Boulder Rock, WA	Apron of granite rock among moss and *Borya* sp.
	CLM1774	MN947570	*Spiculaea ciliata*	Boulder Rock, WA	Apron of granite rock among moss and *Borya* sp.
	CLM1775	MN947571	*Spiculaea ciliata*	Boulder Rock, WA	Apron of granite rock among moss and *Borya* sp.
	CLM1776	MN947573	*Spiculaea ciliata*	Boulder Rock, WA	Apron of granite rock among moss and *Borya* sp.
	CLM1790	MN947564	*Spiculaea ciliata*	Boulder Rock, WA	Apron of granite rock among moss and *Borya* sp.
	CLM1791	MN947563	*Spiculaea ciliata*	Boulder Rock, WA	Apron of granite rock among moss and *Borya* sp.
	CLM1792	MN947562	*Spiculaea ciliata*	Boulder Rock, WA	Apron of granite rock among moss and *Borya* sp.
	CLM1796	MN947561	*Spiculaea ciliata*	Boulder Rock, WA	Apron of granite rock among moss and *Borya* sp.
	CLM1797	MN947560	*Spiculaea ciliata*	Boulder Rock, WA	Apron of granite rock among moss and *Borya* sp.
	CLM1798	MN947559	*Spiculaea ciliata*	Boulder Rock, WA	Apron of granite rock among moss and *Borya* sp.
	CLM1802	MN947555	*Spiculaea ciliata*	East Brookton, WA	Granite rock with moss *Borya* sp.
	CLM1804	MN947554	*Spiculaea ciliata*	East Brookton, WA	Granite rock with moss *Borya* sp.
	CLM1814	MN947553	*Spiculaea ciliata*	East Brookton, WA	Granite rock with moss *Borya* sp.

Tulasnella rosea sp. nov	CLM1820	MN947558	*Spiculaea ciliata*	Canning Mills, WA	unknown
	CLM1827	MN947552	*Spiculaea ciliata*	East Brookton, WA	Granite rock with moss *Borya* sp.
	CLM1828	MN947551	*Spiculaea ciliata*	East Brookton, WA	Granite rock with moss *Borya* sp.
	CLM1829	MN947550	*Spiculaea ciliata*	East Brookton, WA	Granite rock with moss *Borya* sp.
	CLM1830	MN947549	*Spiculaea ciliata*	East Brookton, WA	Granite rock with moss *Borya* sp.
	CLM1831	MN947548	*Spiculaea ciliata*	East Brookton, WA	Granite rock with moss *Borya* sp.
	CLM1844	MN947547	*Spiculaea ciliata*	East Brookton, WA	Granite rock with moss *Borya* sp.
	CLM1847	MN947557	*Spiculaea ciliata*	Canning Mills, WA	unknown
	CLM1848	MN947556	*Spiculaea ciliata*	Canning Mills, WA	unknown

SUPPLEMENTARY TABLE 2. Reference sequences downloaded from GenBank used to construct the ITS phylogenetic tree.

GenBank	**Species**	**Isolate/ clone**	**Origin**	**Hosts/substrates**	**Seq. length**	**References**
KC152437	*Tulasnella violea*	DC293	Germany	Decayed wood	799 bp	Cruz et al. 2014
KC152415	*Tulasnella violea*	DC177	Ecuador	Fallen branch	842 bp	Cruz et al. 2014
AY373292	*Tulasnella eichleriana*	KC852	United States	Unknown	780 bp	McCormick et al. 2004
KC152389	*Tulasnella eichleriana*	DC294	Germany	Decayed wood	768 bp	Cruz et al. 2014
KC152382	*Tulasnella eichleriana*	FO24462a	Germany	Decayed wood	767 bp	Cruz et al. 2014
KC152386	*Tulasnella eichleriana*	FO24462a	Germany	Decayed wood	749 bp	Cruz et al. 2014
KC152384	*Tulasnella eichleriana*	FO24462a	Germany	Decayed wood	767 bp	Cruz et al. 2014
KC152387	*Tulasnella eichleriana*	DC294	Germany	Decayed wood	768 bp	Cruz et al. 2014
KC152396	*Tulasnella eichleriana*	DC294	Germany	Decayed wood	704 bp	Cruz et al. 2014
KC152397	*Tulasnella* sp. ECU5	DC225	Ecuador	Fallen branch	700 bp	Cruz et al. 2014
KC152398	*Tulasnella* sp. ECU5	DC225	Ecuador	Fallen branch	716 bp	Cruz et al. 2014
JN015192	Unassigned; Here identified as *Tulasnella densa* sp. nov	BB0002_2_A	Australia	Unknown	781 bp	Howard & Clements 2011**
KC152401	*Tulasnella* sp. ECU6	DC185	Ecuador	Fallen branch	699 bp	Cruz et al. 2014
KC152402	*Tulasnella* sp. ECU6	DC262	Ecuador	Fallen branch	774 bp	Cruz et al. 2014
KY095117*	*Tulasnella sphagneti*	CLM541	Australia	*Chiloglottis aff. valida*	738 bp	Linde et al. 2017
KY445922	*Tulasnella sphagneti*	CLM583	Australia	*Chiloglottis turfosa*	728 bp	Linde et al. 2017
KY445924	*Tulasnella sphagneti*	13102_1	Australia	*Chiloglottis turfosa*	728 bp	Linde et al. 2017
KY445926	*Tulasnella sphagneti*	13065_1	Australia	*Chiloglottis* sp.	728 bp	Linde et al. 2017
MT214493	*Tulasnella sphagneti*	CLM1952	Australia	*Cryptostylis subulata*	721 bp	This study
MT214494	*Tulasnella sphagneti*	CLM2130	Australia	*Cryptostylis subulata*	727 bp	This study
MT214495	*Tulasnella sphagneti*	CLM2131	Australia	*Cryptostylis subulata*	727 bp	This study

MT214496	*Tulasnella sphagneti*	CLM2132	Australia	*Cryptostylis subulata*	727 bp	This study
KF476575*	*Tulasnella secunda*	CLM009	Australia	*Drakaea elastica*	736 bp	Linde et al. 2014
KF476588	*Tulasnella secunda*	CLM251	Australia	*Drakaea concolor*	736 bp	Linde et al. 2014
KF476568	*Tulasnella secunda*	CLM222	Australia	*Paracaleana minor*	736 bp	Linde et al. 2014
KF476592	*Tulasnella secunda*	CLM253	Australia	*Drakaea confluens*	736 bp	Linde et al. 2014
KF476602	*Tulasnella australiensis* sp. nov	CLM031	Australia	*Arthrochilus oreophilus*	735 bp	Linde et al. 2014
KF476594	Unassigned *Tulasnella*	CLM084	Australia	*Arthrochilus oreophilus*	731 bp	Linde et al. 2014
KF476595	Unassigned *Tulasnella*	CLM085	Australia	*Arthrochilus oreophilus*	731 bp	Linde et al. 2014
KF476596*	*Tulasnella warcupii*	CLM027	Australia	*Arthrochilus oreophilus*	734 bp	Linde et al. 2014
KF476597	*Tulasnella warcupii*	CLM092	Australia	*Arthrochilus oreophilus*	733 bp	Linde et al. 2014
KF476598	*Tulasnella warcupii*	CLM091	Australia	*Arthrochilus oreophilus*	733 bp	Linde et al. 2014
KF476600	*Tulasnella warcupii*	CLM007	Australia	*Arthrochilus oreophilus*	735 bp	Linde et al. 2014
KF476601	*Tulasnella warcupii*	CLM022	Australia	*Arthrochilus oreophilus*	735 bp	Linde et al. 2014
KF476556*	*Tulasnella prima*	CLM159	Australia	*Chiloglottis trilabra*	736 bp	Linde et al. 2014
KF476543	*Tulasnella prima*	CLM309	Australia	*Chiloglottis formicifera*	736 bp	Linde et al. 2014
KF476550	*Tulasnella prima*	CLM306	Australia	*Chiloglottis formicifera*	736 bp	Linde et al. 2014
KF476551	*Tulasnella prima*	CLM308	Australia	*Chiloglottis formicifera*	736 bp	Linde et al. 2014
HM196794	*Tulasnella prima*	CM07.I.5	Australia	*Chiloglottis trapeziformis*	863 bp	Roche et al. 2010
MK430470	*Tulasnella prima*	CL12(5)	Australia	*Cryptostylis ovata*	727 bp	Nguyen et al. 2019
MK430521	*Tulasnella prima*	CS43(17)	Australia	*Cryptostylis ovata*	563 bp	Nguyen et al. 2019
MK430445	Bertram. Here identified as *Tulasnella occidentalis* sp. nov	CB43(9)	Australia	*Cryptostylis ovata*	731 bp	Nguyen et al. 2019

MK430440	Bertram. Here identified as *Tulasnella occidentalis* sp. nov	CB14(3)	Australia	*Cryptostylis ovata*	736 bp	Nguyen et al. 2019
MK430444	Bertram. Here identified as *Tulasnella occidentalis* sp. nov	CB43(6)	Australia	*Cryptostylis ovata*	730 bp	Nguyen et al. 2019
MK430439	Bertram. Here identified as *Tulasnella occidentalis* sp. nov	CB14(1)	Australia	*Cryptostylis ovata*	696 bp	Nguyen et al. 2019
MK430451	*Tulasnella* sp. (Jarrahdale1)	CJ11(8)	Australia	*Cryptostylis ovata*	710 bp	Nguyen et al. 2019
MK430452	*Tulasnella* sp. (Jarrahdale1)	CJ13(3)	Australia	*Cryptostylis ovata*	730 bp	Nguyen et al. 2019
MK430461	*Tulasnella* sp. (Jarrahdale2)	CJ51(7)	Australia	*Cryptostylis ovata*	639 bp	Nguyen et al. 2019
MK430468	*Tulasnella* sp. (Jarrahdale2)	CJ51(17)	Australia	*Cryptostylis ovata*	574 bp	Nguyen et al. 2019
AY373296	*Tulasnella tomaculum*	KC429	United States	Unknown	800 bp	McCormick et al. 2004
KC152380	*Tulasnella tomaculum*	K(M)123675	United Kingdom	Unknown	714 bp	Cruz et al. 2014
MK626568	*Tulasnella* sp. JM 2019a	DAOMC	Canada	Rotten wood	775 bp	Mack and Seifert 2019
MK626686	*Tulasnella* sp. JM 2019a	DAOMC	Canada	Rotten wood	775 bp	Mack and Seifert 2019
MK626687	*Tulasnella* sp. JM 2019a	DAOMC	Canada	Rotten wood	775 bp	Mack and Seifert 2019
JX138568	Unassigned; Here identified as *Tulasnella rosea* sp. nov	MB-2012	Australia	*Spiculaea ciliata*	455 bp	Sommer et al. 2012

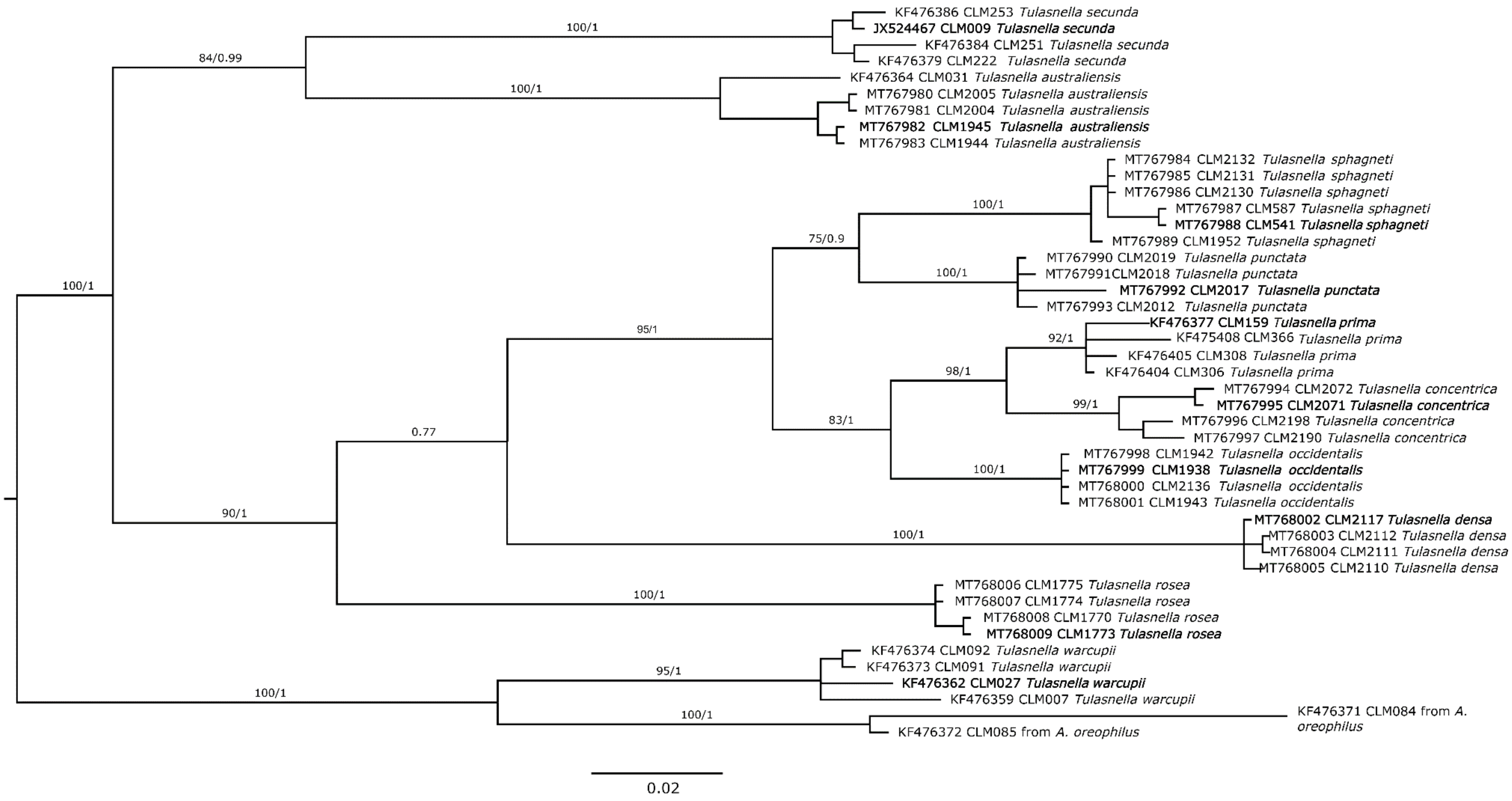
*holotype

**unpublished

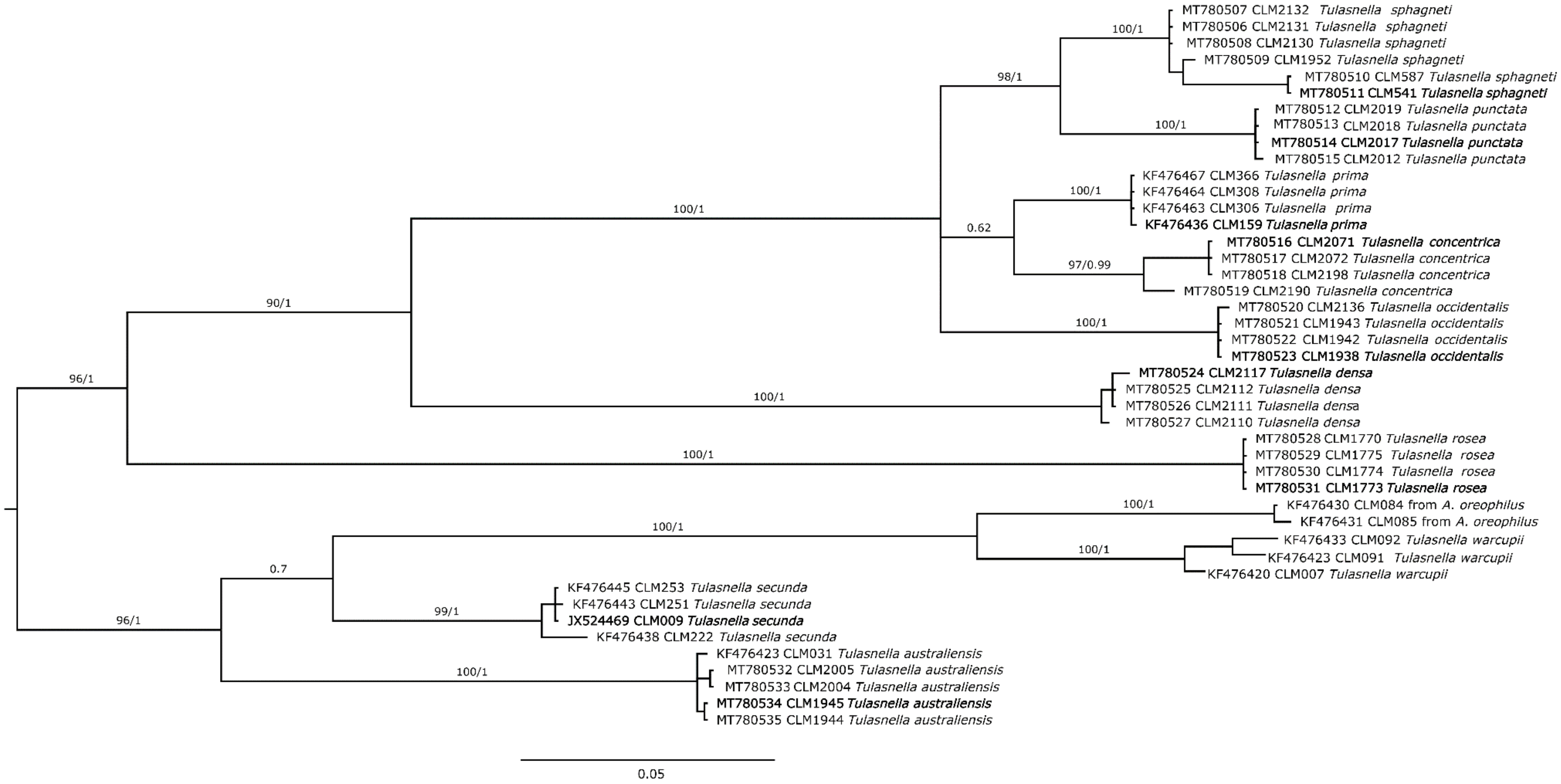
SUPPLEMENTARY TABLE 3. *P*-value of growth measurement comparisons between species within each medium. Shaded cells indicate growth comparisons between two species are significantly ($P < 0.05$) different.

