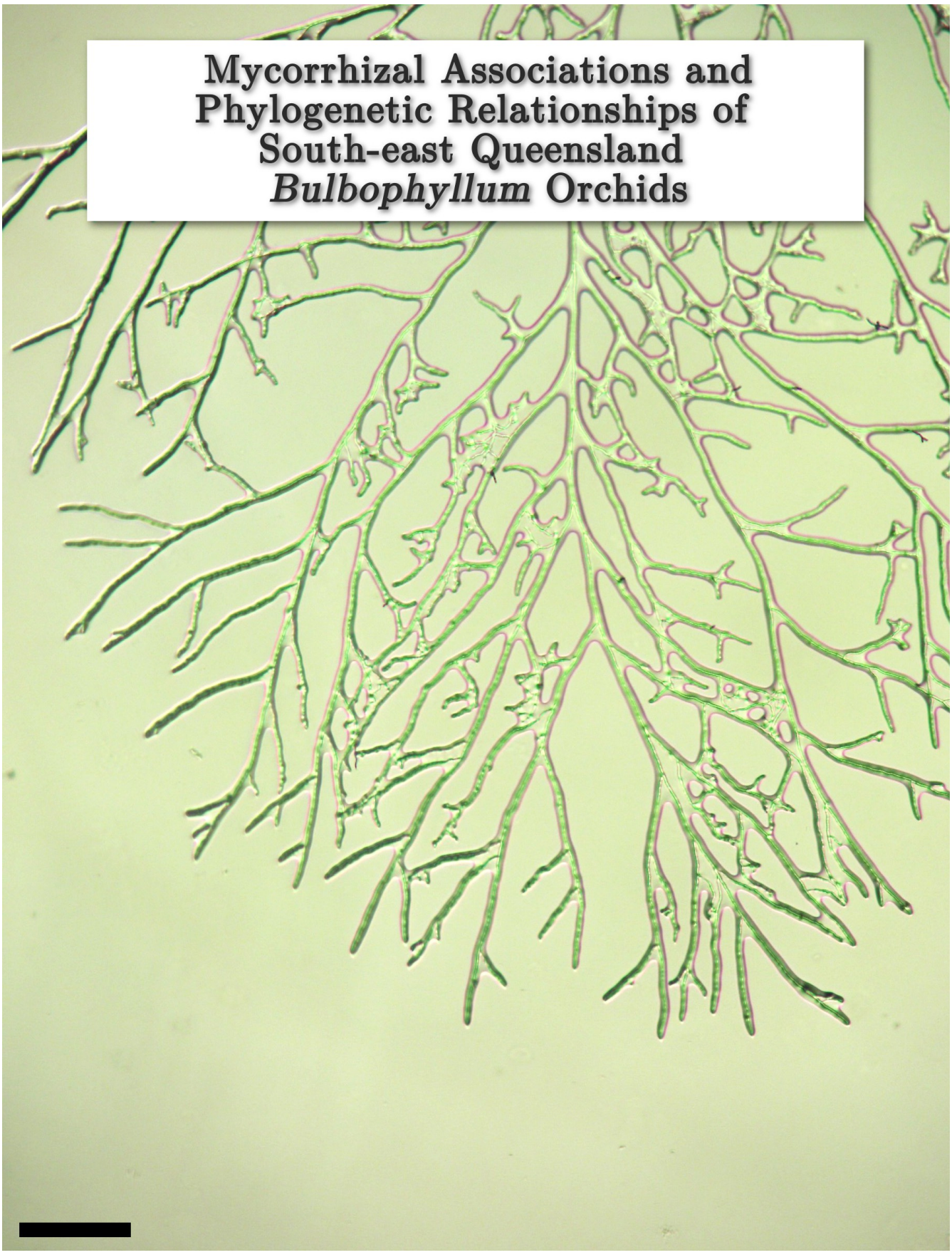
Mycorrhizal Associations and Phylogenetic Relationships of South-east Queensland *Bulbophyllum* Orchids



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2017

Cover image shows hyphae of an endophytic fungus (*Apiognomonia* sp., isolate P3 2.1.2 cultured from roots of the native Australian orchid *Bulbophyllum exiguum*) growing against the inside of an agar plate. Bar is 50μm.

Abstract Throughout their life cycles orchids are reliant on inorganic nutrients provided by mutualistic orchid mycorrhizal fungi (OMF), to which the plants allocate sugars produced in photosynthesis. Orchid seeds usually require a fungal partner to facilitate their germination, and sometimes a sequence of fungal species to promote their growth to maturity. Orchid mycorrhizal (OM) relationships can be very specific, with epiphytic/lithophytic (tree/rock dwelling) orchids often associating with a narrow range of fungi. Additionally, closely-related groups of orchids tend to share common associations with the same lineages of fungi. The genus *Bulbophyllum* is the largest in the Orchidaceae (>2000 spp.) but no study to date has investigated mycorrhizal associations of *Bulbophyllum* in Australia. In this study, fungi were isolated from the roots of the native orchids *B. exiguum, B. bracteatum, B. minutissimum, B. elisae* and *B. shepherdii* at 7 sites in south-east Queensland. Fungi were identified based on internal transcribed spacer (ITS) gene sequences to determine whether these congeneric orchids share OMF partners. Analysis of orchid RuBisCO large subunit (rbcL) gene sequences was also performed to ascertain phylogenetic relationships, and symbiotic seed germination of *B. exiguum* was tested using 4 fungal inocula. In all, 90 fungal isolates were obtained. Molecular identification revealed a diversity of putatively mycorrhizal fungi from the OMF genera *Tulasnella, Serendipita* and *Ceratobasidium,* and dark septate endophytes (DSEs) from the ascomycete order Helotiales. Significantly, 3 orchid spp. (*B. exiguum, B. bracteatum* and *B. elisae*) across 3 sites were found to harbour a single *Tulasnella* sp. that is likely new to science. This indicated narrow OMF specificity and suggested that these orchids may belong to a common sub-clade within *Bulbophyllum*, an observation supported by phylogenetic analysis of rbcL genes and by taxonomic reassignments that have been proposed based solely on morphological features. *B. exiguum* was found to harbour an undescribed *Serendipita* sp. that warrants investigation as a potential agricultural inoculum. *B. shepherdii* harboured a *Ceratobasidium* sp. previously found in Norway, but the plant sampled in this study had been relocated from nearby woodland and thus may not usually associate with this OMF. Isolation of DSE helotialean fungi with highest BLAST matches to ericoid mycorrhizal (ErM) sequences pointed to a possible OMF role for these isolates, however further confirmation is needed to establish whether intracellular nutrient-exchange structures are present. The presence of ErM-like fungi associated with orchids supports recent work suggesting a blurring of functional boundaries between mycorrhizal types. *B. exiguum* seed germination experiments were impeded by overgrowth of fungal contaminants, which were likely endophytes from seed pod tissue, and by poorly-developed seeds that may have resulted from inbreeding. Future studies with seed germination protocols optimised for very small pods are needed to ascertain whether fungal symbionts can stimulate germination in these *Bulbophyllum* spp. These results provide evidence for narrow OMF specificity for *Tulasnella* in some SE Queensland *Bulbophyllum* spp. and further raise the intriguing possibility of DSE forming mycorrhizas with orchids.