Species	¼ PDA	3MN	E-medium	½ FIM
T. australiensis - *T. occidentalis*	0.1619	0.9440	1.0000	0.0017
T. australiensis - *T. punctata*	0.5959	0.7680	0.5145	0.0001
T. australiensis - *T. densa*	0.0025	0.9932	0.9889	0.0002
T. australiensis - *T. concentrica*	0.0025	0.9960	0.0423	<.0001
T. australiensis - *T. rosea*	0.0139	0.9996	0.3483	<.0001
T. occidentalis - *T. punctata*	0.9252	0.9976	0.5822	0.6492
T. occidentalis - *T. densa*	0.2831	0.7085	0.9958	0.8241
T. occidentalis - *T. concentrica*	0.2828	0.7421	0.0525	0.0313
T. occidentalis - *T. rosea*	0.8498	0.8058	0.4103	0.1852
T. punctata - *T. densa*	0.0565	0.4550	0.8498	0.9994
T. punctata - *T. concentrica*	0.0564	0.4885	0.6290	0.4074
T. punctata - *T. rosea*	0.3019	0.5410	1.0000	0.9512
T. densa - *T. concentrica*	1.0000	1.0000	0.1265	0.2603
T. densa - *T. rosea*	0.8214	0.9997	0.7109	0.8300
T. concentrica - *T. rosea*	0.8210	0.9999	0.6773	0.8131

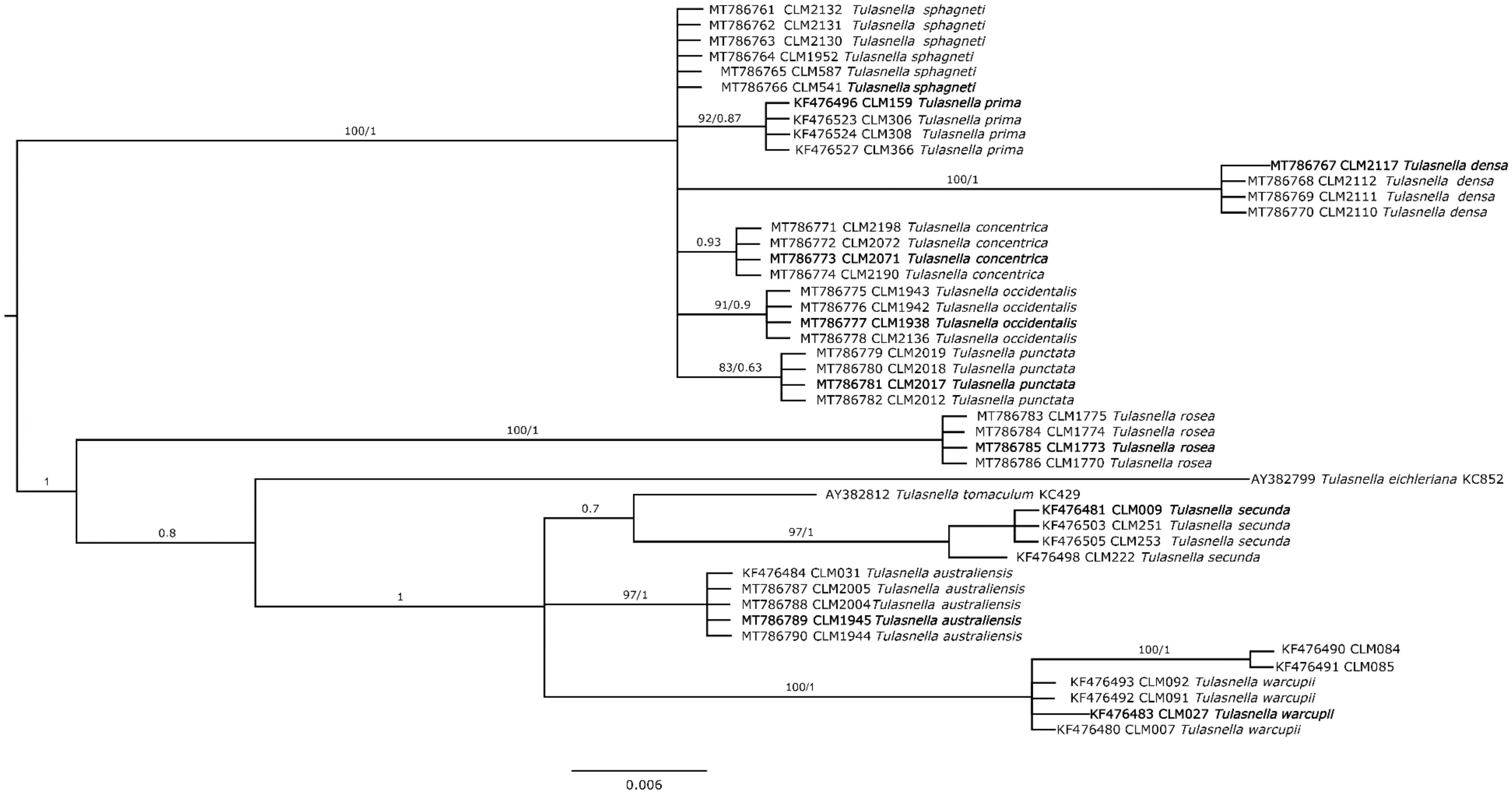
SUPPLEMENTARY FIGURE 1. BI tree for *Tulasnella* using ITS region, complete set of newly generated sequences and reference sequences. Numbers on the branches are bootstrap (>70%) / BPP (>0.70) support values. The new *Tulasnella* taxa are shown with a line for each species. Sequences derived from type cultures shown in bold.



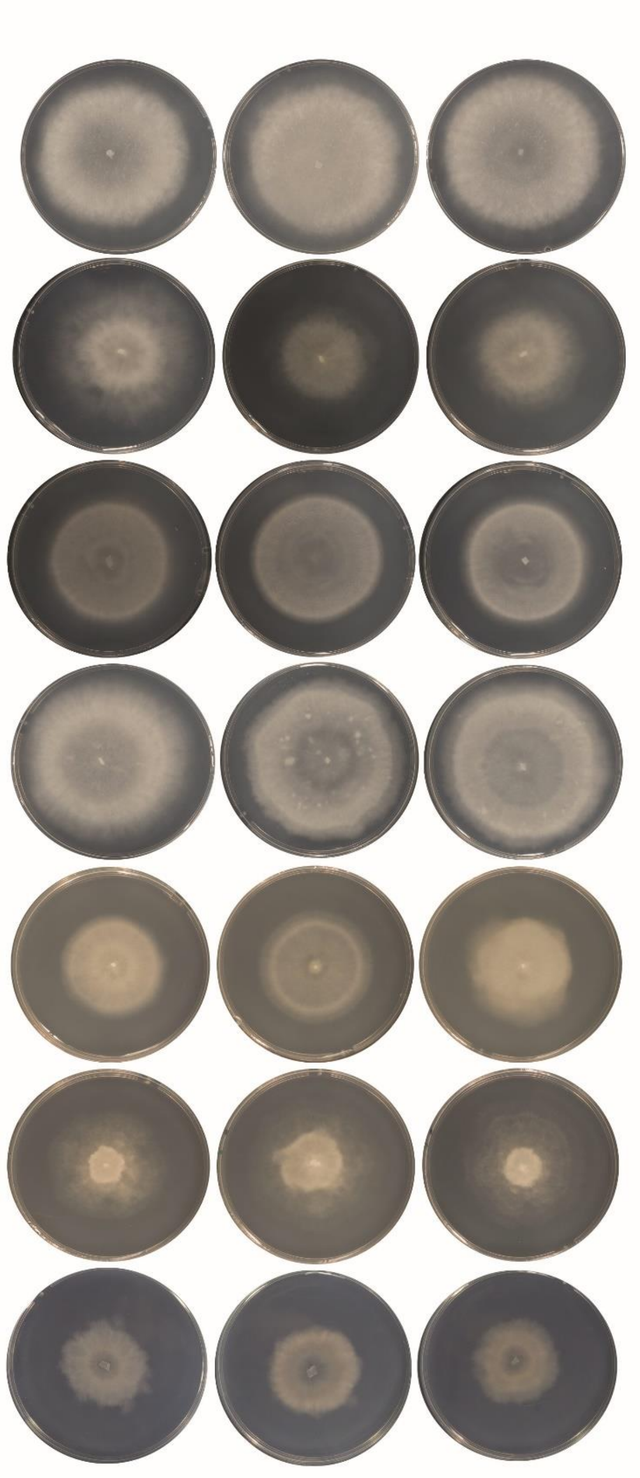
SUPPLEMENTARY FIGURE 2. BI tree for *Tulasnella* using the ATP mitochondrial (*C14436*) locus. Numbers on the branches are bootstrap (>70%) / BPP (>0.70) support values. Sequences derived from type cultures shown in bold.



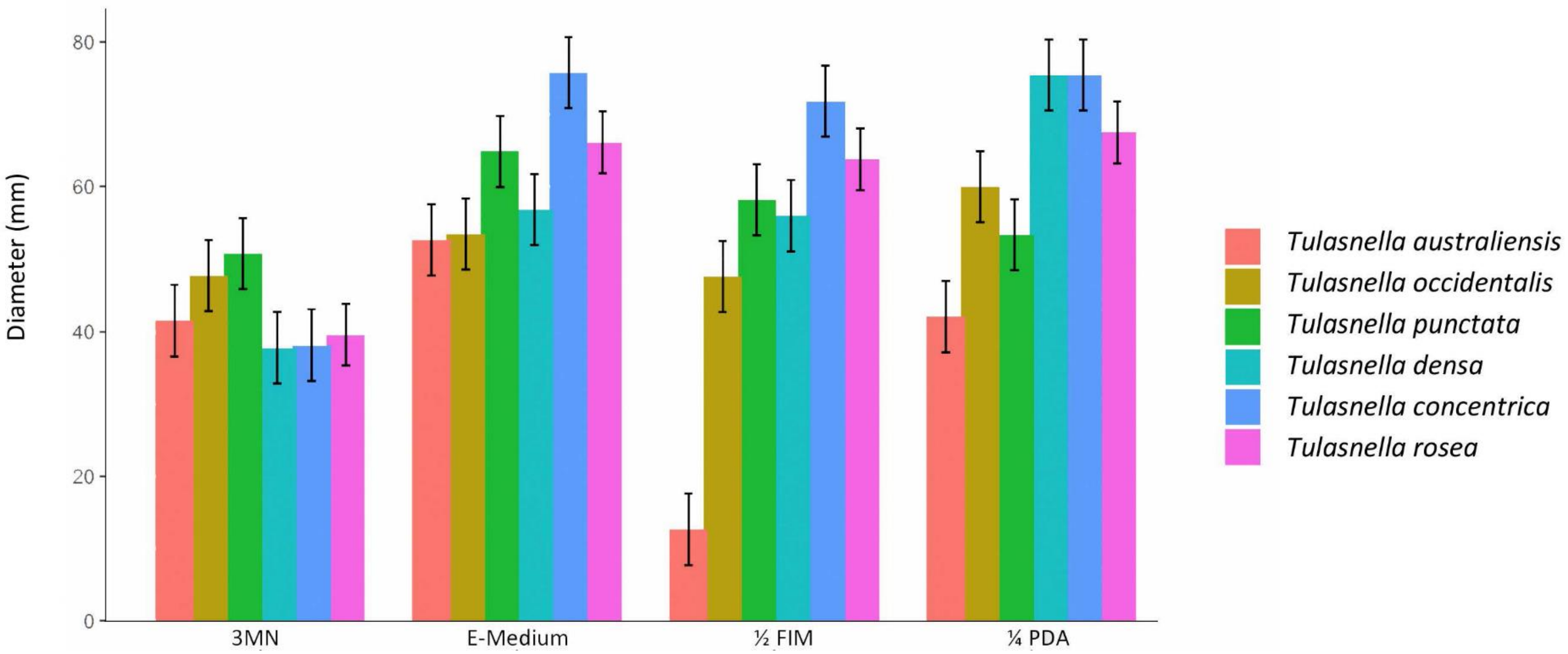
SUPPLEMENTARY FIGURE 3**.** BI tree for *Tulasnella* using the Glutamate synthase (*C4102*) locus. Numbers on the branches are bootstrap (>70%) / BPP (>0.70) support values. Sequences derived from type cultures shown in bold.



SUPPLEMENTARY FIGURE 4**.** BI tree for *Tulasnella* using the mitochondrial large-subunit (mtLSU) locus. Numbers on the branches are bootstrap (>70%) / BPP (>0.70) support values. Sequences derived from type cultures shown in bold.



SUPPLEMENTARY FIGURE 5. *Tulasnella* cultures on the most diagnostic medium (3MN), one row per species. *Tulasnella* species from top to bottom (isolate from left to right, respectively): *Tulasnella prima* (CLM1933-1934-1936) previously described in Linde et al., 2017, *Tulasnella australiensis* (CLM1945-1670-1944), *Tulasnella occidentalis* (CLM1938-1941-1942), *Tulasnella punctata* (CLM2012-2017-2018), *Tulasnella densa* (CLM2118-2110-2112), *Tulasnella concentrica* (CLM2071-2072-2142), *Tulasnella rosea* (CLM1771-1773-1774).



SUPPLEMENTARY FIGURE 6. Colony diameter (mm) of six new *Tulasnella* species on four different media measured after incubation at 22 C for four wk in the dark.

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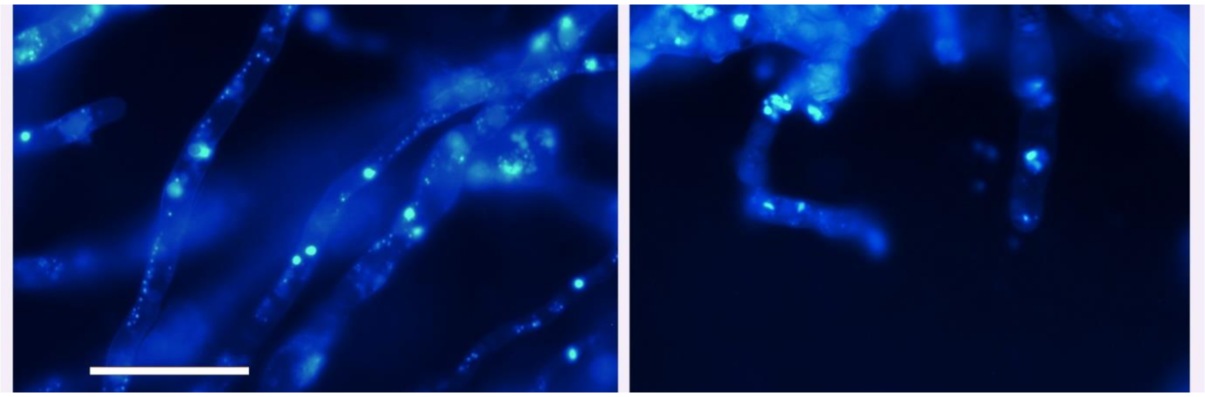

SUPPLEMENTARY FIGURE 7. Fluorescently labelled nuclei of two previously described *Tulasnella* species, *Tulasnella prima* (left) and *Tulasnella warcupii* (right), showing binucleate cells. Scale bar = 20 µm.

CHAPTER FOUR

New species of *Tulasnella* associated with Megastylidinae orchids from Australia

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New species of *Tulasnella* associated with Megastylidinae orchids from Australia

ABSTRACT

Here we used molecular phylogenetic analyses and morphological characteristics to describe two new *Tulasnella* species associated with Australian orchids from the subtribe Megastylidinae. The genus *Tulasnella* has previously been divided into four phylogenetic groups. One of the new species, *Tulasnella inflata,* associates with *Pyrorchis nigricans* from Western Australia and belongs to phylogenetic group III. The second described species, *Tulasnella multinucleata,* associates with the monotypic *Rimacola elliptica* orchid from eastern Australia and belongs to a newly defined phylogenetic group, group V based on phylogenetic analyses of the nuclear ribosomal internal transcribed spacer (ITS) 5.8S region. While these newly described *Tulasnella* species were obtained from the same orchid subtribe, they are distantly related. Binucleate (and occasional trinucleate) hyphal compartments characterise *T. inflata,* whereas *T. multinucleata* has approximately 6-12 nuclei per hyphal compartment. This is the first report of multinucleate hyphal compartments in *Tulasnella,* where previously only binucleate species have been reported.

Keywords: Orchid mycorrhizal fungi, binucleate, multinucleate, *Tulasnella* group V, 2 new taxa.

INTRODUCTION

Fungi belonging to the family Tulasnellaceae are associated with orchids across the world as orchid mycorrhizal fungi (OMF). However, most of the orchids associated *Tulasnella* fungi, remained undescribed. *Tulasnella* is not only an orchid mycobiont, but also associates with Aneuraceae liverworts (Kottke et al., 2008; Krause et al., 2011; Preußing et al., 2010). They can also grow as saprophytes in decayed trees or fallen branches (Cruz et al., 2014; Roberts, 1992, 1993) and may play an important role as ectomycorrhizal fungi in forest trees (Bidartondo et al., 2004; Solis et al., 2017; Tedersoo et al., 2010).

Approximately one hundred *Tulasnella* species are described in the world (www. Indexfungorum.org). Phylogenetic diversity among *Tulasnella* species is high, with four phylogenetic groups classified by Cruz et al., (2014). Recently, a few new *Tulasnella* species has been described using morphology and molecular methods, such as four new *Tulasnella* species from Australian orchids (Linde et al., 2017) that fell into group IV and five species which fell into group II; *T. phuhinrongklaensis* (Rachanarin et al., 2018), *T. tubericola* (Solis et al., 2017) and three *Tulasnella* species from Japan T. *cumulopuntioides, T. dendritica* and *T. ellipsoidea* (Fujimori et al., 2019). Therefore, the newly described species are limited to group II and IV.

Uni-, bi-, tri- and multinucleate cell characteristics are common in *Rhizoctonia*-like fungi such as in Serendipitaceae (Andersen, 1996; Williams & Thilo, 1989), Sebacinaceae (Basiewicz et al., 2012) and Ceratobasidiaceae (Andersen, 1996; Oberwinkler et al., 2013; Veldre et al., 2013). However, little is known about the occurrence of multinucleate cell compartments in Tulasnellaceae, with the only known case is in *T. tubericola*, where trinucleate cells are reported to occur occasionally (Solis et al., 2017).

The development of molecular biology enabled us to identify and delimit fungi without their morphological or teleomorph presence (Schoch et al., 2012). The ITS has been a barcoding standard for fungal delimitation, especially in orchid mycorrhizal fungi (Cruz et al., 2014; Linde et al., 2017; Whitehead et al., 2017). However, fungal discrimination using morphology provides distinctive phenotypic characters which are used to confirm species delimitation using molecular sequencing of multiple loci (Cruz et al., 2011; Shan et al., 2002). The aim of this study is to describe two new *Tulasnella* species associated with Megastylidinae (*Pyrorchis nigricans* and *Rimacola elliptica*) using morphology and phylogenetic methods.

MATERIAL AND METHODS

Study orchids

Pyrorchis (Diurideae, Megastylidinae) is an Australian terrestrial orchid genus where only two species are known; *P. nigricans* which is widespread across Australia and *Pyrorchis forestii* (F.Muell.) D.L.Jones & M.A.Clem which is endemic in southwest Western Australia. *Pyrorchis nigricans* (R.Br.) D.L.Jones & M.A.Clem. grows in various habitats including open forest, woodland and heath across Western Australia, eastern New South Wales, Victoria, South Australia and Tasmania. This species is deciduous and flowering usually occurs after triggered by the fire in the summer, from August to November

(Brundrett, 2014; Jones, 2006). *Rimacola elliptica* (R.Br.) Rupp. (Diurideae, Megastylidinae) grows in unusual moist habitats in dwelling rock cracks, clefts and fissures. As the only member of this genus, *Rimacola* can be described as an epiphytic / litophytic orchid, evergreen, growing in clumps, with few leaves each in each plant. The distribution of *Rimacola* is very narrow in Australia, only endemic in eastern Australia in mountainous regions up to 800 m.a.s.l (Jones, 2006; Pridgeon et al., 2001). No previous research about OMF in monotypic *Rimacola*. Previous research only reported several isolates of Rhizoctonia-like fungi and *T. danica* as well as ericoid mycorrhizal fungi associate with *P. nigricans* (Bonnardeaux et al., 2007).

Orchid collections and fungal isolation

Roots of *Rimacola elliptica* were collected in one population (two plants) in Morton National Park (New South Wales), *Pyrorchis forrestii* from Blue Lake Rd in Western Australia (three plants), and *Pyrorchis nigricans* were obtained from two populations in Pomeroy Rd and Lake Unicup in Western Australia (each three plants sampled). During transportation, all samples were kept moist in damp paper towel and fungal isolations were conducted within a maximum of five days after sampling. All root pieces were washed under tap water to remove any soil and dirt. Roots were dissected to find the presence of pelotons, then washed three times in sterile water. Pelotons and peloton-rich tissues were transferred to ½ half-strength Fungal Isolation Media (½ FIM) (Clements & Ellyard, 1979), supplemented with 50 mg/mL streptomycin sulfate to inhibit bacterial contamination. A small piece of peloton-rich tissue for each plant root sampled (1 cm) was lyophilized and preserved for direct sequencing purposes. Plates were incubated 22 °C in the dark for one to two weeks. Any *Rhizoctonia*-like fungi were transferred to new ½ FIM (supplemented with streptomycin) to obtain pure cultures. Stock cultures were kept on ½ FIM agar slants and stored as agar blocks in sterile miliQ water. Voucher specimens of the fungi were deposited as living cultures (stored metabolically inactive) at the Royal Botanic Gardens Victoria Fungal Culture Collection (RBGV-FCC) and Victorian Plant Pathology Herbarium (VPRI), whereas dried material was lodged at the National Herbarium of Victoria (MEL).

DNA extraction and sequencing

To obtain fungal mycelium for DNA extraction, seven isolates (five Rhizoctonia-like fungi from *P. nigricans* and two from *R. elliptica*) were inoculated in liquid ½ FIM amended with 50 mg/mL streptomycin and incubated for 4 weeks at 22 °C in the dark. Mycelia were harvested from liquid media and lyophilized with free dryer for 24 h. Dried mycelium were stored in the -18 °C prior to DNA extraction using a DNAeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The ITS region were amplified using primer ITS5 and ITS4 (Gardes & Bruns, 1993; White et al., 1990).

For direct sequencing from peloton-rich root tissue, genomic DNA were extracted using a DNeasy Plant Mini Kit (Qiagen, Germany). The ITS region were amplified using *Tulasnella*-specific primer ITS5 and ITS4-Tul (Taylor & McCormick, 2008) after establishing that the majority fungi detected in all three orchid species are *Tulasnella*. PCR products were cloned using a pCR4-TOPO cloning vector (Thermofisher, USA) and transformed to competent *Escherichia coli* DH10B. Direct sequencing will detect any *Tulasnella* symbionts which might not be able to culture by conventional isolation.

Phylogenetic analyses

To confirm the isolates are new isolates and have not been described elsewhere, all obtained *Rhizoctonia*-like fungi sequences were matched on BLASTN on NCBI GenBank (http://www.ncbi.nlm.nih.gov/). All possible closely-related as well as distantly-related *Tulasnella* sequences were downloaded from GenBank, these included the representatives of *Tulasnella* phylogenetic groups based on Cruz et al. (2014). Sequencing results were checked and manually edited using Sequencher v.5.4.1 (Genes Codes Corp., Michigan, USA). Due to the difficulties in aligning different phylogenetic groups in *Tulasnella*, the initial sequence alignment was undertaken only for the most conserved 5.8S region of the ITS (175 bp). Phylogenetic trees were constructed using Bayesian inference analysis using MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003) as implemented in Geneious 10.2.6. Node support was assessed with Bayesian Posterior Probabilities (BPP) for 1 200 000 generations and trees was sampled every 120 generations with a 10% burn-in. Additionally, a RAxML Maximum Likelihood (ML) analysis (Stamatakis et al., 2008) phylogenetic tree was constructed using 1,000 pseudoreplicates of nonparametric bootstrapping. Both BPP and ML analyses were generated using Geneious v.10.2.6 (Kearse et al., 2012) and visualised using

FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). The 5.8S ITS phylogenetic tree was rooted with *Serendipita vermifera* (GenBank accession number DQ983816).

After constructing the *Tulasnella* phylogeny using the 5.8S ITS, *Tulasnella* sequences were clustered into separate phylogenetic group clades, following the methods and results of Cruz et al. (2014). For each phylogenetic group represented by our isolates, a separate full ITS sequence alignment (approximately 640 bp) was undertaken using similar parameters and analyses (Bayesian inference and Maximum likelihood) as above. Both phylogenetic trees were midpoint-rooted and visualised using FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Morphological and culture characteristics

To assess cultural growth and morphological features, stock isolates were initially grown on ½ FIM agar (with streptomycin) and incubated for 2-3 weeks at 22 °C in the dark. A small agar block (5 mm²) was transferred to each of four different media, with three replicates per isolate per medium. The different media used to visualise morphological and cultural characteristics were; ½ FIM (Clements & Ellyard, 1979), 3MN (Wright et al., 2010) +A-Z vitamin (Wyeth Consumer Healthcare, Baulkham Hills, Australia, E-Medium (Caldwell et al., 1991) and ¼ PDA (Potato Dextrose Agar, Alpha Biosciences, USA), all supplemented with streptomycin sulfate 50 mg/mL. The examined characteristics included the shape of colony margin, the colour of colony, growth uniformity, any visible concentric zones and colony textures and other distinctive features. All isolates were incubated 22°C in the dark and all colony diameters were measured with calipers with two perpendicular measurements after four weeks. Statistical analysis was performed using a linear-mix model ("LMERTEST" package) in R (R Core Team, 2019) and a one-way ANOVA was used to determine significance differences in colony growth between both *Tulasnella* species within each medium.

Microscopic characteristics and fluorescence staining of fungal nuclei

Nuclei staining was examined based on (Yang et al., 1991b), with Hoechst dye 33342 (ThermoFisher Scientific, USA) as the staining agent. To prepare the inoculum for staining, glass slides were covered with a thin layer of PDA. PDA-covered glass slide was laid above the damp whatman filter paper in the sterile petri dish to keep the humidity during incubation. After the PDA dried up, 1 mm² of agar block mycelium was inoculated on the middle of the slide. All procedures were prepared aseptically in the air-flow cabinet. Petri dishes were sealed and incubated 22 °C, 3 weeks in the dark. After incubation, slides were air-dried in a laminar airflow cabinet for approximately 15 minutes. To make the working solution of Hoechst dye, a buffer pH 7.8 consists of 0.1 M KH_2PO_4 and 0.1 M NaOH was prepared for best visualisation of Rhizoctonia fungi (Fang et al., 2013; Yang et al., 1991a). Nuclei examination was conducted using a Leica fluorescence microscope DM5500B at 100X magnification. The number of nuclei was counted for at least 20 cells / compartments per isolate. The photographs of cells for each isolate were taken using an Automated Upright Microscope Leica DM5500B Camera (Leica Microsystems, Germany).

RESULTS

To support our phylogenetic analysis, we included a total of 193 *Tulasnella* sequences available from GenBank covering distantly and closely-related *Tulasnella*, with 10 *Tulasnella* sequences from group I, 71 sequences from group II, 36 sequences from group III, 63 sequences from group IV, four sequences of *T. irregularis*, six unassigned *Tulasnella* sequences and three sequences included to our proposed new *Tulasnella* group—hereafter named group V (Table S1). Group classification is based on Cruz et al., (2014). From our fungal isolation and direct sequencing, we obtained seven isolates and 25 clones from three Megastylidinae orchids (Table 1).

The ITS 5.8 *Tulasnella* phylogeny constructed from *Tulasnella* sequences and clones from this study, as well as sequences from GenBank (Figure 1), formed seven main clades; the previously described *Tulasnella* phylogenetic groups I - IV, a *T. irregularis* clade and two unassigned groups – one of which is here described as group V. Group IV clustered in a well-supported clade, separate to all other *Tulasnella* clades. *Tulasnella* group II is sister to *T. irregularis*.

Tulasnella sequences of isolates/clones obtained from *P. nigricans* and *P. forestii* all clustered within *Tulasnella* group II and III (Table 1). *Tulasnella* described here as *T. inflata*, is a well-supported clade with BPP = 1 and 100 % Bootstrap support clustered to *Tulasnella* group III. No sequence variation was detected within five T. *inflata* isolates (0 %). In addition, another two clades were obtained from *Pyrorchis*, OTU S and OTU Q, but we were not able to obtain living isolates of these OTUs (Figure 2). The percentage sequence variation between *T. inflata* and other *Tulasnella* species in this group is high, ranging from 19.0 – 44.6 % (Table 2).

Our newly described *T. multinucleata* obtained from *R. elliptica* clustered in *Tulasnella* group V (the new *Tulasnella* phylogenetic group from this study, Figure 3). Sequence divergence within *T. multinucleata* was 1 % (Table 3). *T. multinucleata* is sister to *Tulasnella* sp. CH01 (GenBank accession KP973894) with sequence divergence between them greater than 6%. *Tulasnella* sp. Gno5C-1 and *Tulasnella* sp. clone DC2 (GenBank accession number JX514389 and MK239039 respectively) can be considered as one OTU with sequence divergence between them less than 4% (Table 3).

TAXONOMY

Tulasnella inflata Arifin, T.W. May & Linde, sp. nov.

Typification: AUSTRALIA. WESTERN AUSTRALIA, *C. Linde* CLM1788, isolated from *Pyrorchis nigricans*, collected from Pomeroy Road, 13 August 2016 (**holotype** RBGV-FCC 200012, preserved in a metabolically inactive state. Isotypes: preserved in a metabolically inactive state, MEL 2470320).

Etymology: Refers to the morphology of various swollen cells in this species.

Hosts: *Pyrorchis nigricans.*

Cultural morphology: On ¼ PDA, 72-78 mm diameter, with very thin aerial mycelium, hyphae mostly submerged, regular radial growth, no concentric zonation, even margin, underside and upside off white, in ¼ PDA mycelium showed most dense growth compared to other media. On ½ FIM and E-medium showed similar features with ¼ PDA (just less dense). On 3MN, there are obvious aerial mycelium growing around the edge of the colony (Figure 4).

Micromorphology: Hyphae 3-5 µm diameter, 35-45 µm long, cylindrical, terminal elements clavate and occasionally subglobose, swollen cells sometimes much shorter and fusoid (10-20 µm long and 6-7 µm diameter); septate; lacking clamp connections, branched usually at right angles with constriction at the base; mostly binucleate but occasionally trinucleate (Figure 5).

Other speciments examined: *C. Linde* CLM1760, isolated from *Pyrorchis nigricans* collected from Pomeroy Road, WA, Australia, August 2016 (RBGV-FCC 200013). *C. Linde* CLM1761, isolated from *Pyrorchis nigricans* collected from Pomeroy Road, WA, Australia, August 2016 (RBGV-FCC 200014). *C. Linde* CLM1762, isolated from *Pyrorchis nigricans* collected from Pomeroy Road, WA, Australia, August 2016 (RBGV-FCC 200015).

Notes: This species grew faster than *T. multinucleata*, with the diameter of the colonies more than 65 mm after four weeks on all media tested (Figure S1). Compared to all *Tulasnella* we described (including six species described from Chapter Three), growth of *T. inflata* was comparatively fast and uniform in all four media tested.

Tulasnella multinucleata Arifin, T.W. May & Linde, sp. nov.

Typification: AUSTRALIA: NEW SOUTH WALES, *A. Arifin* CLM2222, isolated from *Rimacola elliptica*, collected from Morton National Park, 1 September 2018 (**holotype** RBGV-FCC 190931, preserved in a metabolically inactive state. Isotypes: VPRI 43509, preserved in a metabolically inactive state, MEL 2469731).

Etymology: Refers to the only multinucleate cell compartment within genus *Tulasnella*.

Host: Roots of *Rimacola elliptica*.

Cultural morphology: On ¼ PDA, 43-62 mm diameter, with cottony to cobwebby, well-developed off white aerial mycelium, particularly over the inoculum block (where cream in colour), with irregular, dendritic radial growth; with sparse submerged mycelium extending beyond area of aerial mycelium; no concentric zonation; margin very uneven; underside cream under inoculum block, otherwise off white. On ½ FIM, similar to PDA, but with less aerial mycelium and submerged mycelium more extensive. On MN and E-medium, similar to PDA (Figure 4).

Micromorphology: Hyphae 7-10 µm diameter, 50-100 µm long, cylindrical, sometimes short and swollen, swollen at one end or fusoid (to 15 µm in diameter), these cells in short, irregular chains; terminal elements sometimes clavate or subglobose; septate, lacking clamp connections; branched, branches usually at right angles and with constriction at base of branch hypha, but sometimes not constricted and/or with branch not at a right angle; sometimes the direction of chains of swollen cells changes, with one section of the chain arising from the side of the adjacent cell in the chain; multinucleate, with 6-12 nuclei per cell (Figure 6).

Other speciment examined: *A. Arifin* CLM2223, isolated from *Rimacola elliptica* collected from Morton National Park, NSW, Australia, September 2018 (RBGV-FCC 190932).

Notes: Compared to all species described (including six species from Chapter Three), *T. multinucleata* in culture has the most irregular growth, with dendroid growth on most media and a very uneven margin, as well as the most well-developed aerial mycelium. *T. australiensis* (Chapter Three) also has somewhat irregular growth on ¼ PDA but the dendritic growth is finer, does not have obvious dendritic growth on the other three media. In addition, *T. australiensis* also has very low growth rate on ½ FIM, no more than 20 mm in four weeks, compared to at least 50 mm for *T. multinucleata*. Morphologically, T. *multinucleata* is the only species that is multinucleate.

DISCUSSION

In *Tulasnella*, species delimitation using ITS gave a similar topology compared to combined nuclear and mitochondrial loci based on Linde et al. (2014). The ITS region has been widely-used to delimit cryptic species of fungi (Schoch et al., 2012), using a universal sequence divergence threshold is 3 % (Nilsson et al., 2008). However, several Basidiomycota fungi have been delimited with a range of sequence divergence thresholds. For example, the minimum sequence divergence is 2 % in *Cortinarius* (Stefani et al., 2014), 4-6 % in *Serendipita* (Whitehead et al., 2017) and ~3.3-5.7 % in *Tulasnella* (Linde et al., 2017). For *Tulasnella inflata* the sequence divergence with its closest relative was 19 % (*T*. sp. AL.KSE from an Australian terrestrial orchid *Thelymitra epipactoides* (Diurideae; Thelymitrinae) (Reiter et al., 2018)). *Tulasnella multinucleata* from *R. elliptica* showed sequences divergences 5.7-6.4 % with its closest relative *Tulasnella* CH01 (GenBank accession number KP973894, Table 3).

Australian terrestrial orchids within the Drakaeinae (Linde et al., 2017; Linde et al., 2014) (Chapter Two) and Cryptostylidinae (Nguyen et al., 2019) (Chapter One) all associate with *Tulasnella* symbionts classified as belonging to group IV. In contrast, orchids within the Megastylidinae associate with *Tulasnella* from phylogenetic groups II, III and group V in this study. Previously, *Pyrorchis* was shown to associate with a broad range of Rhizoctonia-like fungi including *T. danica* KC388 (Bonnardeaux et al., 2007) which belongs to *Tulasnella* group II and ericoid mycorrhizal fungi. In our study, *Pyrorchis* also associates with *Tulasnella* group II (Table 1, Figure S2 clones only, data not shown—but see Figure 2 in Chapter Two). As an orchid genus with a widespread distribution, *Pyrorchis* has the ability to utilise a broad range of fungi (distantly related *Tulasnella* group II and III) which may play an important role for the orchids to adapt and grow in various habitats (Bonnardeaux et al., 2007; De Long et al., 2013).

A recent study by Freitas et al. (2020) revealed the diversity of four new *Tulasnella* species obtained from Brazilian epiphytic orchids. The sequences from the Freitas et al. (2020) study are not yet available on GenBank, but their phylogeny which includes *T. pruinosa* and *T. asymmetrica* suggests that three of their *Tulasnella* species belongs to group III and could be closely-related to *T. inflata*. It is interesting that despite the geographical distance (America vs. Australia), different habitats (e.g. terrestrial vs. epiphytic and temperate vs. tropical orchids) and distantly related orchid hosts (Laeliinae vs. Megastylidinae), these orchids associate with closely related *Tulasnella* symbionts. However, the phylogenetic tree that delimit the four *Tulasnella* species in (Freitas et al., 2020) used an insufficient number of reference sequences and failed to include known closely related *Tulasnella* taxa e.g. those described in Reiter et al. (2018), Cruz et al. (2014) and Nontachaiyapoom et al. (2010).