Declaration

I certify that the work reported in this thesis is wholly my own except where otherwise noted. I also certify that this work is original and has not been previously submitted for assessment in any other course of study at any other university.

Jed Calvert Honours candidate Date

Endorsed by

Dr John D.W. Dearnaley Supervisor Date

Acknowledgements

Thank you to my supervisor, Dr John Dearnaley, who has given me the opportunity to learn about two fascinating groups of organisms and explore some beautiful forest landscapes in the process, as well as to greatly improve my writing and laboratory skills. You have been patient, knowledgeable, supportive and a great guy to be around. Thank you also to Shirali. You help me in a million different ways, and your care and flexibility have meant that my research never feels overwhelming or burdensome.

At USQ I would also like to thank Dr Ant Young, who has provided some very interesting conversations and made helpful suggestions to improve my writing. Thank you also to Dr Levente Kiss, whose insights into the world of Mycology and academic machinations have been inspiring and instructive.

Finally, I would like to thank my mum and dad, who have always been full of love and support, never pushing me in any direction I didn't want to walk myself. I can't believe how lucky I am to have two people as lovely as you as my parents.

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List of Abbreviations

OM – orchid mycorrhiza

OMF – orchid mycorrhizal fungi

ErM – ericoid mycorrhiza

ErMF – ericoid mycorrhizal fungi

AMF – arbuscular mycorrhizal fungi

C – carbon

N – nitrogen

P – phosphorus

K – potassium

NH_4^+ – ammonium

ANOVA – analysis of variance

ITS – internal transcribed spacer gene

rbcL – Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) large subunit gene

NCBI – National Center for Biotechnology Information

NaClO – sodium hypochlorite

bp – base pairs

AGRF – Australian Genome Research Facility

DSE – dark septate endophyte

PDA – potato dextrose agar

PCR – polymerase chain reaction

BLAST – basic local alignment search tool

MEGA – Molecular Evolutionary Genetics Analysis

GIMP – GNU Image Manipulation Program

GRI – germination rate index

DRI – developmental rate index

Glossary of Technical Terms

anamorph	Mould-like asexual form of a fungus
Ascomycetes	One of two large divisions of the 'higher fungi', members of which possess an 'ascus', a microscopic sac-like sexual structure containing nonmotile spores called ascospores
backbone alignment	An assembly of gene sequences included for comparison in a phylogenetic analysis along with the sequences of interest
Basidiomycetes	The other division of the 'higher fungi', including most mushrooms, which reproduce sexually via the formation of specialised club-shaped cells called basidia
dehiscence	The splitting open of a mature seed pod along built-in lines of weakness
endophyte	A micro-organism that lives inside a living plant for at least part of its life cycle without causing apparent disease
hypha	Filamentous vegetative structure of fungi (pl. hyphae)

mutualism	The manner whereby two organisms of different species have a relationship in which each individual benefits from the activity of the other
mycelium	Collective term for a mass of hyphae (pl. mycelia)
peloton	Fungal hyphae coiled inside an orchid cell; site of nutrient exchange
protocorm	Embryonic pre-seedling stage of a germinated orchid
septum	Wall between two fungal cells (pl. septa)
symbiosis	Any kind of close, long-term interaction between two organisms. Symbioses may be mutualistic, commensalistic, or parasitic
teleomorph	Sexual or 'fruiting' form of a fungus (e.g. mushroom, puffball)

1. Introduction

1.1 Orchid mycorrhizal fungi (OMF)

Mycorrhizal fungi (Greek: *myco* = fungus, *rhiza* = root) form mutualistic associations with the roots of most higher land plants and with the substrate-bound tissues of many mosses, hornworts and liverworts (Smith & Read 2008). Mycorrhizas, a term that refers to both the plant and fungal components of such mutualisms, usually involve the transfer of inorganic nutrients from fungus to plant and sugars from plant to fungus (Fuhrer 2005). The nature and quantities of transferred compounds varies from group to group (Garcia et al. 2016). It is estimated that 80% of land plant species, representing 92% of plant families, are mycorrhizal (Wang & Qui 2006).

For the 27,801 recognised species in Orchidaceae, one of the largest flowering plant families (The Plant List 2017; Cribb et al. 2003), mycorrhizal associations are crucial throughout the life cycle (Bailarote et al. 2012). A defining aspect of the life history of orchids is that their tiny seed germinates into an achlorophyllous, embryo-like protocorm, which relies on the coiled hyphae (pelotons) of a cell-penetrating fungus for the organic C, P and N necessary for it to develop into an adult plant (Batygina, Bragina & Vasilyeva 2003). Until recently the orchid mycorrhiza (OM) relationship has been described as an obligate parasitism that only benefits the plant (Rasmussen & Rasmussen 2014). However, an accumulating body of evidence suggests the flow of nutrients is bidirectional, with the fungal partner receiving N in the form of ammonium (NH_4^+) (Fochi et al. 2017) as well as fixed C in the form of photosynthate from the orchid (Cameron, Leake & Read 2006; Látalová & Baláž 2010). This, as with other forms of mycorrhiza, is indication of a truly mutualistic symbiosis.

OMF species all appear to possess the capacity for independent existence (García, Onco & Susan 2006; Rasmussen & Rasmussen 2009). However, there is some indication that their distribution in soil is more dependent upon their orchid hosts than their designation as

unspecialised saprotrophs (litter and wood rotters) might suggest (Waud et al. 2016; Voyron et al. 2017). Some researchers have raised the issue of culture bias, whereby only easily cultivable mycorrhizal fungi are isolated from a plant in a culture-dependent study, and a conclusion is reached that the plant only associates with cultivable fungi (Read & Perez-Moreno 2003; Vrålstad 2004). Many uncultivable OMF are therefore likely to be undocumented.