T. multinucleata is the most distinctive species in *Tulasnella* with 6-12 nuclei in one cell compartment. Multiple nuclei per cell compartment may provide an adaptive advantage over fungi which are uni- or binucleate. Multinucleate fungi were shown to allow different sets of genes to use in substrate utilisation, which lead to fungal phenotypic adaptation (Jinks, 1952) and contributed to virulence in a fungal pathogen (Ma et al., 2010; Rep & Kistler, 2010). A number of filamentous Ascomycota harbours up to 100 nuclei per hyphal compartment, such as in *Neurospora crassa, Aspergillus nidulans, Sclerotinia sclerotiorum* and *Fusarium oxysporum* (Roper et al., 2011). Although little is known about the role of multinucleate in OMF, *T. multinucleata* provides a unique study opportunity to explore the mechanisms affected by multinucleasm. The host of *T. multinucleata*, *R. elliptica* grows in very different habitats compared to other Australian orchids—as a lithophyte growing on cliffs, and is narrowly distributed in eastern Australia (Jones, 2006). The advantage/disadvantage of being multinucleate, should therefore be explored for T. multinucleata. Although our sampling is very limited (only one population), this study reveals the possibilities of unearthed *Tulasnella* groups and species, that need to be explored and described.

These newly established species contribute to the number of described *Tulasnella* taxa in the world, providing accepted names to species assisting in the accurate identification of fungal taxa, especially for those that play a significant role as mycobiont partners for many endangered orchid species. Mostly, these newly described species will assist future ecological and conservation studies in Australia, thereby having the opportunity to deal with the same taxonomic identity for *Tulasnella* across studies.

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Table 1. *Tulasnella* isolates and clones used in this study. All isolates used for species descriptions are written in bold.

No.	Species/OTUs	Isolate/clone	Orchid host	Origin	Habitat	Group
1	***T. multinucleata* sp. nov.**	**CLM2222***	***Rimacola elliptica***	**Morton National Park, NSW**	**Growing on the cliff**	**V**
2	***T. multinucleata* sp. nov.**	**CLM2223**	***Rimacola elliptica***	**Morton National Park, NSW**	**Growing on the cliff**	**V**
3	*T. multinucleata* sp. nov.	CL072.2_clone1	*Rimacola elliptica*	Morton National Park, NSW	Growing on the cliff	V
4	*T. multinucleata* sp. nov.	CL072.2_clone2	*Rimacola elliptica*	Morton National Park, NSW	Growing on the cliff	V
5	*T. multinucleata* sp. nov.	CL072.1_clone3	*Rimacola elliptica*	Morton National Park, NSW	Growing on the cliff	V
6	*T. multinucleata* sp. nov.	CL072.1_clone4	*Rimacola elliptica*	Morton National Park, NSW	Growing on the cliff	V
7	*T. multinucleata* sp. nov.	CL072.1_clone5	*Rimacola elliptica*	Morton National Park, NSW	Growing on the cliff	V
8	*T. multinucleata* sp. nov.	CL072.1_clone6	*Rimacola elliptica*	Morton National Park, NSW	Growing on the cliff	V
9	*T. multinucleata* sp. nov.	CL072.1_clone7	*Rimacola elliptica*	Morton National Park, NSW	Growing on the cliff	V
10	*Tulasnella* OTU Q	BLR2_clone1	*Pyrorchis forrestii*	Blue lake Rd, WA	Jarrah forest	III
11	*Tulasnella* OTU Q	BLR2_clone2	*Pyrorchis forrestii*	Blue lake Rd, WA	Jarrah forest	III
12	*Tulasnella* OTU Q	BLR3_clone2	*Pyrorchis forrestii*	Blue lake Rd, WA	Jarrah forest	III
13	*Tulasnella* OTU S	CL042.2_clone1	*Pyrorchis nigricans*	West of lake Unicup, WA	Jarrah forest	III
14	*Tulasnella* OTU S	CL042.2_clone2	*Pyrorchis nigricans*	West of lake Unicup, WA	Jarrah forest	III
15	*Tulasnella* OTU S	CL042.2_clone3	*Pyrorchis nigricans*	West of lake Unicup, WA	Jarrah forest	III
16	*Tulasnella* OTU S	CL042.2_clone4	*Pyrorchis nigricans*	West of lake Unicup, WA	Jarrah forest	III
17	***T. inflata* sp. nov.**	**CLM1759**	***Pyrorchis nigricans***	**Pomeroy Rd, WA**	**Jarrah forest**	**III**
18	***T. inflata* sp. nov.**	**CLM1760**	***Pyrorchis nigricans***	**Pomeroy Rd, WA**	**Jarrah forest**	**III**
19	***T. inflata* sp. nov.**	**CLM1761**	***Pyrorchis nigricans***	**Pomeroy Rd, WA**	**Jarrah forest**	**III**

20	***T. inflata* sp. nov.**	**CLM1762**	***Pyrorchis nigricans***	**Pomeroy Rd, WA**	**Jarrah forest**	**III**
21	***T. inflata* sp. nov.**	**CLM1788***	***Pyrorchis nigricans***	**Pomeroy Rd, WA**	**Jarrah forest**	**III**
22	Unassigned *Tulasnella*	BLR1_clone1	*Pyrorchis forrestii*	Blue lake Rd, WA	Jarrah forest	II
23	Unassigned *Tulasnella*	BLR1_clone2	*Pyrorchis forrestii*	Blue lake Rd, WA	Jarrah forest	II
24	Unassigned *Tulasnella*	BLR1_clone3	*Pyrorchis forrestii*	Blue lake Rd, WA	Jarrah forest	II
25	Unassigned *Tulasnella*	BLR1_clone4	*Pyrorchis forrestii*	Blue lake Rd, WA	Jarrah forest	II
26	Unassigned *Tulasnella*	CL037.1_clone1	*Pyrorchis nigricans*	Pomeroy Rd, WA	Jarrah forest	II
27	Unassigned *Tulasnella*	CL037.2_clone1	*Pyrorchis nigricans*	Pomeroy Rd, WA	Jarrah forest	II
28	Unassigned *Tulasnella*	CL037.3_clone1	*Pyrorchis nigricans*	Pomeroy Rd, WA	Jarrah forest	II
29	Unassigned *Tulasnella*	CL037.3_clone2	*Pyrorchis nigricans*	Pomeroy Rd, WA	Jarrah forest	II
30	Unassigned *Tulasnella*	CL042.2_clone3	*Pyrorchis nigricans*	West of lake Unicup, WA	Jarrah forest	II
31	Unassigned *Tulasnella*	CL042.3_clone1	*Pyrorchis nigricans*	West of lake Unicup, WA	Jarrah forest	II
32	Unassigned *Tulasnella*	CL042.3_clone2	*Pyrorchis nigricans*	West of lake Unicup, WA	Jarrah forest	II

*Holotype

Table 2. Maximum percentage pairwise sequence divergence between species, within species, as well as minimum and maximum percentage pairwise sequence divergence between species, for the ITS region of *Tulasnella* group III.

	Max. within species	*Tulasnella* sp. AL.KSE	***T. inflata* sp.nov.**	*Tulasnella* sp. DC271	*T.* *'asymmetrica'*	*T. pruinosa*	*T.* cf. *pinicola*	*T.* OTU S
Tulasnella sp. AL.KSE	0.0							
***T. inflata* sp.nov.**	0.0	19.0						
Tulasnella sp. DC271	1.6	19.7-20.0	19.5-19.6					
T. 'asymmetrica'	1.6	21.7-23.2	20.2-21.2	16.3-18.7				
T. pruinosa	5.1	24.3-26.1	21.3-26.0	22.4-27.3	20.9-25.7			
T. cf. *pinicola*	0.4	23.8-24.0	19.1-19.2	21.4-22.3	19.5-21.0	12.9-18.0		
Tulasnella OTU S	0.9	44.7-45.0	42.7-43.0	41.3-42.4	42.4-43.7	43.6-46.8	43.2-43.6	
Tulasnella OTU Q	0.8	44.4-44.8	44.5-44.6	42.7-43.8	43.6-44.5	44.0-47.4	44.2-44.5	31.3-31.8

Table 3. Maximum percentage pairwise sequence divergence between species, within species, as well as minimum and maximum percentage pairwise sequence divergence between species, for the ITS region of *Tulasnella* group V.

	Max. within species	*T*. sp. Gno5C-1	*T*. sp. CH01	*T*. sp. DC2
JX514389 *Tulasnella* sp. Gno5C-1	-			
KP973894 *Tulasnella* sp. CH01	-	6.47		
MK239039 *Tulasnella* sp. DC2	-	3.91	6.3	
***T. multinucleata* sp. nov**	1.0	6-7.2	5.7-6.4	6.6-7.4

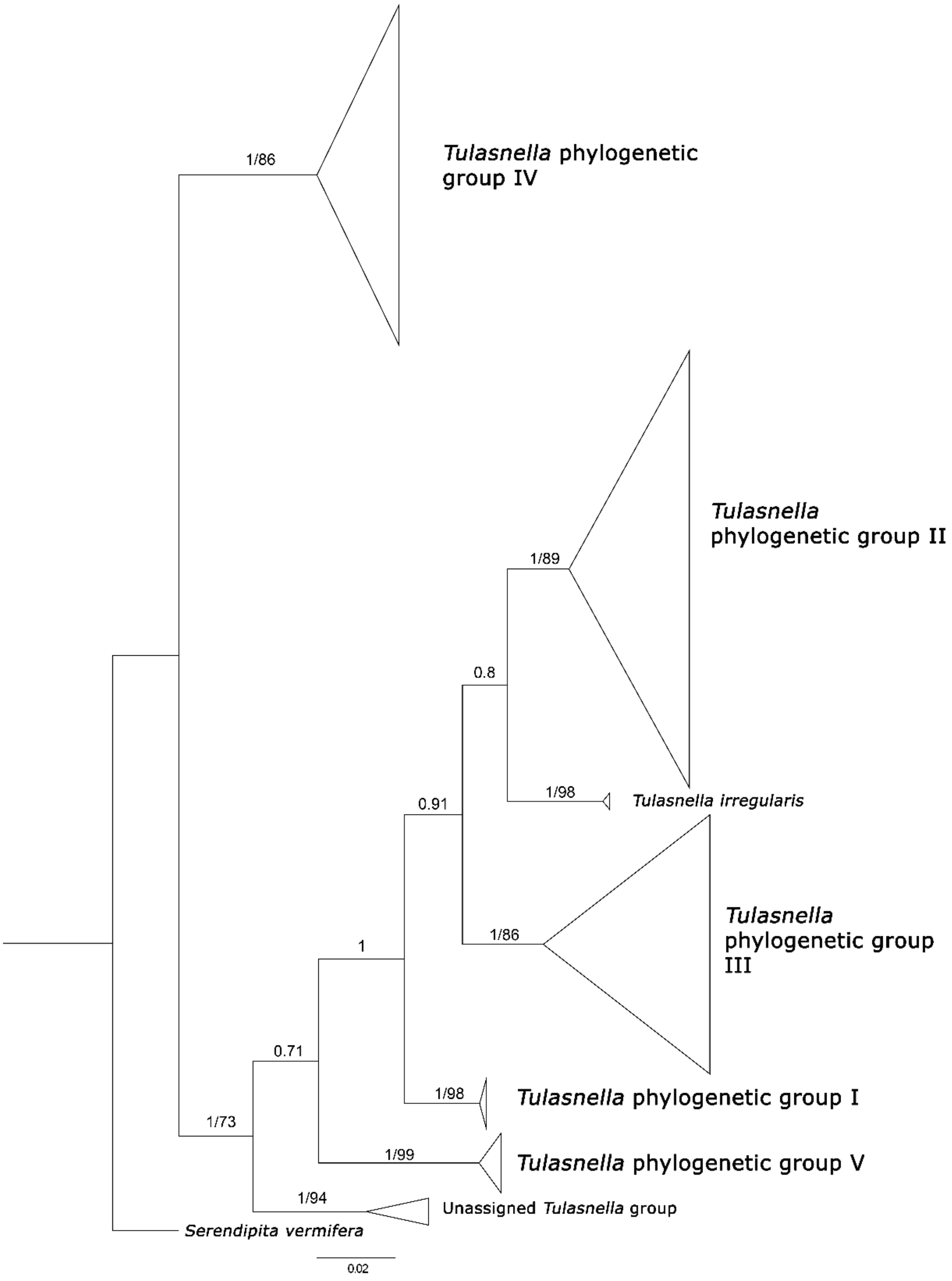

Figure 1. MrBayes phylogenetic tree for *Tulasnella* using the ITS 5.8S region. The phylogenetic groups I-IV are based on a previous study by Cruz et al. (2014). New sequences of *Tulasnella* clones/isolates from *Pyrorchis* and *Rimacola* are included in group II+III and the new phylogenetic group V, respectively. Numbers on the branches are Bayesian posterior probability / bootstrap support values. Posterior probabilities of ≥0.70 and Bootstrap values ≥70% are shown.

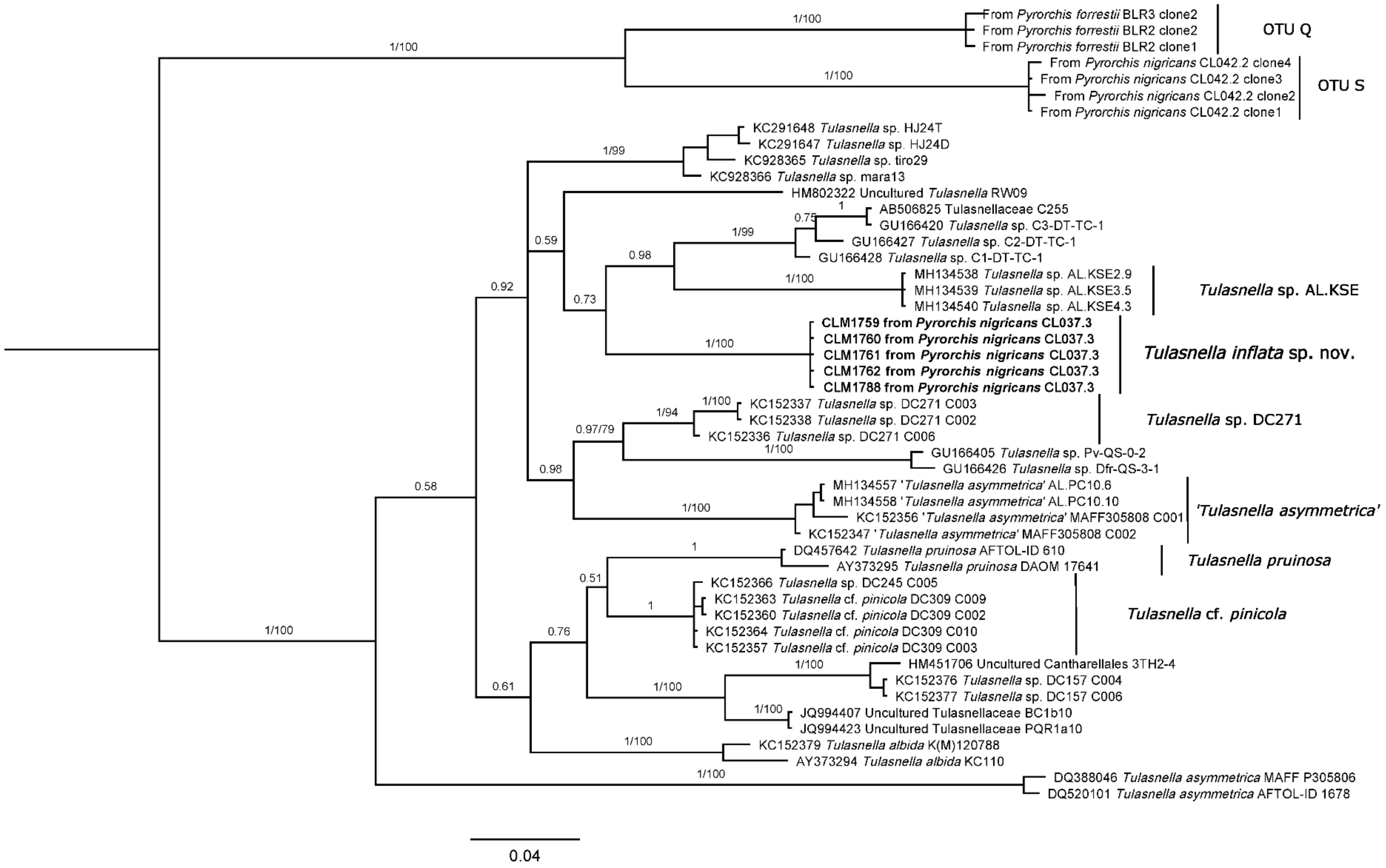

Figure 2. Midpoint-rooted MrBayes phylogenetic tree for *Tulasnella* phylogenetic group III using the ITS region. Numbers above the branches are Bayesian posterior probability/percentage bootstrap support. Bootstrap values ≥70% are shown. *Tulasnella* described in this study are shown in bold.

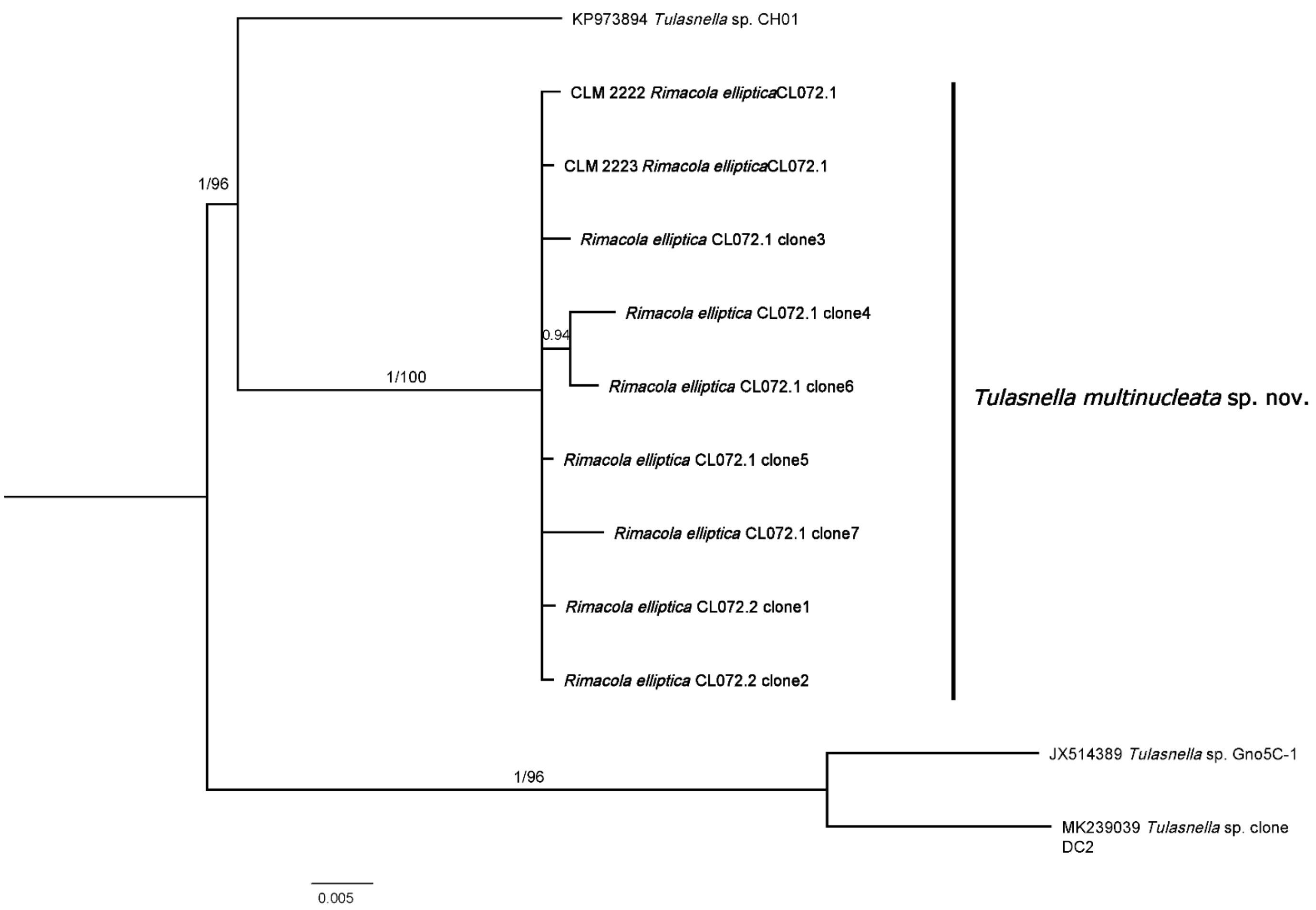

Figure 3. Midpoint-rooted MrBayes phylogenetic tree for *Tulasnella* phylogenetic group V using the ITS region. Numbers above the branches are Bayesian posterior probability/percentage of bootstrap support. *Tulasnella* described in this study are shown in bold.

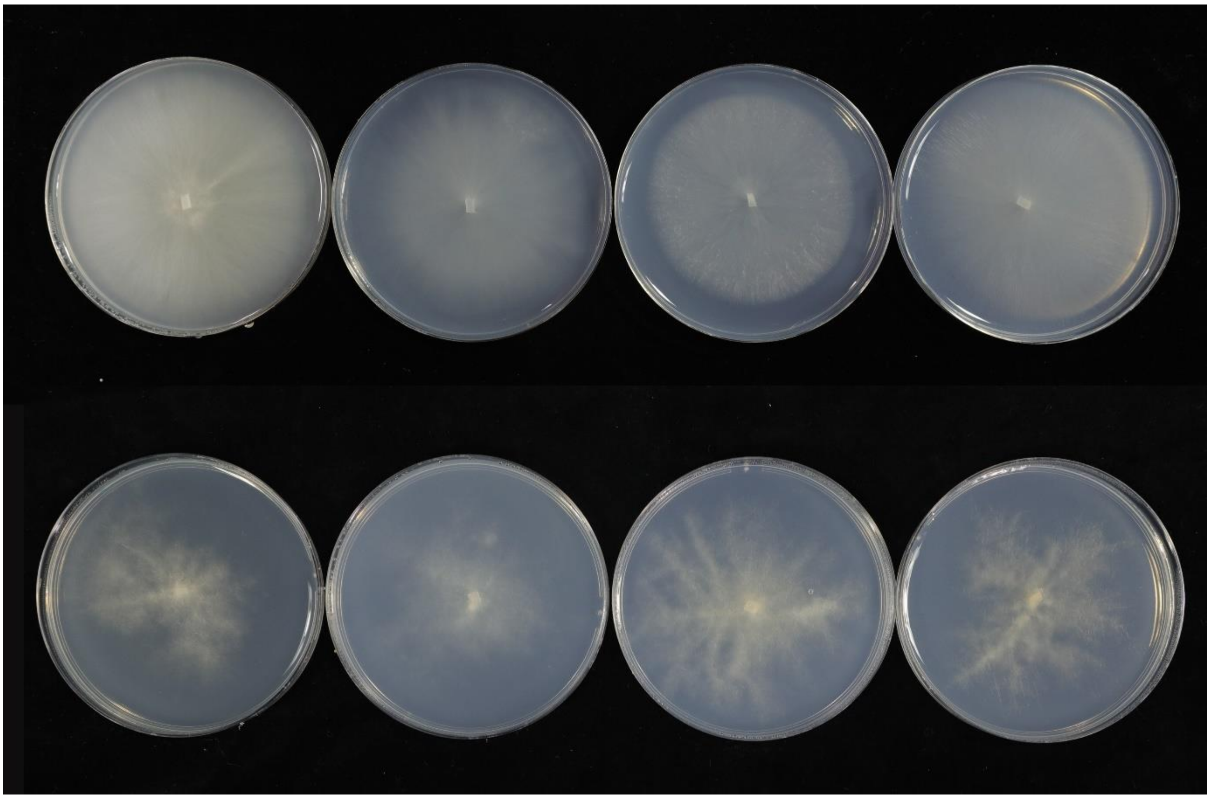

Figure 4. *Tulasnella* cultures (from left to right) on ¼ PDA, ½ FIM, 3MN+vitamin, and E-medium; all supplemented with streptomycin sulfate 50 mg/mL. TOP: *Tulasnella inflata* (CLM1788); BOTTOM*: Tulasnella multinucleata* (CLM2222).

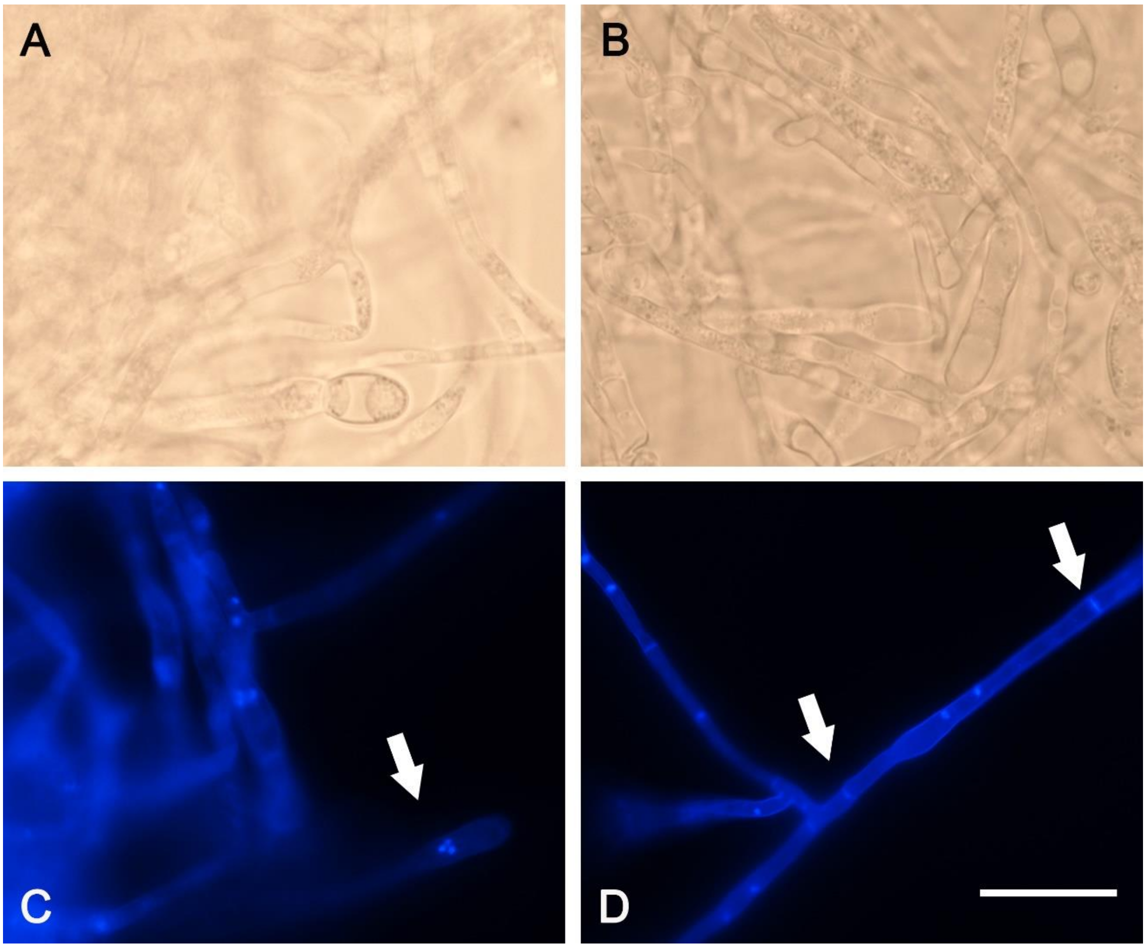

Figure 5. Micromorphological features of *Tulasnella inflata* showing swollen cell compartments (A-B), occasionally trinucleate (C, white arrows) with mostly binucleate (D) cell compartments separated by septa (D, white arrows). Bar = 20 µm.

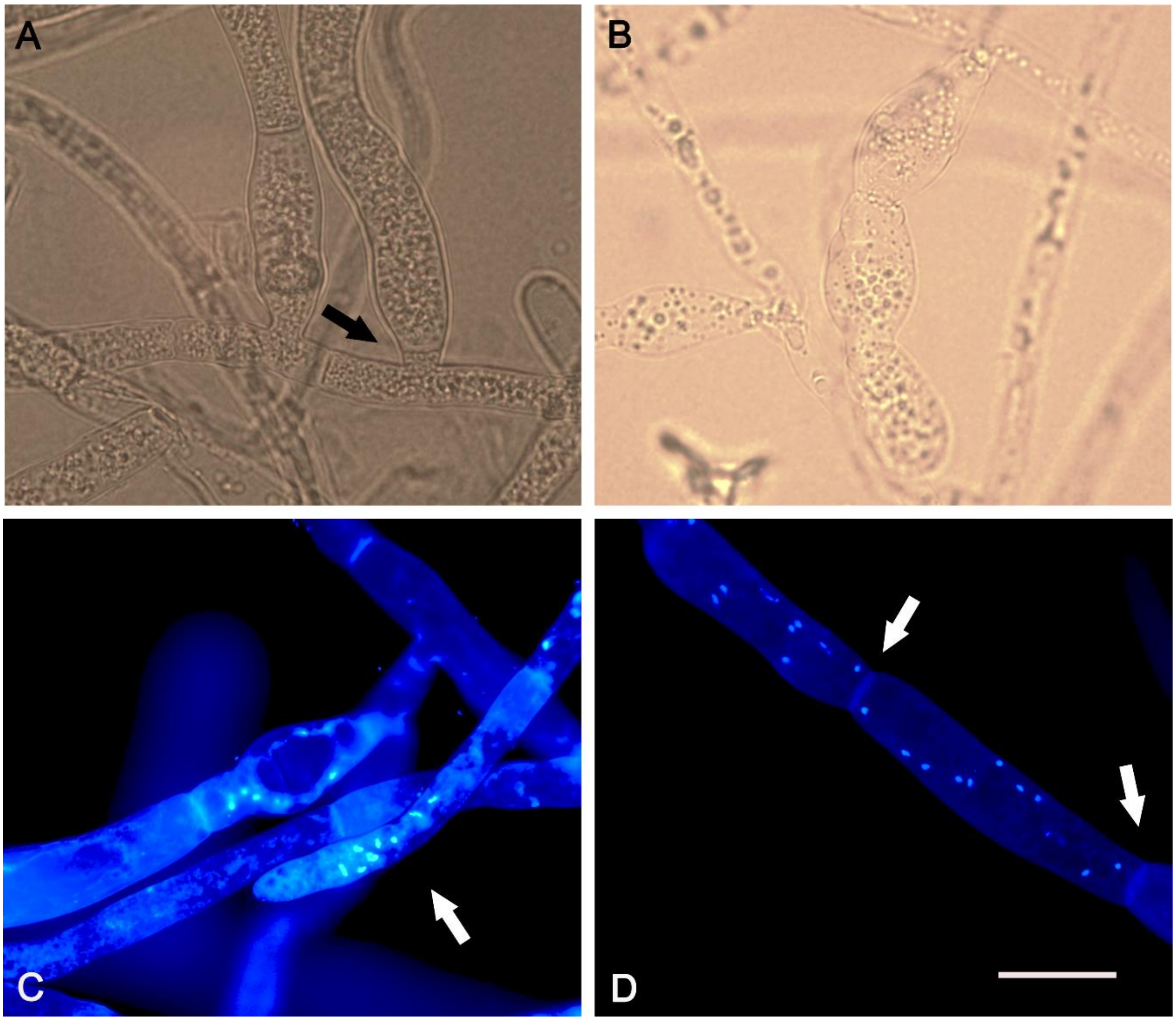

Figure 6. Micromorphological features of *Tulasnella multinucleata* showing branches mostly at right angle (A), swollen cell chains (B), multinucleate cell compartment, cylindrical hyphae and terminal end (C, white arrow), with septate (D, white arrows). Bar = 20 µm.

SUPPLEMENTARY MATERIALS

Table S1. Reference sequences used to construct the ITS 5.8S phylogenetic tree.

No.	GenBank	Species	Isolate/clone	Origin	Hosts/substrates	Group	References
1	KC152437	*Tulasnella violea*	DC293	Germany	Decayed wood	IV	Cruz et al. 2014
2	KC152415	*Tulasnella violea*	DC177	Ecuador	Fallen branch	IV	Cruz et al. 2014
3	AY373292	*Tulasnella eichleriana*	KC852	United States	Unknown	IV	McCormick et al. 2004
4	KC152389	*Tulasnella eichleriana*	DC294	Germany	Decayed wood	IV	Cruz et al. 2014
5	KC152397	*Tulasnella* sp. ECU5	DC225	Ecuador	Fallen branch	IV	Cruz et al. 2014
6	KC152398	*Tulasnella* sp. ECU5	DC225	Ecuador	Fallen branch	IV	Cruz et al. 2014
7	JN015192	*Tulasnella* sp.	BB0002_2_A	Australia	Terrestrial orchid	IV	Howard & Clements 2011**
8	MT036524	*Tulasnella* sp.	CLM2112	Australia	*Cryptostylis hunteriana*	IV	Arifin et al. 2020**
9	MT036520	*Tulasnella* sp.	CLM2117	Australia	*Cryptostylis hunteriana*	IV	Arifin et al. 2020**
10	KC152401	*Tulasnella* sp. ECU6	DC185	Ecuador	Fallen branch	IV	Cruz et al. 2014
11	KC152402	*Tulasnella* sp. ECU6	DC262	Ecuador	Fallen branch	IV	Cruz et al. 2014
12	KY095117*	*Tulasnella sphagneti*	CLM541	Australia	*Chiloglottis aff. valida*	IV	Linde et al. 2017
13	KY445922	*Tulasnella sphagneti*	CLM583	Australia	*Chiloglottis turfosa*	IV	Linde et al. 2017
14	KY445924	*Tulasnella sphagneti*	13102_1	Australia	*Chiloglottis turfosa*	IV	Linde et al. 2017
15	KY445926	*Tulasnella sphagneti*	13065_1	Australia	*Chiloglottis sp.*	IV	Linde et al. 2017
16	MT214493	*Tulasnella sphagneti*	CLM1952	Australia	*Cryptostylis subulata*	IV	Arifin et al. 2020**
17	KF476575*	*Tulasnella secunda*	CLM009	Australia	*Drakaea elastica*	IV	Linde et al. 2014
18	KF476588	*Tulasnella secunda*	CLM251	Australia	*Drakaea concolor*	IV	Linde et al. 2014
19	KF476568	*Tulasnella secunda*	CLM222	Australia	*Paracaleana minor*	IV	Linde et al. 2014
20	KF476592	*Tulasnella secunda*	CLM253	Australia	*Drakaea confluens*	IV	Linde et al. 2014

21	MT003731	*Tulasnella* sp.	CLM1944	Australia	*Cryptostylis erecta*	IV	Arifin et al. 2020**
22	MT003730	*Tulasnella* sp.	CLM1945	Australia	*Cryptostylis erecta*	IV	Arifin et al. 2020**
23	MT003714	*Tulasnella* sp.	CLM2005	Australia	*Cryptostylis erecta*	IV	Arifin et al. 2020**
24	KF476602	*Tulasnella* sp.	CLM031	Australia	*Arthrochilus oreophilus*	IV	Linde et al. 2014
25	KF476594	Unassigned *Tulasnella*	CLM084	Australia	*Arthrochilus oreophilus*	IV	Linde et al. 2014
26	KF476595	Unassigned *Tulasnella*	CLM085	Australia	*Arthrochilus oreophilus*	IV	Linde et al. 2014
27	KF476596*	*Tulasnella warcupii*	CLM027	Australia	*Arthrochilus oreophilus*	IV	Linde et al. 2014
28	KF476599	*Tulasnella warcupii*	CLM028	Australia	*Arthrochilus oreophilus*	IV	Linde et al. 2014
29	KF476600	*Tulasnella warcupii*	CLM007	Australia	*Arthrochilus oreophilus*	IV	Linde et al. 2014
30	KF476601	*Tulasnella warcupii*	CLM022	Australia	*Arthrochilus oreophilus*	IV	Linde et al. 2014
31	KF476543	*Tulasnella prima*	CLM309	Australia	*Chiloglottis formicifera*	IV	Linde et al. 2014
32	KF476550	*Tulasnella prima*	CLM306	Australia	*Chiloglottis formicifera*	IV	Linde et al. 2014
33	KF476551	*Tulasnella prima*	CLM308	Australia	*Chiloglottis formicifera*	IV	Linde et al. 2014
34	HM196794	*Tulasnella prima*	CM07.I.5	Australia	*Chiloglottis trapeziformis*	IV	Roche et al. 2010
35	MK430470	*Tulasnella prima*	CL12(5)	Australia	*Cryptostylis ovata*	IV	Nguyen et al. 2019
36	MK430473	*Tulasnella prima*	CL13(6)	Australia	*Cryptostylis ovata*	IV	Nguyen et al. 2019
37	MK430521	*Tulasnella prima*	CS43(17)	Australia	*Cryptostylis ovata*	IV	Nguyen et al. 2019
38	MK430445	*Tulasnella* sp.	CB43(9)	Australia	*Cryptostylis ovata*	IV	Nguyen et al. 2019
39	MK430439	*Tulasnella* sp.	CB14(1)	Australia	*Cryptostylis ovata*	IV	Nguyen et al. 2019
40	MT008096	*Tulasnella* sp.	CLM1938	Australia	*Cryptostylis ovata*	IV	Arifin et al. 2020**
41	MT008092	*Tulasnella* sp.	CLM1942	Australia	*Cryptostylis ovata*	IV	Arifin et al. 2020**
42	MT008091	*Tulasnella* sp.	CLM1943	Australia	*Cryptostylis ovata*	IV	Arifin et al. 2020**
43	MT008074	*Tulasnella* sp.	CLM2136	Australia	*Cryptostylis ovata*	IV	Arifin et al. 2020**
44	MK430451	*Tulasnella* sp. (Jarrahdale1)	CJ11(8)	Australia	*Cryptostylis ovata*	IV	Nguyen et al. 2019