Plant dependency upon mycorrhizal fungi varies throughout the Orchidaceae. It has been proposed that green photosynthetic orchids, which represent the majority of species (Dearnaley, Martos & Selosse 2012), are to some extent mixotrophic, being dependent upon their OMF for some C as adults as well as embryonically (Selosse & Roy 2009; Selosse & Martos 2014). Available research suggests that the fungal partners of green orchids belong largely to a number of clades from the Basidiomycete genera *Tulasnella, Serendipita* and *Ceratobasidium* (García et al. 2006; Whitehead et al. 2017). Mixotrophy is a common evolutionary stepping-stone to obligate myco-heterotrophy, a state seen in some orchids that are totally reliant on their fungal partner for organic C throughout their lifespan (Bidartondo 2005). Genes coding for RuBisCO—a C fixation enzyme central to photosynthesis—in the achlorophyllous orchid *Corallorhiza striata* contain mutations such as frameshifts and stop codons (Barrett & Freudentein 2008); evidence for total loss of photosynthetic ability. The fungal groups that associate with these non-green orchids are separate from the majority of OMF, being ectomycorrhizal or saprotrophic species from a wide range of clades (Taylor & Bruns 1999; Smith & Read 2008).

Historically, *Tulasnella, Serendipita* and *Ceratobasidium* have been included in the *Rhizoctonia* group, a polyphyletic form genus containing distantly-related fungi that share morphological features (Smith & Read 2008). Recent phylogenetic analyses have placed OMF of green orchids in the fungal orders Cantharellales and Sebacinales, other members of which

are non-mycorrhizal endophytes, saprotrophs, pathogens and ectomycorrhizal fungi (Veldre et al. 2013). There are also reports of green orchids forming mycorrhizal associations with members of the Pucciniomycotina ('rust') basidiomycetes (Kottke et al. 2010), as well as with ascomycete fungi (Selosse et al. 2004; Waterman et al. 2011). Recent work on the so-called dark septate endophytes (DSE), a group of ascomycetes known to form mycorrhizas with plants in the Ericaceae, suggests that they may be widespread in their mycorrhizal associations (Mandyam & Jumpponen 2005), raising the possibility that DSEs could also associate with plants in the Orchidaceae. However, DSE to orchid nutrient transfer has not yet been demonstrated.

Generally speaking, *Rhizoctonia*-type OMF (Figure 1) are characterised by right-angled, constricted hyphal branches, formation of barrel-shaped monilioid cells, slow growth, complex dolipore septa, and by the difficulty of inducing their teleomorphs *in vitro* (García, Onco & Susan 2006). Since the advent of fungal DNA barcoding, molecular classification has resulted in a reshuffling of older taxonomic groupings and reduced reliance on visual identification (Schoch et al. 2012). Nevertheless, *Rhizoctonia*-type OMF must be morphologically identified in order to isolate them in culture, and the above characteristics serve as a guide (Pereira et al. 2014). A notable feature of *Tulasnella, Serendipita* and *Ceratobasidium* OMF is that they *can* be axenically cultured in-vitro. Other mycorrhizal groups, such as arbuscular (Lalaymia et al. 2012) and ectomycorrhizal fungi (Szuba 2015) are currently challenging or impossible to maintain in pure culture without the presence of live host plant roots. According to a functional model of OMF nutrient transfer proposed by Dearnaley & Cameron (2017), hyphae penetrate the orchid cell wall and grow into an invagination of the plasma membrane (Figure 2). An interfacial matrix, which along with the membrane facilitates transport of nutrient molecules, also lies between peloton and orchid cell cytosol (Paduano et al. 2011).

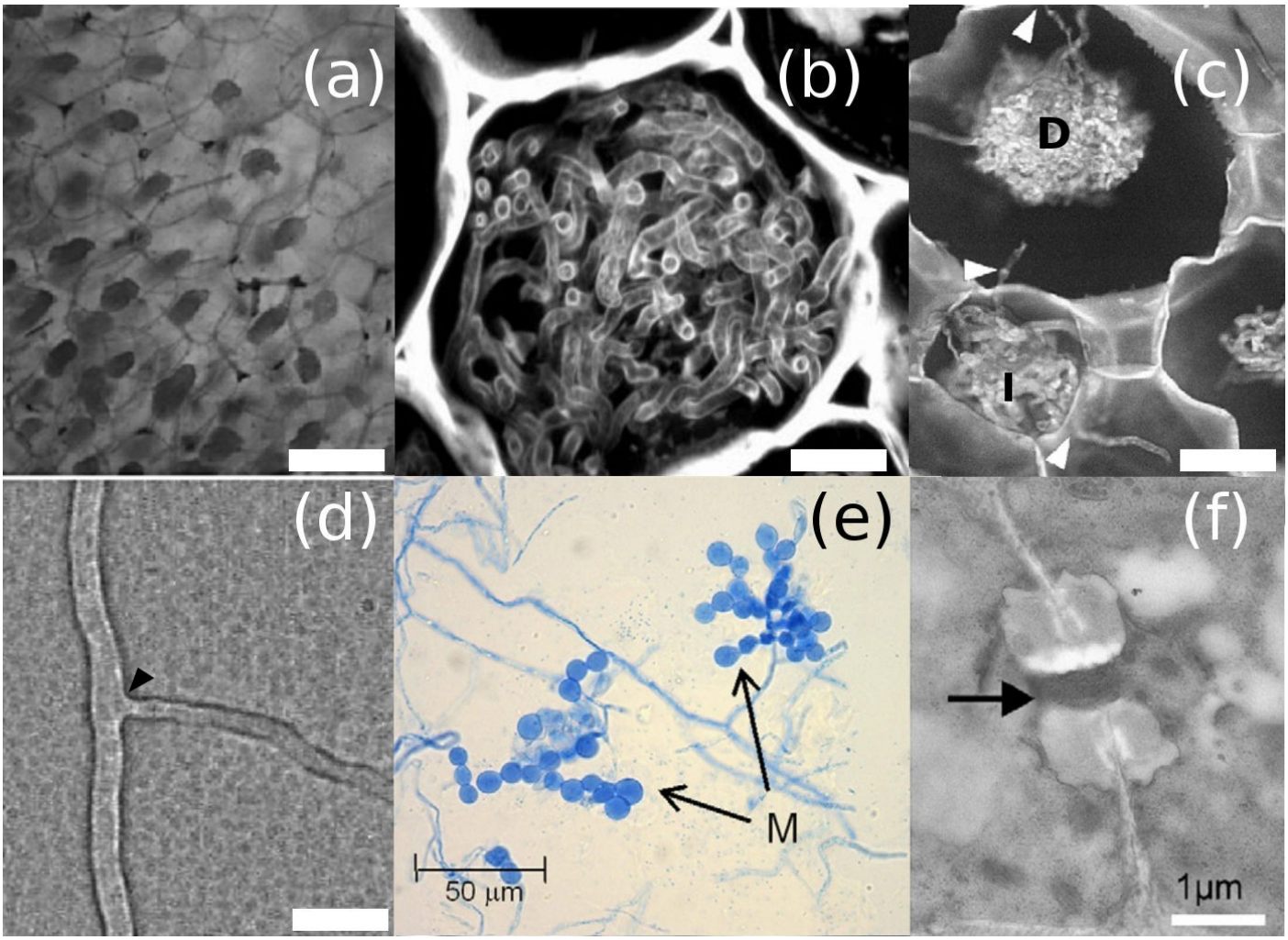

Figure 1 **(a)** Pelotons inside cortical root cells of the Chilean orchid *Chloraea* sp. Bar is 250µm. **(b)** Intact peloton inside a root cell of the rare Borneo orchid *Paphiopedilum sanderianum.* Bar is 25µm. **(c)** Disintegrating (D) and intact (I) pelotons in cortical root cells (bar is 25µm) and **(d)** right-angled branching hypha (arrowhead) (bar is 15µm) of OM from the Eurasian orchid *Spiranthes spiralis*. **(e)** *Tulasnella* sp. forming monilioid cells (M) in culture. **(f)** Dolipore septum of OM fungus cultured from *S. spiralis* roots. Images: (a) & (d) from Pereira et al. (2014); (b) from Peterson & Massicotte (2004); (c) & (f) from Tondello et al. (2012); (e) from Steinfort et al. (2010).

The intracellular dialogue between plant cell and peloton may be for as brief a period as 24 hours (Hadley & Williamson 1971). Subsequent lysis and digestion of entire pelotons appears to constitute another major means of fungus to plant nutrient transfer (Figure 2) (Kuga, Sakamoto & Yurimoto 2014). Orchids possess genes for fungus-specific hydrolytic enzymes, such as chitinases and β-1,3-glucanases, as part of a system for controlling fungal colonisation (Amian et al. 2011). Rasmussen & Rasmussen (2014) have explored the notion that OMF

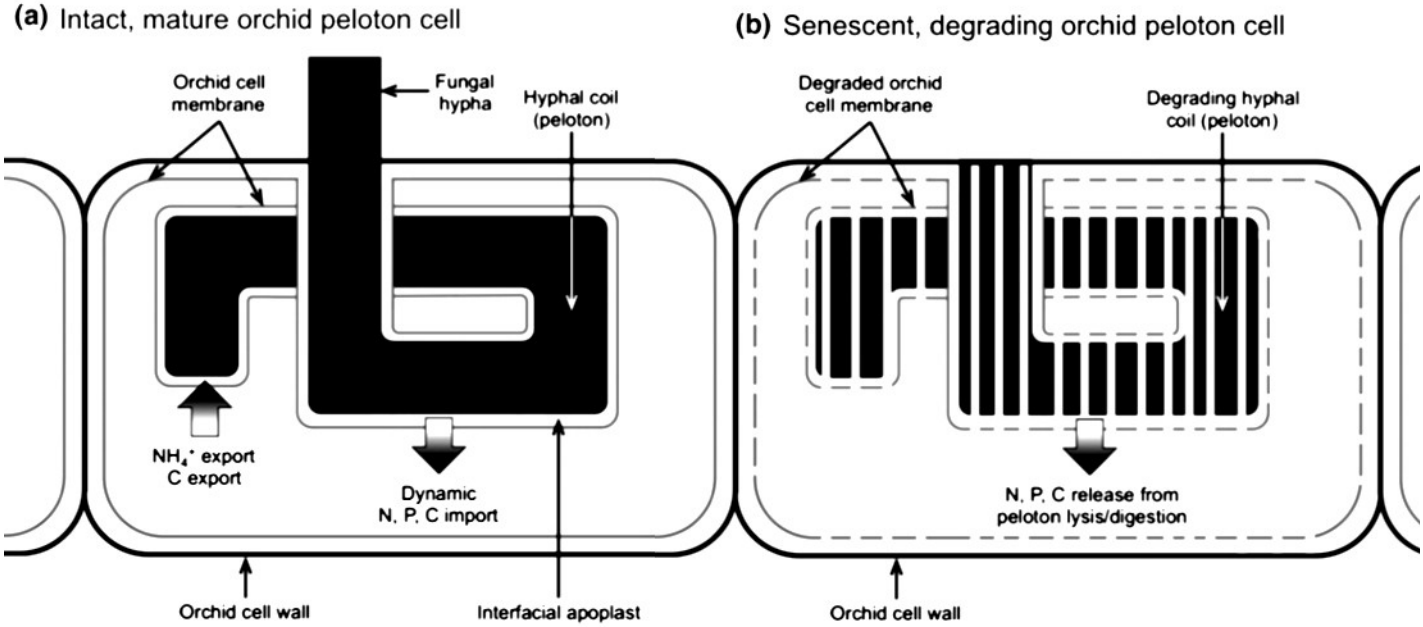

Figure 2 Model of OM nutrient exchange proposed by Dearnaley & Cameron (2017). Orchid root cells **(a)** containing coiled fungal hyphae (pelotons) import N, P & C from the fungus via the cell membrane and interfacial matrix (apoplast); NH_4^+ is exported to the fungus from the embryonic plant, and C is exported when the orchid develops photosynthetic capacity. When pelotons collapse after ~24hrs **(b)**, digestion of hyphae provides the orchid cell with N, P & C. (Figure adapted from Dearnaley & Cameron 2017.)

could have evolved from fungal pathogens that provoked but survived such defensive measures, limiting hyphal necrosis and establishing a stable basis for nutrient exchange. Colonised orchid root cells contain higher amounts of other defensive enzymes, including glutamate dehydrogenase and peroxidases, than do uncolonised cells (Rasmussen 2002). The ability of cells containing digested pelotons to be recolonised provides the orchid with a constant supply of hyphal compounds (Peterson & Massicotte 2004).

1.2 Epiphytic orchids and OMF

Most research on OMF has focused on terrestrial (ground-dwelling) orchids, but approximately 70% of orchid species globally and 18% in Australia are epiphytic or lithophytic (tree- or rock-dwelling) (Jones 2006). DNA and seed germination studies have identified mycorrhizal partners of selected epiphytic orchids (Nontachaiyapoom, Sasirat &

Manoch 2011; Sathiyadash et al. 2014; Khamchatra et al. 2016) but these cover only a small subset of a very diverse group of plants.