45	MK430452	*Tulasnella* sp. (Jarrahdale1)	CJ13(3)	Australia	*Cryptostylis ovata*	IV	Nguyen et al. 2019
46	MK430461	*Tulasnella* sp. (Jarrahdale2)	CJ51(7)	Australia	*Cryptostylis ovata*	IV	Nguyen et al. 2019
47	MK430468	*Tulasnella* sp. (Jarrahdale2)	CJ51(17)	Australia	*Cryptostylis ovata*	IV	Nguyen et al. 2019
48	AY373296	*Tulasnella tomaculum*	KC429	USA	Unknown	IV	McCormick et al. 2004
49	KC152380	*Tulasnella tomaculum*	K(M)123675	United Kingdom	Unknown	IV	Cruz et al. 2014
50	MK626568	*Tulasnella* sp. JM 2019a	DAOMC	Canada	Rotten wood	IV	Mack and Seifert 2019
51	MK626686	*Tulasnella* sp. JM 2019a	DAOMC	Canada	Rotten wood	IV	Mack and Seifert 2019
52	MK626687	*Tulasnella* sp. JM 2019a	DAOMC	Canada	Rotten wood	IV	Mack and Seifert 2019
53	JX138568	*Tulasnella* sp.	MB-2012	Australia	*Spiculaea ciliata*	IV	Sommer et al. 2012
54	MN947569	*Tulasnella* sp.	CLM1773	Australia	*Spiculaea ciliata*	IV	Arifin et al. 2020**
55	MN947570	*Tulasnella* sp.	CLM1774	Australia	*Spiculaea ciliata*	IV	Arifin et al. 2020**
56	MT008124	*Tulasnella* sp.	CLM2012	Australia	*Cryptostylis subulata*	IV	Arifin et al. 2020**
57	MT008122	*Tulasnella* sp.	CLM2017	Australia	*Cryptostylis subulata*	IV	Arifin et al. 2020**
58	MT008121	*Tulasnella* sp.	CLM2018	Australia	*Cryptostylis subulata*	IV	Arifin et al. 2020**
59	MT008119	*Tulasnella* sp.	CLM2020	Australia	*Cryptostylis subulata*	IV	Arifin et al. 2020**
60	MT036547	*Tulasnella* sp.	CLM2071	Australia	*Cryptostylis leptochila*	IV	Arifin et al. 2020**
61	MT036546	*Tulasnella* sp.	CLM2072	Australia	*Cryptostylis leptochila*	IV	Arifin et al. 2020**
62	MT036545	*Tulasnella* sp.	CLM2142	Australia	*Cryptostylis leptochila*	IV	Arifin et al. 2020**
63	MT036533	*Tulasnella* sp.	CLM2198	Australia	*Cryptostylis leptochila*	IV	Arifin et al. 2020**
64	AJ313442	*Epulorhiza sp.*	Am8	Singapore	*Arachnis* 'Maggie oei'	II	Ma et al. 2003
65	AY373297	*Tulasnella danica*	KC 388	USA	Unknown	II	McCormick et al. 2004
66	KT164598	*Epulorhiza anaticula*	13O004	South Korea	*Platanthera chlorantha*	II	Han et al. 2015
67	EU218891	*Epulorhiza anaticula*	UAMH 5428	USA	Unknown	II	Taylor & McCormick 2008

68	EF176466	*Epulorhiza* sp.	Kings_Park_P25	Australia	*Pyrorchis nigricans*	II	Bonnardeaux et al. 2007
69	EF176465	*Epulorhiza* sp.	Kings_Park_P24	Australia	*Pyrorchis nigricans*	II	Bonnardeaux et al. 2007
70	EF176469	*Epulorhiza* sp.	Kings_Park_P30	Australia	*Pyrorchis nigricans*	II	Bonnardeaux et al. 2007
71	LC175307	*Tulasnella dendritica*	T273	Japan	*Spiranthes sinensis*	II	Fujimori et al. 2019
72	LC175311	*Tulasnella dendritica*	T300	Japan	*Spiranthes sinensis*	II	Fujimori et al. 2019
73	FJ613194	*Epulorhiza* sp.	YK-2009	China	*Cymbidium faberi*	II	Yang et al. 2009**
74	FJ786661	Uncultured *Tulasnella*	PA193	China	*Paphiopedilum armeniacum*	II	Yuan et al. 2010
75	GQ241779	Uncultured Tulasnellaceae	PM413	China	*Paphiopedilum micranthum*	II	Yuan et al. 2010
76	GQ241799	Uncultured Tulasnellaceae	PM10	China	*Paphiopedilum micranthum*	II	Yuan et al. 2010
77	GQ241801	Uncultured Tulasnellaceae	PM7	China	*Paphiopedilum micranthum*	II	Yuan et al. 2010
78	JF691162	Uncultured Tulasnellaceae	FM072.1	Reunion island	Orchid root	II	Martos et al. 2012
79	JF691200	Uncultured Tulasnellaceae	FM105.1	Reunion island	Orchid root	II	Martos et al. 2012
80	JF691203	Uncultured Tulasnellaceae	FM106.1	Reunion island	Orchid root	II	Martos et al. 2012
81	JQ771180	*Epulorhiza* sp.	CJ7	China	*Cymbidium*	II	Wang et al. 2012
82	KX587484	Uncultured Tulasnellaceae	43-11	China	*Dendrobium strongylanthum*	II	Xing et al. 2016**
83	MF427707	*Tulasnella phuhinrongklaensis*	CMU-CR41	Thailand	*Phalaenopsis pulcherrima*	II	Rachanarin et al. 2018
84	MF427708*	*Tulasnella phuhinrongklaensis*	CMU-CR42	Thailand	*Phalaenopsis pulcherrima*	II	Rachanarin et al. 2018
85	MH005847	Uncultured Tulasnellaceae	OTU8	China	Orchid root	II	Xing et al. 2018**
86	MH348613	Tulasnellaceae sp.	SSCDO-4	China	*Dendrobium catenatum*	II	Shao 2018**
87	MK142818	*Tulasnella* sp.	CMUFS1	Thailand	*Epipactis flava*	II	Suwannarach & Lumyong 2018**
88	MK142822	*Tulasnella* sp.	CMUFS2	Thailand	*Epipactis flava*	II	Suwannarach & Lumyong 2018**

89	NR160569	*Tulasnella dendritica*	MAFF 244709	Japan	*Spiranthes sinensis*	II	Fujimori et al. 2019
90	DQ178100	Uncultured *Tulasnella*	C2.1.3	Ecuador	*Pleurothallis lilijae*	II	Suarez et al. 2006
91	DQ388041	*Tulasnella calospora*	MAFF P305801	Australia	*Acianthus exsertus*	II	Suarez et al. 2006
92	DQ388042	*Tulasnella calospora*	MAFF P305802	Australia	*Diuris maculata*	II	Suarez et al. 2006
93	GU732309	Uncultured *Tulasnella*	DC42_C05	Ecuador	Decayed wood	II	Cruz et al. 2011
94	GU732324	Uncultured *Tulasnella*	DC5_C10	Ecuador	Decayed wood	II	Cruz et al. 2011
95	GU732327	Uncultured *Tulasnella*	DC24_C03	Ecuador	Decayed wood	II	Cruz et al. 2011
96	GU732345	Uncultured *Tulasnella*	DC42_C02	Ecuador	Decayed wood	II	Cruz et al. 2011
97	HM451585	Uncultured Cantharellales	1TC2_8	Ecuador	Orchid root	II	Herrera et al. 2011**
98	HM451675	Uncultured Cantharellales	3TB2_1	Ecuador	Orchid root	II	Herrera et al. 2011**
99	HM451698	Uncultured Cantharellales	3TG1_4	Ecuador	Orchid root	II	Herrera et al. 2011**
100	HM451699	Uncultured Cantharellales	3TG1_5	Ecuador	Orchid root	II	Herrera et al. 2011**
101	JF691000	Uncultured Tulasnellaceae	FM004.1	Reunion island	Orchid root	II	Martos et al. 2012
102	JF691009	Uncultured Tulasnellaceae	FM007.1	Reunion island	Orchid root	II	Martos et al. 2012
103	HM802310	Uncultured *Tulasnella*	RW03	New Zealand	*Nematoceras iridescens*	II	Watkins et al. 2011**
104	EF176473	*Epulorhiza* sp.	Kings_Park_D03	Australia	*Disa bracteata*	II	Bonnardeaux et al. 2007
105	EF160068	*Epulorhiza* sp.	Kings_Park_Dm01	Australia	*Diuris corymbosa*	II	Bonnardeaux et al. 2007
106	AY373291	*Tulasnella deliquescens*	DAOM 47.8	USA	Unknown	II	McCormick et al. 2004
107	DQ388044	*Tulasnella calospora*	MAFF P305804	Australia	*Thelymitra* sp.	II	Suarez et al. 2006
108	FJ613169	*Tulasnella calospora*	H0302-8	China	*Cymbidium faberi*	II	Yang et al. 2009**
109	JQ713573	*Epulorhiza* sp.	M-1	China	*Ascocentrum himalaicum*	II	Tan & Guo 2012
110	LC175313	*Tulasnella ellipsoidea*	T289	Japan	*Spiranthes sinensis*	II	Fujimori et al. 2019

111	LC175322	*Tulasnella cumulopuntioides*	T164b	Japan	*Spiranthes sinensis*	II	Fujimori et al. 2019
112	LC175324	*Tulasnella cumulopuntioides*	T304	Japan	*Spiranthes sinensis*	II	Fujimori et al. 2019
113	KX929166	*Tulasnella tubericola*	EP15	Spain	*Quercus ilex* subsp. ballota	II	Solis et al. 2017
114	KX774347	*Tulasnella tubericola*	EP3	Spain	*Quercus ilex* subsp. ballota	II	Solis et al. 2017
115	KX774346	*Tulasnella tubericola*	EP2	Spain	*Quercus ilex* subsp. ballota	II	Solis et al. 2017
116	KX774345	*Tulasnella tubericola*	EP1	Spain	*Quercus ilex* subsp. ballota	II	Solis et al. 2017
117	KU664577	*Tulasnella* sp.	7 MM-2016	USA	*Goodyera pubescens*	II	McCormick et al. 2016
118	KC291620	*Tulasnella calospora*	HB2E	Brazil	*Hoffmannseggella cinnabarina*	II	Bocayuva et al. 2012**
119	KC291619	*Tulasnella calospora*	HB2C	Brazil	*Hoffmannseggella cinnabarina*	II	Bocayuva et al. 2012**
120	KC154065	*Epulorhiza* sp.	OM23	Brazil	Orchid root	II	Noguieira et al. 2012**
121	KC154064	*Epulorhiza* sp.	OM24	Brazil	Orchid root	II	Noguieira et al. 2012**
122	JQ655466	*Tulasnella calospora*	BUH0004	India	*Eulophia epidendreae*	II	Sathiyadash et al. 2012**
123	JF907598	*Epulorhiza epiphytica*	AERO_3.2	Brazil	Orchid root	II	Almeida et al. 2014
124	HQ833210	*Tulasnella calospora*	CG035	China	*Cymbidium goeringi*	II	Gui et al. 2010**
125	FJ594935	*Tulasnella calospora*	H1401-30	China	*Cymbidium*	II	Li et al. 2008**
126	FJ594934	*Tulasnella calospora*	H1401-29	China	*Cymbidium*	II	Li et al. 2008**
127	EF393625	*Epulorhiza* sp.	Yuhui CH02X2-1	China	*Cymbidium faberi*	II	Yang et al. 2007**
128	DQ790804	Uncultured mycorrhizal fungus	isolate 19	Australia	*Diuris daltonii*	II	Smith et al. 2010
129	DQ790798	Uncultured mycorrhizal fungus	isolate 13	Australia	*Diuris punctata*	II	Smith et al. 2010
130	AY373290	*Tulasnella bifrons*	BPI 724849	USA	Unknown	II	McCormick et al. 2004
131	AY373307	*Tulasnella* sp.	186	USA	*Tipularia discolor*	II	McCormick et al. 2004
132	AY373273	*Tulasnella* sp.	149	USA	*Goodyera pubescens*	II	McCormick et al. 2004
133	AY373270	*Tulasnella* sp.	241	USA	*Goodyera pubescens*	II	McCormick et al. 2004
134	AY373267	*Tulasnella* sp.	148	USA	*Goodyera pubescens*	II	McCormick et al. 2004

135	MH861654	*Tulasnella irregularis*	CBS 574.83	Australia	Type of *T. irregularis* Warcup &PHB Talbot	Unassigned	Vu et al. 2019
136	GU166423	*Tulasnella irregularis*	C3-DT-TC-2	Thailand	*Cymbidium* Golden Elf×*Grammatophyllum measuresianum*	Unassigned	Nontachaiyapoom et al. 2010
137	GU166413	*Tulasnella irregularis*	D1-KT-TC-1	Thailand	*Cymbidium* Golden Elf×*Grammatophyllum measuresianum*	Unassigned	Nontachaiyapoom et al. 2011
138	EU218889	*Tulasnella irregularis*	CBS 574.83	Australia	Type of *T. irregularis* Warcup &PHB Talbot	Unassigned	Taylor & McCormick 2008
139	DQ388046	*Tulasnella asymmetrica*	MAFF P305806	Australia	*Thelymitra luteocilium*	III	Suarez et al. 2006
140	DQ520101	*Tulasnella asymmetrica*	AFTOL-ID 1678	Australia	Unknown	III	Garnica & Weiss 2009
141	MH134557	*"Tulasnella asymmetrica"*	AL.PC10.6	Australia	*Thelymitra epipactoides*	III	Reiter et al. 2018
142	MH134558	*"Tulasnella asymmetrica"*	AL.PC10.10	Australia	*Thelymitra epipactoides*	III	Reiter et al. 2018
143	KC152347	*"Tulasnella asymmetrica"*	MAFF305808_C002	Australia	Terrestrial orchid	III	Cruz et al. 2014
144	KC152356	*"Tulasnella asymmetrica"*	MAFF305808_C001	Australia	Terrestrial orchid	III	Cruz et al. 2014
145	HM451706	Uncultured Cantharellales	3TH2_4	Ecuador	Orchid	III	Herrera et al. 2014
146	KC152376	*Tulasnella* sp. ECU3	DC157 C004	Ecuador	Decaying wood	III	Cruz et al. 2014
147	KC152377	*Tulasnella* sp. ECU3	DC157 C006	Ecuador	Decaying wood	III	Cruz et al. 2014
148	KC152336	*Tulasnella* sp.	DC271 C006	Ecuador	Fallen branch	III	Cruz et al. 2014
149	KC152337	*Tulasnella* sp.	DC271 C003	Ecuador	Fallen branch	III	Cruz et al. 2014
150	MH134538	*Tulasnella* sp.	AL.KSE2.9	Australia	*Thelymitra epipactoides*	III	Reiter et al. 2018
151	MH134539	*Tulasnella* sp.	AL.KSE3.5	Australia	*Thelymitra epipactoides*	III	Reiter et al. 2018
152	MH134540	*Tulasnella* sp.	AL.KSE4.3	Australia	*Thelymitra epipactoides*	III	Reiter et al. 2018
153	AB506825	Tulasnellaceae	C255	Japan	*Cymbidium dayanum*	III	Ogura-Tsujita et al. 2012

154	GU166420	*Tulasnella* sp.	C3-DT-TC-1	Thailand	*Cymbidium* Golden Elf×*Grammatophyllum measuresianum*	III	Nontachaiyapoom et al. 2011
155	GU166427	*Tulasnella* sp.	C2-DT-TC-1	Thailand	Cymbidium Miniature	III	Nontachaiyapoom et al. 2011
156	GU166428	*Tulasnella* sp.	C1-DT-TC-1	Thailand	*Cymbidium* Golden Elf	III	Nontachaiyapoom et al. 2011
157	KC152366	*Tulasnella* sp.	DC245 C005	Ecuador	Unknown	III	Cruz et al. 2014
158	KC152379	*Tulasnella albida*	K(M)120788	England	Basidiomata	III	Cruz et al. 2014
159	AY373294	*Tulasnella albida*	KC 110	USA	Unknown	III	McCormick et al. 2004
160	KC152363	*Tulasnella cf. pinicola*	DC309 C009	Germany	Basidiomata	III	Cruz et al. 2014
161	KC152364	*Tulasnella cf. pinicola*	DC309 C010	Germany	Basidiomata	III	Cruz et al. 2014
162	KC152357	*Tulasnella cf. pinicola*	DC309 C003	Germany	Basidiomata	III	Cruz et al. 2014
163	KC152360	*Tulasnella cf. pinicola*	DC309 C002	Germany	Basidiomata	III	Cruz et al. 2014
164	DQ457642	*Tulasnella pruinosa*	AFTOL-ID 610	Unknown	Unknown	III	Matheny et al. 2006
165	AY373295	*Tulasnella pruinosa*	DAOM 17641	Unknown	Unknown	III	McCormick et al. 2004
166	GU166405	*Tulasnella pruinosa*	Pv-QS-0-2	Thailand	*Paphiopedilum vilosum*	III	Nontachaiyapoom et al. 2011
167	GU166426	*Tulasnella pruinosa*	Dfr-QS-3-1	Thailand	*Dendrobium friedericksianum*	III	Nontachaiyapoom et al. 2011
168	JQ994407	Uncultured Tulasnellaceae	BC1b10	USA	*Piperia yadonii*	III	Pandey et al. 2013
169	JQ994423	Uncultured Tulasnellaceae	PQR1a10	USA	*Piperia yadonii*	III	Pandey et al. 2013
170	KC291647	*Tulasnella* sp.	HJ24D	Brazil	*Hadrolaelia jongheana*	III	Bocayuva et al. 2013**
171	KC291648	*Tulasnella* sp.	HJ24T	Brazil	*Hadrolaelia jongheana*	III	Bocayuva et al. 2013**
172	KC928365	*Tulasnella* sp.	tiro29	Brazil	*Encyclia ghillanyi*	III	Almeida et al. 2017**
173	KC928366	*Tulasnella* sp.	mara13	Brazil	*Encyclia ghillanyi*	III	Almeida et al. 2017**
174	HM802322	Uncultured *Tulasnella*	RW09	New Zealand	*Singularybas oblongus*	III	Watkins 2011
175	KC152322	*Tulasnella* sp. DC245	DC294	Germany	Unknown	I	Cruz et al. 2014