Yoder, Zettler & Stewart (2000) observed differences in biological characteristics when comparing epiphytic and terrestrial orchid species: smaller seeds, higher seedling water content after fungal colonisation, higher water loss rates, and much faster germination, all of which speak of a need to maximise intake of water while it is available. In this respect the OM relationship may be more important to epiphytic orchids as a means of accessing water than it is for soil-bound terrestrials. Additionally, the diversity of epiphytic communities means that orchids often share a substrate with other epiphytes such as mosses and liverworts. In Costa Rica, Osorio-Gil, Forero-Montaña & Otero (2008) found that adult *Ionopsis utricularioides* orchids growing epiphytically on moss-covered guava trees had higher rates of root-cell OM colonisation than plants growing on non-mossy trees. Orchid co-occurrence with mosses may be facilitated by the water-retaining properties of heavily-colonised substrates, which create microenvironments more amenable to fungal growth (Osorio-Gil et al. 2008).

In Australia little research has focused on epiphytic orchids and their fungal partners. The rare epiphyte *Sarcochilus weinthalii*, native to north-east New South Wales and south-east Queensland, was found to have narrow OM specificity, mainly associating with an undescribed species of *Ceratobasidium* (Graham & Dearnaley 2012). Another study established *Ceratobasidium* spp. to be present in the roots of three epiphytic orchids, *Sarcochilus hillii*, *S. parviflorus* and *Plectorrhiza tridentata* in south-eastern Australia (Gowland et al. 2007). Interestingly, these three orchid species were found to prefer living on trees with moderate to high moss cover (see above reference to Osorio-Gil et al. 2008 in Costa Rica). Of the few epiphytic orchid genera and their OMF that have been studied in Australia, one prominent group that has been so far neglected is *Bulbophyllum*.

1.3 The genus *Bulbophyllum*

More than 2000 species are included in the orchid genus *Bulbophyllum* Thouars, making it the largest genus in the Orchidaceae and the second largest in the Angiosperms after the pea genus *Astragalus* (Frodin 2004). Most species are epiphytes, but the group is morphologically very diverse (Fischer et al. 2007). A hinged labellum (lower part of the flower—a landing platform for pollinating insects) is one character common to the approximately 32 native Australian *Bulbophyllum* spp., all of which occur in Queensland (Jones 2006). Eleven of these were listed in the 2016 *Census of the Queensland Flora* as being of conservation concern: 1 near-threatened, 9 vulnerable, and 1 endangered (Jessup 2016).

Jones (2006) has deemed the size of the *Bulbophyllum* genus 'unwieldy' and has divided the Australian portion into 11 genera based on floral and vegetative structure. However, molecular phylogeneticists working on Orchidaceae have cautioned against this practice. There is evidence of mis-classifications due to widespread convergent evolution resulting in similar morphology in disparate lineages (Carlsward et al. 2006). A commonly-used modern method of inferring plant relationships is to phylogenetically analyse chloroplast DNA sequences (Górniak, Paun & Chase 2010), comparing them to existing archived sequences such as those held in the NCBI GenBank database (Benson et al. 2012). A recent phylogenetic study by Chase et al. (2015) based on plastid DNA sequences and a low-copy nuclear gene for xanthine dehydrogenase (*Xdh*) has placed *Bulbophyllum* in the subfamily Epidendroideae, tribe Malaxideae, subtribe Dendrobiinae and sister clade to *Dendrobium*.

No Australian studies to date have identified the OM partner of a native *Bulbophyllum* orchid. Globally, the only evidence in the literature of *Bulbophyllum* OMF identification comes from Réunion Island in the Indian Ocean east of Madagascar. Six fungal DNA sequences identified as belonging to the genus *Serendipita* were isolated from root samples by Martos & Selosse (2008 unpub.) from *B. macrocarpum*, *B. nutans* and *B. longiflorum*

(GenBank accessions FJ514083 (*Bmac*), FJ514084 (*Bmac*), FJ514085 (*Bmac*), FJ514086 (*Bmac*), FJ514078 (*Bnut*) & FJ514090 (*Blon*), respectively). The latter 2 sequences were used as additional alignment data in a published study of OM partner preference for two Epidendroid orchid species in the tribe Neottieae, which were found to associate primarily (~75%) with fungi belonging to *Serendipita* (Těšitelová et al. 2015). The authors proposed that most or all orchids in the genus *Neottia* associate primarily with fungi in the family Sebacinales—partially or fully mycoheterotrophic species with Clade A, and primarily autotrophic species with *Serendipita* (also termed Clade B). In 2012, Martos et al. published a review of epiphytic OMF stating that *Bulbophyllum* had been observed partnering with fungi in the Sebacinales and Tulasnellaceae. However, no reference or data was presented in the article to support that statement.

1.4 OMF evolution, ecology and conservation

Symbioses—particularly endosymbioses—appear to have been a defining feature in the evolution of life from a very early stage. Several prominent models implicate them in the origins of the eukaryotes (Alberts et al. 2015; López-García et al. 2017). From an ecological and evolutionary standpoint the OM relationship has been investigated in terms of how it relates to orchid speciation, and the reasons and mechanisms for plant-fungus specificity.

Research in South Africa has indicated that closely-related orchids associate with the same groups of mycorrhizal fungi irrespective of where they are growing (Waterman et al. 2011). In contrast to the influence of flower-specific pollinating insects, which can cause reproductive isolation and therefore speciation, orchid-specific OMF are unlikely to be drivers of speciation (Waterman & Bidartondo 2008). However, the diversity of fungal partners among varied orchid communities may be a means of resource partitioning. This would ensure that different

orchid species growing in close proximity in the soil derive nutrients from OMF that exploit different underground resources (Waud et al. 2016).

With regard to variation in OM specificity, a Western Australian study focused on two terrestrial orchids with rapidly-spreading, weed-like ecology (Bonnardeaux et al. 2007). Both species formed associations with a diverse range of OMF compared to much narrower specificity in rarer, patchily-distributed orchids. This suggests that high partner specificity may be a factor that limits orchid distribution, especially in varied landscapes. Narrow OMF specificity is thus an aspect that must be considered in the conservation of rare orchids.

Some orchids rely on different fungal species to progress to different stages of their life cycles (Xu & Mu 1990), with a succession of OMF species colonising root cells as the plant matures. In addition to establishing which OMF can trigger the seed germination of an endangered orchid species, it is also prudent to observe plants into adulthood to establish whether multiple OMF are required for the orchid's further development (Khamchatra et al. 2016). Seed germination experiments are therefore helpful in (i) verifying the status of putatively mycorrhizal fungal species, and (ii) elucidating any fungus-dependent developmental shifts in the life cycle of the orchid.

More Orchidaceae species are designated as threatened on the IUCN Red List than species from any other plant family (Ercole et al. 2013). Since the 1970s, land clearing rates in Australia have been highest in south-east Queensland and north-east New South Wales, with Queensland having the highest proportional deforestation rates of any Australian state or territory from 1995 to 2005 (Bradshaw 2012). Recent Queensland Government data released in the 2015-16 Statewide Landcover and Trees Study (SLATS) showed that woody vegetation in Queensland was cleared at a rate of nearly 400,000 ha/year, 33% faster than in 2014-15 (Dept. of Science, Information Technology & Innovation 2017). As a result, much of Queensland's remaining native vegetation is highly fragmented, resulting in major population

declines of native plant species, including orchids (McAlpine et al. 2009). *Ex situ* conservation (propagation outside a species' natural range), which is widely viewed as a necessary procedure in the conservation of threatened orchids, requires an understanding of their fundamental ecological requirements (Martínez-García et al, 2005; Liu et al. 2006; Wade et al. 2016). Establishing the details of mycorrhizal associations can therefore provide essential information to assist the conservation of orchids located in Queensland's disappearing forests.

1.5 Project hypotheses and overview

This project will test the following hypotheses:

1. Five south-eastern Queensland *Bulbophyllum* orchids (Figure 3) associate with the same group of OMF as the *Bulbophyllum* on Réunion Island studied by Martos & Selosse (2008 unpub.): *Serendipita* (family Sebacinaceae, Clade B). This is is relevant to the evolutionary and biogeographical study of OM specificity.
2. *Bulbophyllum exiguum* exhibits OMF specificity across multiple sites in south-east Queensland. This is relevant to the ecology and evolution of smaller landscape-scale epiphytic OM specificity.
3. OMF cultures isolated from adult *B. exiguum* plants are able to stimulate germination and are required for developmental shifts to proceed. This may have practical implications for *ex situ* conservation of native *Bulbophyllum* should they become of conservation concern.
4. Five SE Queensland *Bulbophyllum* orchids belonging to 3 new genera proposed by Jones (2006)—*Adelopetalum*, *Oxysepala* and *Oncophyllum*— have shared, genus-specific OMF partners that differ from those of other *Bulbophyllum* spp. This will test current Australian *Bulbophyllum* taxonomy and may have implications for the validity of Jones' morphology-based reclassification.

5. Chloroplast DNA sequences of 5 SE Queensland *Bulbophyllum* orchids belonging to new genera proposed by Jones (2006)—*Adelopetalum*, *Oxysepala* and *Oncophyllum*—do not exhibit phylogenetic clustering, but belong to different clades. This will provide comparative data for Hypothesis 4.

The investigation will consist of three distinct phases. Phase 1 will focus on the isolation, culturing and molecular identification of OMF from 5 *Bulbophyllum* species. Phase 2 will consist of seed germination experiments aimed at confirming the mycorrhizal status and developmental role of OMF isolated from *B. exiguum*. Phase 3 will comprise the collection and analysis of orchid chloroplast DNA from 5 *Bulbophyllum* species for phylogenetic reconstruction.

This project aims to clarify fundamental aspects of the ecology and life history of Australian representatives of an under-studied but prominent orchid genus. Results will contribute to the body of knowledge in terms of orchid mycorrhizal specificity, biogeography, taxonomy and evolution, and may have practical applications for future *ex situ* conservation efforts.