176	KC152321	*Tulasnella* sp.	DC294 C003	Germany	Unknown	I	Cruz et al. 2014
177	KC152320	*Tulasnella* sp.	DC294 C002	Germany	Unknown	I	Cruz et al. 2014
178	HM451636	uncultured Cantharellales	2TB3_5	Ecuador	Unknown	I	Herrera et al. 2011**
179	HM451502	uncultured Cantharellales	1EA2_6	Ecuador	Orchid	I	Herrera et al. 2011**
180	HM451601	uncultured Cantharellales	1TF1_3	Ecuador	Orchid	I	Herrera et al. 2011**
181	HM451632	uncultured Cantharellales	2TB3_11	Ecuador	Orchid	I	Herrera et al. 2011**
182	HM451615	uncultured Cantharellales	1TG2_4	Ecuador	Orchid	I	Herrera et al. 2011**
183	HM451531	uncultured Cantharellales	1EE2_2	Ecuador	Orchid	I	Herrera et al. 2011**
184	DQ178117	Uncultured *Tulasnella*	C3MN3	Ecuador	*Stelis superbiens*	I	Suarez et al. 2006
185	JX138569	*Tulasnella* sp.	13 MB-2012	Australia	*Lyperanthus serratus*	Unassigned	Sommer et al. 2012
186	KC440181	Uncultured Tulasnellaceae	ece5r1_4OF_a10_080	Costa Rica	*Epidendrum firmum*	Unassigned	Kartzinel et al. 2013
187	JX998901	Uncultured Tulasnellaceae	T040MIR15R1	Costa Rica	*Epidendrum firmum*	Unassigned	Kartzinel et al. 2013
188	JX998890	Uncultured Tulasnellaceae	T026EF10070613R4	Costa Rica	*Epidendrum firmum*	Unassigned	Kartzinel et al. 2013
189	JX998862	Uncultured Tulasnellaceae	T140EF10070613R2	Costa Rica	*Epidendrum firmum*	Unassigned	Kartzinel et al. 2013
190	KX587492	Uncultured Tulasnellaceae	37-43	China	*Dendrobium aphyllum*	Unassigned	Xing et al. 2017
191	MK239039	*Tulasnella* sp.	DC2	South Africa	*Disa cornuta*	New group	Khambule et al. 2018**
192	KP973894	*Tulasnella* sp.	CH01	Brazil	*Cyrtopodium hatschbachii*	New group	de Carvalho et al. 2015**
193	JX514389	*Tulasnella* sp.	Gno5C-1	Taiwan	*Acanthephippium striatum*	New group	Jiang 2012**
194	DQ983816	*Serendipita vermifera*	MAFF305838	Australia	*Caladenia tesselata*	Outgroup	Desmukh et al. 2006

*holotype

**unpublished

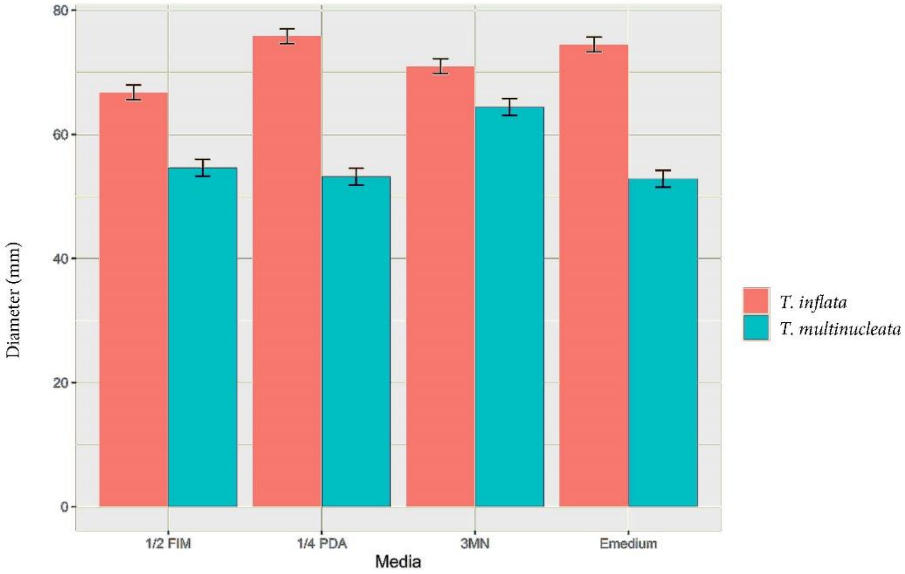

Figure S1. Colony diameter (mm) of two new *Tulasnella* species on four different media measured after incubation at 22 °C for four weeks in the dark. The colony diameters are significantly different (*P* < 0.001) between two species within each media (Error bars indicate Standard Error).

CHAPTER FIVE

Orchid specificity at subtribe level: Thelymitrinae and Megastylidinae

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ABSTRACT

Tulasnella are fungi that form mycorrhizal associations with some orchids. The orchid subtribe Megastylidinae and Thelymitrinae mostly associated with distinct *Tulasnella* phylogenetic groups compared to *Tulasnella* symbionts in Cryptostylidinae and Drakaeinae. While all Cryptostylidinae and Drakaeinae associate with *Tulasnella* phylogenetic group IV, Megastylidinae and Thelymitrinae mostly have fungal partners in *Tulasnella* phylogenetic group II and III. However, a few exceptions were noted in the orchid genera *Lyperanthus* and *Rimacola* (Megastylidinae) which have two unassigned *Tulasnella* groups and *Calochilus* (Thelymitrinae) that associate with two OTUs in *Tulasnella* phylogenetic group IV. Thus, at the subtribe level, Megastylidinae and Thelymitrinae have multiple association with a broad range of *Tulasnella* groups, whereas fungal associations ofthe Drakaeinae and Cryptostylidinae are limited to *Tulasnella* phylogenetic group IV species.

INTRODUCTION

Photosynthetic orchids commonly associate with Rhizoctonia-like fungi of the Sebacinaceae, Ceratobasidiaceae and Tulasnellaceae (Rasmussen, 2002; Rasmussen & Rasmussen, 2009). Based on their relationships with orchid mycorrhizal fungi (OMF), orchids that are specialists associate with a small number of fungal associations; such as in *Drakaea* (Phillips et al., 2011), *Chiloglottis* (Roche et al., 2010; Ruibal et al., 2017) and *Caladenia* (Whitehead et al., 2017). In contrast, many orchid genera can be categorized as generalists since they associate with a broad range of OMF e.g. *Microtis* (De Long et al., 2013), *Dactylorhiza* (Jacquemyn et al., 2012), *Tipularia* (McCormick et al., 2004) and *Orchis* (Jacquemyn et al., 2010). However, whether an orchid is a generalist or specialist in their mycorrhizal associations does not correlate with the orchid's distribution (ie. rarity) (Davis et al., 2015; McCormick & Jacquemyn, 2014; Phillips et al., 2011).

In the recent classification of Orchidaceae, the subtribe Megastylidinae includes seven genera; *Burnettia* Lindl., *Leporella* A.S.George, *Lyperanthus* R.Br., *Megastylis* (Schltr.) Schltr., *Pyrorchis* D.L.Jones & M.A.Clements, *Rimacola* Rupp and *Waireia* D.L.Jones, Molloy & M.A.Clements (Chase et al., 2015; Weston et al., 2014). Several studies investigated the diversity of OMF in Megastylidinae (Bonnardeaux et al., 2007; Sommer et al., 2012) and Thelymitrinae (Reiter et al., 2018) and showed Tulasnellaceae as the main symbiont in these orchid subtribes. However, these studies are limited to only a few species in the Thelymitrinae and Megastylidinae and the OMF of several other genera are still largely unknown. Furthermore, these orchids have a wide distribution across Australia and The Pacific, as well as occur in various habitats, suggesting that there may be many fungal symbiont taxa that have not been recognized previously. This chapter is a preliminary study into the OMF diversity of Thelymitrinae (*Calochilus*) and five genera of Megastylidinae, focussing on Tulasnellaceae fungi as the main symbiont.

MATERIALS AND METHODS

Study species and sampling

Five genera (seven species) of Megastylidinae were sampled across Australia in this study; two monotypic genera *Rimacola elliptica* and *Leporella fimbriata, Lyperanthus* (*L. serratus, L. suaveolens*), *Pyrorchis* (*P. nigricans, P. forestii*) and *Megastylis gigas*. In addition, four *Calochilus* species (*C. paludosus, C. platychilus, C. robertsonii* and *C. gracillimus*) were collected from several populations in NSW and Victoria (Table 1). All four *Calochilus* species sampled belong to one subgenus (Core *Calochilus* clade) based on infrageneric classification in Nargar et al. (2018).

Fungal isolations

Please see Chapter Two for detailed methods.

Fungal DNA extraction, PCR amplification and sequencing

Please see Chapter Two for detailed methods.

Phylogenetic analyses

Sequencing results were checked and manually edited using Sequencher v.5.4.1 (Genes Codes Corp., Michigan, USA). All obtained sequences from fungal cultures and clones were matched on BLASTN on NCBI GenBank (http://www.ncbi.nlm.nih.gov/). All *Tulasnella* sequences as well as their closely-related sequences from GenBank were selected and grouped based on their corresponding *Tulasnella* phylogenetic groups (Cruz et al., 2014). ITS phylogenetic trees (ITS1+5.8S+ITS2) were constructed for each phylogenetic group since the ITS regions of each group is divergent and could not be aligned. Phylogenetic trees were constructed using Bayesian inference analysis using MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003). Node support was assessed with Bayesian Posterior Probabilities (BPP) for 1,100,000 generations and trees were sampled every 200 generations with a 25% burn-in. Convergence of runs was confirmed when the average standard deviation of the split frequencies was <0.01 and the effective sample sizes >200. Furthermore, Maximum Likelihood (ML) RAxML (Stamatakis et al., 2008) phylogenetic tree inference was conducted using 1,000 bootstrap replicates under the GTR + GAMMA model. Both BPP and ML analyses were conducted using Geneious v.10.2.6 (Kearse et al., 2012) and visualised using FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). All trees were midpoint rooted.

RESULTS

In total, we obtained 13 isolates and 71 clones from five genera in Megastylidinae and *Calochilus* (Thelymitrinae) (Table 1). Five phylogenetic trees from different *Tulasnella* groups were constructed; phylogenetic group II, III, IV and two unassigned groups. All *Tulasnella* sequences from four *Calochilus* species (Thelymitrinae) were identified as OTU (Operational taxonomic unit) O and OTU T, both are sister clades and grouped into *Tulasnella* phylogenetic group IV (Figure 1).

Most *Tulasnella* obtained from Megastylidinae belonged to *Tulasnella* Group II and III based on the classification by Cruz et al. (2014), as well as two unassigned groups. *Megastylis gigas* (Megastylidinae) associated with one OTU from *Tulasnella* Group II (Figure 2) and two *Pyrorchis* species associated with five *Tulasnella* OTUs (representing Group II and III; see Figure 2 and Figure 3 respectively). *Lyperanthus serratus* and *L. suaveolens* (Megastylidinae) associated with one *Tulasnella* OTU which is clustered into an unassigned phylogenetic group (Figure 4, closest GenBank match to *Tulasnella* sp. 13 MB-2012—GenBank accession number JX138569). *Rimacola* associated with a single *Tulasnella* OTU (another unassigned phylogenetic group), and *Leporella* did not associate with *Tulasnella*, but with *Ceratobasidium* (data not shown, closest GenBank match *Ceratobasidium* sp. RGDAL1, accession number GQ175314).

DISCUSSION

Drakaeinae and Cryptostylidinae subtribes have a “constrained” associations with closely related lineages in *Tulasnella* phylogenetic group IV. *Tulasnella* from the Megastylidinae orchids did not share the same fungi as the Drakaeinae and Cryptostylidinae orchids. In contrast, Megastylidinae, which are more closely related to Drakaeinae than Cryptostylidinae (Weston et al., 2014), associates with multiple *Tulasnella* phylogenetic groups (II, III and two unassigned groups), but not group IV. Two genera in Megastylidinae (*Rimacola* and *Lyperanthus*) associate with two different unassigned *Tulasnella* phylogenetic groups and none of species from these groups have been described. *Megastylis gigas* associates with *Tulasnella* group II and *Pyrorchis* associates with *Tulasnella* in both groups (II and III). Although we only have one genus with four species of *Calochilus* in subtribe Thelymitrinae, it associates with *Tulasnella* phylogenetic group IV. Meanwhile, the sister genus *Thelymitra* associates with *Tulasnella* phylogenetic group III (Reiter et al., 2018 - '*Tulasnella asymmetrica*' which clusters in group III). Thus multiple "gains" in fungal associations were made by Thelymitrinae and Megastylidinae from the ancestral Cryptostylidinae associations, most of which were apparently lost again in the Drakaeinae.

Our results show that Cryptostylidinae and Drakaeinae are specialists in their fungal associations (group IV only), whereas Thelymitrinae and Megastylidinae are more generalist (associate with multiple *Tulasnella* groups). Furthermore, despite Cryptostylidinae and Drakaeinae being not closely related subtribes (Weston et al., 2014), they have in common associations with *Tulasnella* group IV, even sharing *Tulasnella* species/OTUs. Therefore, Cryptostylidinae and Drakaeinae appears to show some phylogenetic signal for at least the *Tulasnella* group they associate with.

Interestingly, a *Tulasnella* OTU from *Megastylis* has very high sequence similarity with *T. phuhinrongklaensis* (94.5-96 %) and *T. dendritica* (96-97 %) (Figure 2) and likely is *T. dendritica* based on a 3.3-5.7 % sequence divergence species delimitation in *Tulasnella* (Linde et al., 2017). Sequence divergence between *T. dendritica* and *T. phuhinrongklaensis* is only 3-4 %. This, however, also questions the validity of the recently described *T. phuhinrongklaensis* (Rachanarin et al., 2018) as a novel species because the earliest validly published name should be retained (Turland et al., 2018). A comprehensive examination of all described species and published sequences is, therefore, necessary for *Tulasnella* phylogenetic group II.

Calochilus (Thelymitrinae) associates with two *Tulasnella* OTUs, OTU O and OTU T which fell into phylogenetic group IV and closely related with *Tulasnella* obtained from Cryptostylidinae and Drakaeinae. However, the fungal associations of *Calochilus* might be greater since we only sampled four closely related *Calochilus* species from eastern Australia, all of which are classified into the core *Calochilus* clade (Nargar et al., 2018). *Calochilus* appears to have demanding fungal growth requirements as we only successfully cultivate five isolates from *C. robertsonii* and none from the three other species.

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Table 1. The number of orchid plants analysed and the number of *Tulasnella* isolates and clones from Australian / New Caledonia orchids of the subtribe Thelymitrinae and Megastylidinae.

No	Host Taxon	Subtribe	Locality	State/Country	Plant identification number	Number of plants	Number of Isolates	Number of Clones	*Tulasnella* Group (Cruz et al. 2014)
1	*Calochilus paludosus* R.Br.	Thelymitrinae	Bulahdelah	New South Wales	CL080	1	0	4	IV
2	*Calochilus platychilus* D.L.Jones.	Thelymitrinae	Gipsy Point	Victoria	CL094	3	0	6	IV
3	*Calochilus robertsonii* Benth.	Thelymitrinae	Oallen Ford Rd	New South Wales		3	5	—	IV
4	*Calochilus gracillimus* Rupp.	Thelymitrinae	Sanctuary Point	New South Wales	CL134	3	0	8	IV
5	*Leporella fimbriata* (Lindl.) A. S. George.	Megastylidinae	Eukaba	South Australia		4	0	0	Not *Tulasnella*
6	*Leporella fimbriata* (Lindl.) A. S. George.	Megastylidinae	Pomeroy Rd	Western Australia		3	0	0	Not *Tulasnella*
7	*Leporella fimbriata* (Lindl.) A. S. George.	Megastylidinae	Canning Mills	Western Australia		3	0	0	Not *Tulasnella*
8	*Lyperanthus suaveolens* R.Br.	Megastylidinae	Black Mountains	Australian Capital Territory	CL071	2	1	6	Unassigned
9	*Lyperanthus suaveolens* R.Br.	Megastylidinae	Mallacoota	Victoria	CL095	3	0	3	Unassigned
10	*Lyperanthus serratus* Lindl.	Megastylidinae	Hazeldene	Western Australia		3	—	16	Unassigned
11	*Pyrorchis nigricans* (R.Br.) D.L.Jones & M.A.Clem.	Megastylidinae	Pomeroy Rd	Western Australia	CL037	3	5	4	II and III
12	*Pyrorchis nigricans* (R.Br.) D.L.Jones & M.A.Clem.	Megastylidinae	Lake Unicup	Western Australia	CL042	3	—	6	II and III
13	*Pyrorchis forestii* (F.Muell.) D.L.Jones & M.A.Clem.	Megastylidinae	Blue Lake Rd	Western Australia		3	—	5	II and III
14	*Rimacola elliptica* (R.Br.) Rupp.	Megastylidinae	Morton National Park	New South Wales	CL072	2	2	7	V (Chapter 4)
15	*Megastylis gigas* (Rchb. f.) Schltr.	Megastylidinae		New Caledonia		2	—	6	II
							13	71	