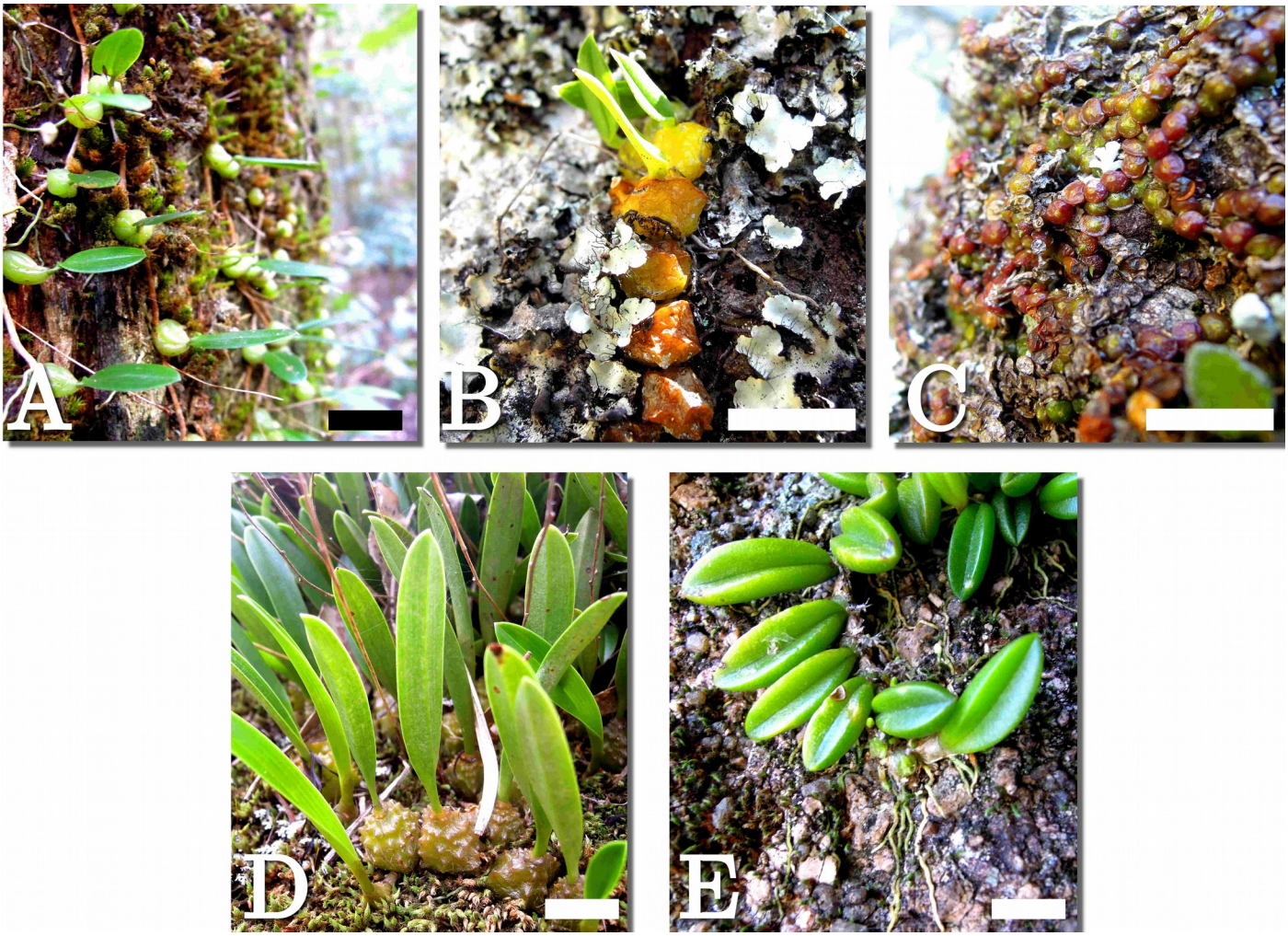

Figure 3 The five *Bulbophyllum* spp. investigated in this study. Jones' (2006) revised genera are listed in brackets. (A) *Bulbophyllum* (*Adelopetalum*) *exiguum,* (B) *Bulbophyllum* (*Adelopetalum*) *bracteatum,* (C) *Bulbophyllum* (*Oncophyllum*) *minutissimum,* (D) *Bulbophyllum* (*Adelopetalum*) *elisae,* (E) *Bulbophyllum* (*Oxysepala*) *shepherdii.* Bars are 15mm.

2. Materials & Methods

2.1 Identification of orchid mycorrhizal fungi

2.1.1 Collection sites

Root samples were taken from *Bulbophyllum exiguum, B. minutissimum, B. bracteatum, B. shepherdii* and *B. elisae* at 7 sites in south-east Queensland (Figure 4). Four of the sites are Queensland National Parks for which collection permits were obtained. Three are on private land.

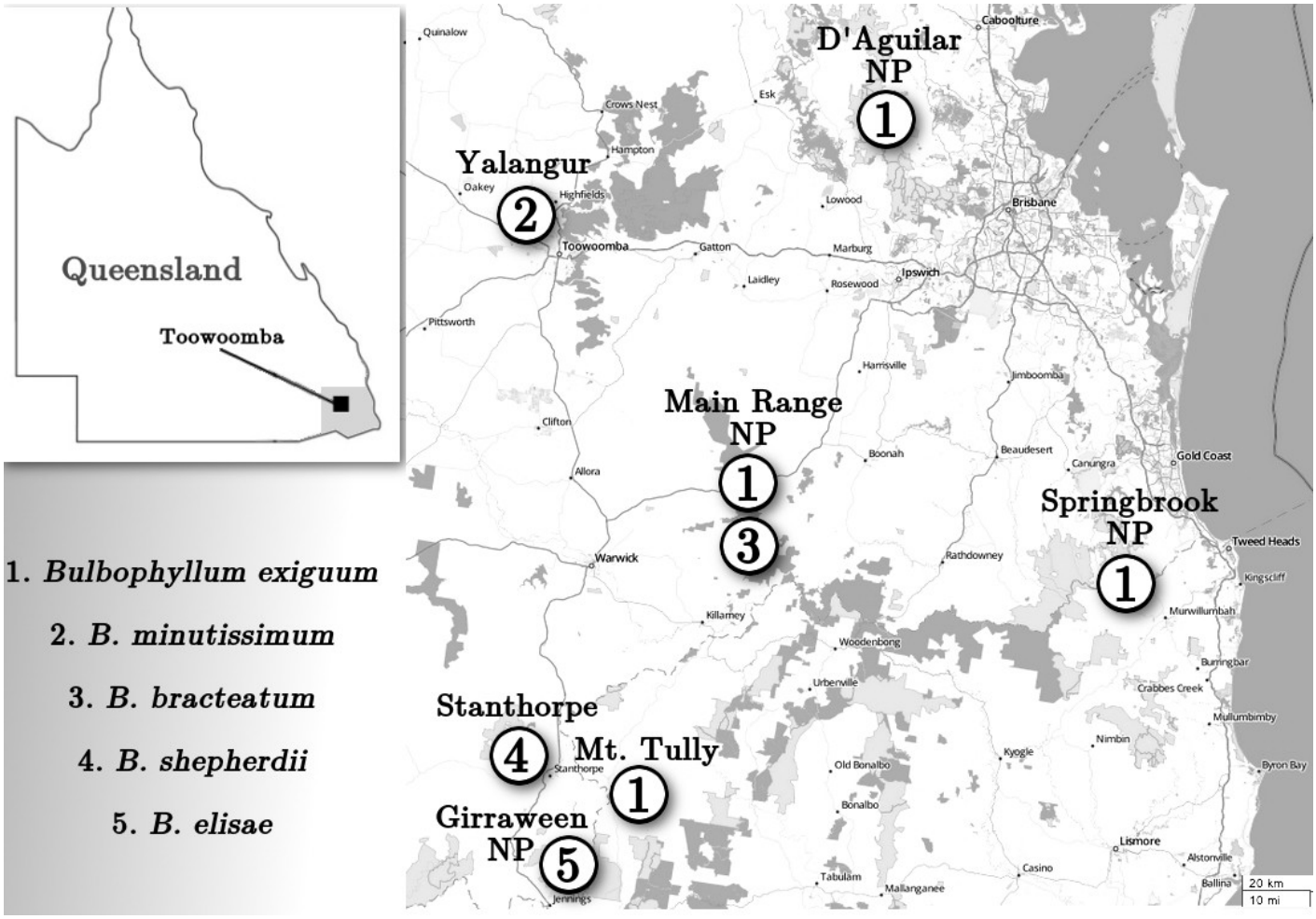

Figure 4 Collection sites for fungal and orchid DNA in south-east Queensland. (Map data: openstreetmap.org.)

The site at D'Aguilar NP consisted of subtropical rainforest with the sampled *B. exiguum* colony growing on the trunk of a 15m tree identified as *Rhodamnia* sp. The *B. exiguum* colony at Main Range NP was growing on the side of a basalt boulder in a moist, shaded gully near Queen Mary's Falls. At Mount Tully *B. exiguum* colonies were growing inside the crack of a large, split granite boulder close to the summit. At Springbrook NP *B. exiguum* were found growing on the trunk of a >20m tree identified as *Acacia melanoxylon*.