—: not attempted; 0: failed

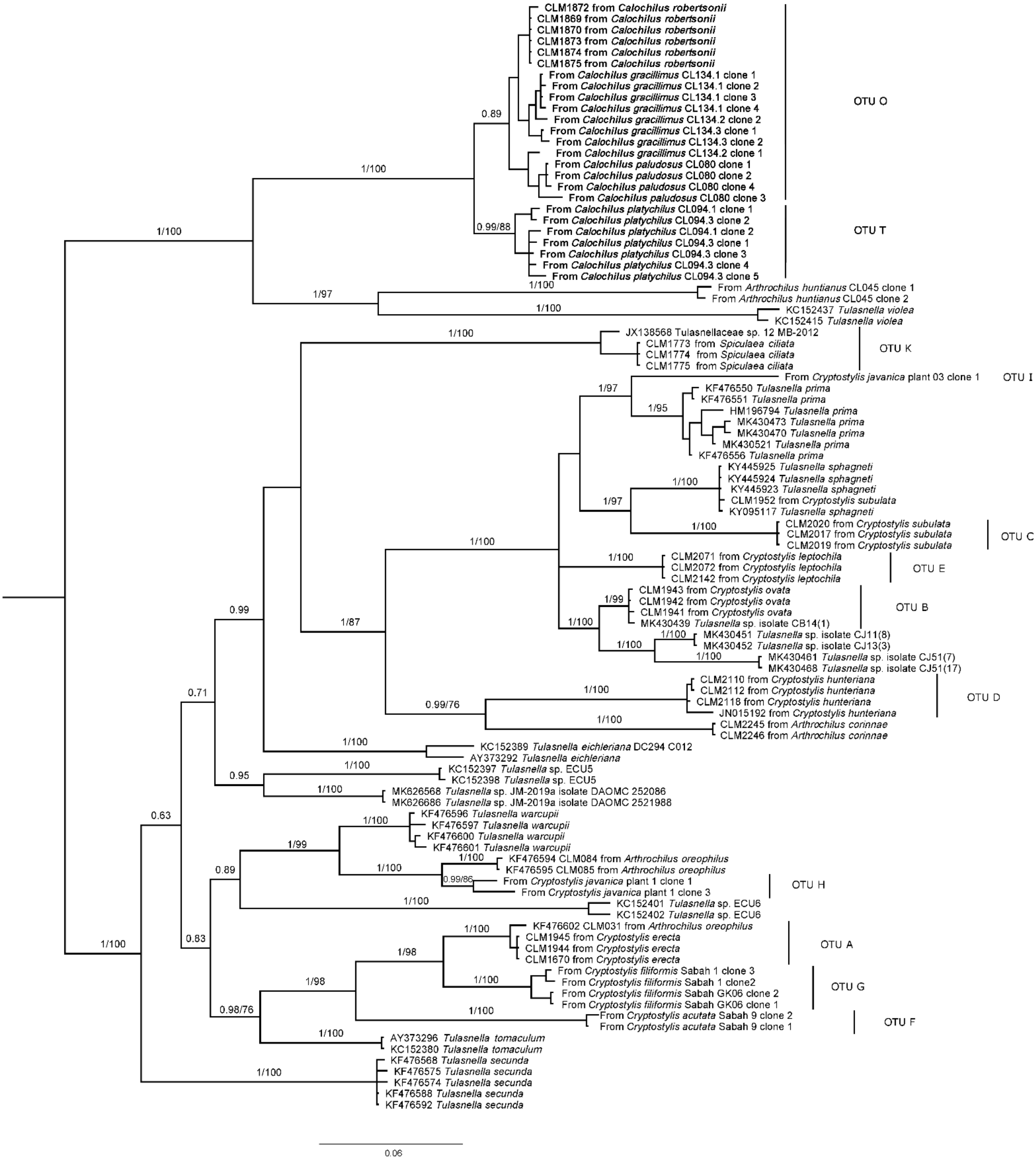

Figure 1. Midpoint rooted Bayesian inference phylogenetic tree for *Tulasnella* phylogenetic group IV associated with *Calochilus* orchids (shown in bold), as well as other *Tulasnella* taxa from *Cryptostylis* and *Drakaeinae*, obtained for the internal transcribed spacer (ITS). The numbers above the branches are Bayesian posterior probabilities / bootstrap support.

Figure 2. Midpoint rooted Bayesian inference phylogenetic tree for *Tulasnella* phylogenetic group II associated with *Megastylis* and *Pyrorchis* orchids (shown in bold), obtained for the internal transcribed spacer (ITS). The numbers above/under the branches are Bayesian posterior probabilities.

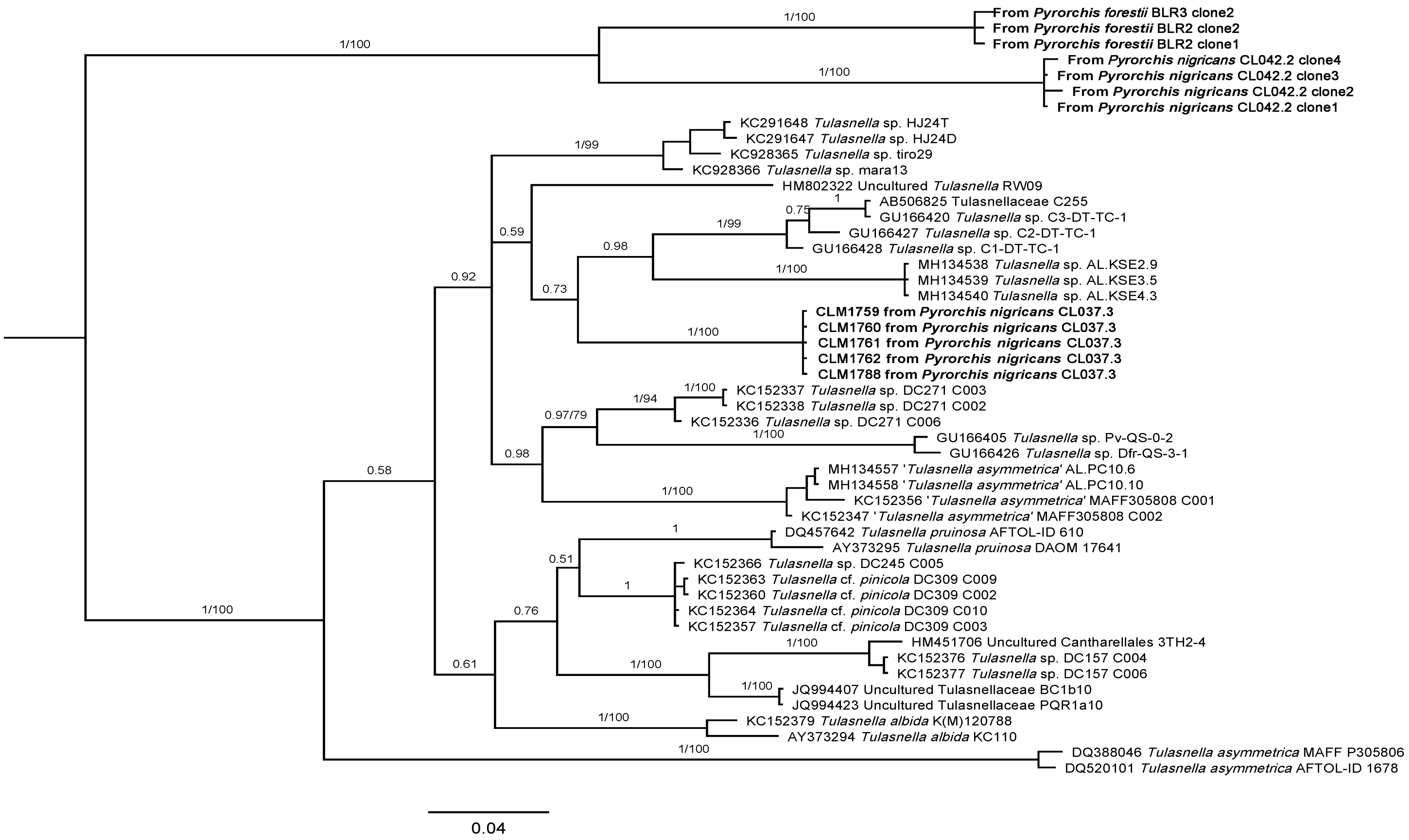

Figure 3. Midpoint rooted Bayesian inference phylogenetic tree for *Tulasnella* phylogenetic group III associated with *Pyrorchis* orchids (shown in bold), obtained for the internal transcribed spacer (ITS). The numbers above the branches are Bayesian posterior probabilities / bootstrap supports.

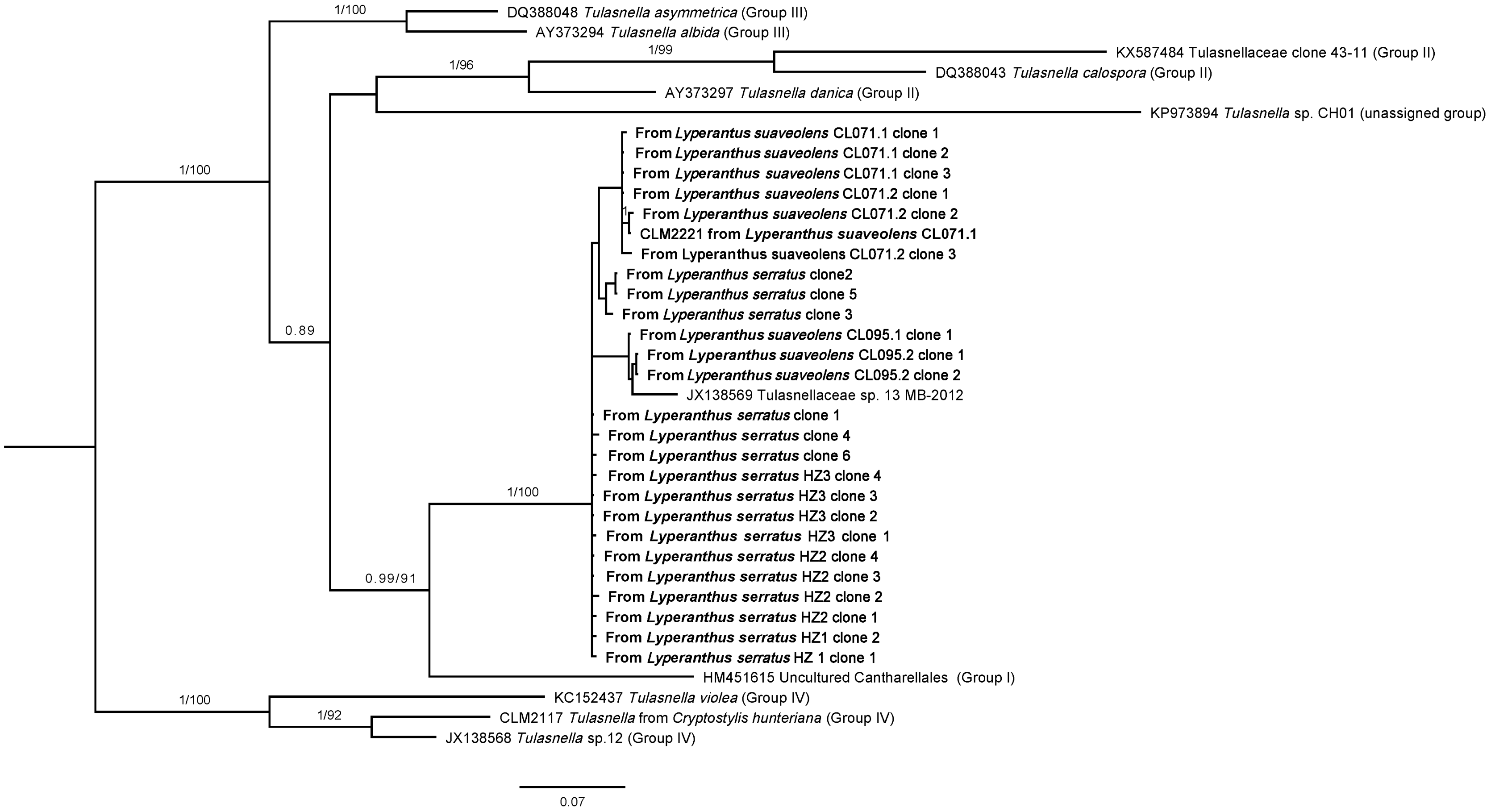

Figure 4. Midpoint rooted Bayesian inference phylogenetic tree for *Tulasnella* with *Lyperanthus* orchids (shown in bold), obtained for the internal transcribed spacer (ITS). The numbers above the branches are Bayesian posterior probabilities / bootstrap supports.

THESIS SYNTHESIS

Tulasnella might have roles as both an orchid mycorrhizal (OMF) and ectomycorrhizal fungus (ECM) in some orchids with seemingly insufficient photosynthetic capacity

The main association of *C. hunteriana* with OMF, not ECM suggests that *C. hunteriana* is not fully mycoheterotrophic (Chapter One). It is likely that the green coloration of the fleshy flowering stem contributes to some level of photosynthesis, as observed in the orchid genus *Corallorhiza* (Barrett et al., 2014). The trophic mode of *C. hunteriana* likely represents an intermediate state in the evolution towards full mycoheterotrophy and that this shift from autotrophy to full mycoheterotrophy is accompanied by a loss or shift in mycorrhizal partners, as previously suggested by (Jacquemyn & Merckx, 2019). The distinctive mycorrhizal pattern of OMF association in *C. hunteriana* differs to that observed in other mycoheterotrophic orchids, which typically associate with ECM (Dearnaley & Le Brocque, 2006; Girlanda et al., 2006; Kennedy et al., 2011). Moreover, the opposite, where green orchids associate with ECM fungi, has also been observed (Jacquemyn et al., 2017). Interestingly, a similar pattern is observed in another leafless Australian orchid, *Arthrochilus huntianus*. This orchid associates with one *Tulasnella* OTU that is closely related to *Tulasnella* OTUs that associate with Drakaeinae and Cryptostylidinae orchids (Chapter Two). While it is possible that *A. huntianus* presents a similar anomaly to *C. hunteriana* in that its primary mycorrhizal association is with OMF, a greater number of individuals are required to be tested from a variety of populations to confirm this association. The research by Gebauer et al. (2016) using stable isotopes of hydrogen and oxygen shows that partial mycoheterotrophy in mature orchid is widespread in green Rhizoctonia-associated orchids, which likely applies for both *C. hunteriana* and *A. huntianus*. However, the nutritional status of the orchids deserves more detailed investigations to reveal the extent of mycoheterotrophy able to support the orchids, such as previously Cameron et al. (2006) investigated in an experiment on *Goodyera repens*. A closer look at the orchid phylogenies reveal that *A. huntianus* is in a separate clade compared to other *Arthrochilus* species, whereas *C. hunteriana* is clustered together with other autotrophic Australian *Cryptostylis* (Peakall et al., 2020, *in prep*). Similarly, in *Neottia* (Lallemand et al., 2019; Yagame et al., 2016) and *Cymbidium* (Ogura-Tsujita et al., 2012), mycoheterotrophic and autotrophic orchids within one genus clustered in separate clades, with fungal shifts being linked to their trophic status.

Apart from their role as OMF, some *Tulasnella* lineages also play an important role as ectomycorrhizal fungi in forest trees and liverworts (Bidartondo et al., 2003; Bidartondo et al., 2004; Solis et al., 2017; Tedersoo et al., 2010). Little is known about how ectomycorrhizal *Tulasnella* evolved, and whether the 'ECM' *Tulasnella* and 'OMF' *Tulasnella* are sister lineages such as in Sebacinales. In Sebacinales, generally Group A Sebacinales is known as ECM and symbionts in mycoheterotrophic orchids, but Group B (Serendipitaceae) are symbionts of autotrophic orchids (Weiss et al., 2016). A similar pattern may also occur in Tulasnellaceae. Research on stable-isotopes and identification of mycorrhizal fungi from trees nearby mycoheterotrophic orchids may further elucidate the OMF and ECM patterns in mycoheterotrophic orchids (Bidartondo et al., 2003).

Contrasting patterns of phylogenetic congruency in orchid subtribes Drakaeinae and Cryptostylidinae

Topological congruency between two interacting phylogenies can be affected not only by coevolution but also phylogenetic niche conservatism. Coevolution is not expected in orchid-mycorrhizal associations because OMF can survive without the orchids. This interaction is also similar with sexually-deceptive orchids and their pollinators where the orchids need pollinators but pollinators do not necessarily rely on the orchids. In Australian sexually-deceptive orchids and their *Tulasnella* fungi interactions, Drakaeinae shows some degree of congruency whereas there is not a significant congruency between Cryptostylidinae and *Tulasnella*. Generally, sexually-deceptive orchids show specialisation in their pollinators that likely drive an increase in speciation through rapid diversification (Breitkopf et al., 2015; Phillips et al., 2020). Australian *Cryptostylis* has unusual pollinator sharing—one pollinator species for five Australian *Cryptostylis* (Coleman, 1927, 1928a, 1928b, 1929, 1930a, 1930b) that potentially have lower diversification rates compared to other sexually-deceptive orchid genera (e.g. *Chiloglottis* and *Drakaea*), possibly explaining the absence of phylogenetic niche conservatism in Cryptostylidinae. This suggests that rather than coevolution, phylogenetic niche conservatism may drive the phylogenetic congruency in Drakaeinae, thus conserve their fungal associations.

‘Constrained relationships’ between *Tulasnella* and orchid subtribes Drakaeinae and Cryptostylidinae, but not in Thelymitrinae and Megastylidinae

Currently, there are only six *Tulasnella* species recorded in Australia (Linde et al., 2017; Warcup & Talbot, 1967, 1980), and approximately 100 species recorded globally. The addition of eight new formally described *Tulasnella* species (Chapters Three and Four) from three subtribes of Australian orchids—Cryptostylidinae, Drakaeinae and Megastylidinae, practically doubles the number of *Tulasnella* described in Australia. Six species of our newly described *Tulasnella* are closely related, despite the fact that their orchid hosts are distantly related - some in Cryptostylidinae and some in Drakaeinae. Drakaeinae and Cryptostylidinae have fungal associations restricted to *Tulasnella* phylogenetic IV, whereas other closely related orchid subtribes Thelymitrinae and Megastylidinae associate with a number *Tulasnella* phylogenetic groups (Chapter Two) that also show broad phylogenetic variation within the groups. It is also interesting to reveal the evolutionary relationships of *Tulasnella* in Thelymitrinae and Megastylidinae, which have broader *Tulasnella* associations than Cryptostylidinae and Drakaeinae. Molecular detection can be performed, however, to describe a species we need culturable isolates. In *Tulasnella*, sometimes fungal isolations from orchid tissues can be challenging and several OTUs are not even culturable, e.g. in *Calochilus* (Diurideae; Thelymitrinae), no successful isolations have been made so far (Chapter Two). It is likely that additional *Tulasnella* diversity may remain undescribed due to these limitations in culturability.

A milestone of *Tulasnella* diversity in Australia as well as globally

Macro- and micromorphological characteristics from eight newly described *Tulasnella* support our molecular data to delimit fungal species. The most distinct characteristic is the different number of nuclei. All *Tulasnella* obtained from Drakaeinae and Cryptostylidinae have binucleate cells (Chapter Three, Arifin et al. (2020)), whereas *Tulasnella* that associate with Megastylidinae orchids have cells with more than two nuclei (occasionally trinucleate) and multinucleate (Chapter Four). The discovery of the distantly related *Tulasnella* group from Megastylidinae which shows multinucleasm is a striking phenomenon and has not been described elsewhere for *Tulasnella*. Moreover, species delimitation is very important to answer evolutionary questions in orchid and mycorrhizal fungi that may arise in the future. Four *Tulasnella* species have been described by Linde et al. (2017) from several Drakaeinae genera and surprisingly most of them are also shared with Cryptostylidinae (Chapter One). Definitive and formal names will prevent confusion, especially when the similar fungi that we described here are detected in other orchid species in the future.

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