B. bracteatum sampled at Queen Mary's Falls, Main Range NP were growing on basalt boulders at the top of a ~20m cliff face. *B. minutissimum*, sampled at a private property in Yalangur QLD, were growing in a dense mat over basalt slabs at the top of a steep, forested hill. The *B. elisae* colony sampled in Girraween NP was growing on the side of a large granite boulder in open *Eucalyptus* woodland.

B. shepherdii, sampled at a private property west of Stanthorpe, had been taken from its natural setting in a woodland and moved ~2km by the owner of the property to a granite outcrop. As such, the root-associated fungi reported here for *B. shepherdii* are included only for completeness. They cannot be taken as representative of the orchid's natural mycobionts.

2.1.2 Root sampling

~2cm root lengths (roots are approximately 1-2mm in diameter) were cut from 3 to 5 individual plants (Appendix A) at each site using sterilised forceps and scissors. Care was taken to select only roots that were in contact with the substrate, as previous studies have found higher levels of fungal colonisation in such roots (Chomicki, Bidel & Jay-Allemand 2014). Samples were placed in 1.5mL centrifuge tubes and kept on ice while in transit to the laboratory at USQ.

2.1.3 Isolation and culturing of mycorrhizal fungi

To kill any root surface-dwelling micro-organisms, roots were surface-sterilised by 1 minute immersion in commercial bleach (0.05% NaClO) and rinsed 3 times in sterilised distilled water. In a sterile laminar flow chamber, each root was finely sliced and squashed with an ethanol- and flame-sterilised scalpel blade to release pelotons. Sterile distilled water was mixed with the crushed root and divided between 3 x 90mm petri dishes (3 replicates per root), and cooled, molten potato dextrose agar (PDA) (Bacto Labs, Liverpool NSW) poured to cover the plate. Plates were sealed with parafilm and incubated at 21°C in the dark. Every 14 hours they were checked for fungal growth by light microscopy. Colonies were assessed for similarity to *Rhizoctonia* fungi in terms of slowness of radial growth (<2mm/day), right-angled hyphal branch angles and the absence of spores as per Garcia, Onco & Susan (2006). *Rhizoctonia*-like colonies were cut from the agar and sub-cultured onto a new plate of PDA, this step being

repeated until a number of pure candidate isolates, without contamination from bacteria or other fungi, were obtained. Collection, isolation and sub-culturing commenced on the 14th of February and concluded on the 5th of July 2017.

2.1.4 Fungal DNA extraction, polymerase chain reaction (PCR) and internal transcribed spacer (ITS) gene sequencing

Total DNA was extracted when fungal colonies reached 2cm in diameter, at which point sufficient tissue was available for the DNA extraction process. Each isolate was sub-cultured prior to DNA extraction in case plates became contaminated on opening.

In a sterile laminar flow chamber, DNA was extracted from approximately $3mm^3$ of mycelial tissue using a commercial kit (Extract-N-Amp, Sigma-Aldrich, Castle Hill NSW). PCR targeting the internal transcribed spacer (ITS) ribosomal DNA region was then carried out. The mitochondrial cytochrome c oxidase subunit 1 (CO1) gene, used as a species barcode to identify animals, is difficult to amplify from the DNA of many groups of fungi, which has led to ITS sequences being favoured for the DNA barcoding of fungi (Schoch et al. 2012). The ITS1 and ITS2 regions consist of relatively fast-evolving spacer DNA sequences that lie between the large (LSU) and small (SSU) subunit nuclear ribosomal RNA genes, plus the 5.8S rRNA gene, which lies in the middle (Figure 5).

Initially, the universal fungal ITS primers ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990) were used for PCR reactions (Table 1). These primers gave a low (successful in ~45% of isolates) amplification rate, so 6 OMF- and *Tulasnella*-specific primers ITS1OFa, ITS1OFb, ITS4-OF, ITS4-Tul, ITS4-Tul2a, ITS4-Tul2b (Taylor & McCormick 2008) were used thereafter. These primers yielded more consistent amplification (~85%).

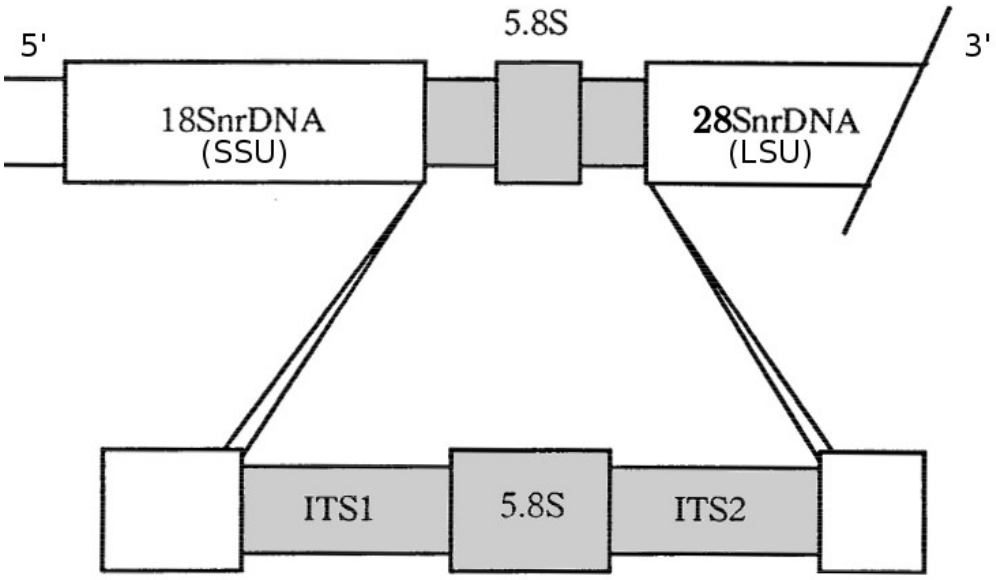

Figure 5 Structure of the fungal nuclear ribosomal RNA genes. The ITS region is highlighted in grey. (Figure adapted from Bena et al. 1998.)

Table 1 Nucleotide sequences and specificity of fungal PCR primers used in this study.

Primer name	Specificity	Sequence (5' - 3')
ITS1F *forward*	Fungi	CTTGGTCATTTAGAGGAAGTAA
ITS4 *reverse*	Fungi	TCCTCCGCTTATTGATATGC
ITS1OFa *forward*	OMF	AACTCGGCCATTTAGAGGAAGT
ITS1OFb *forward*	OMF	AACTTGGTCATTTAGAGGAAGT
ITS4-OF *reverse*	OMF	GTTACTAGGGGAATCCTTGTT
ITS4-Tul *reverse*	*Tulasnella*	CCGCCAGATTCACACATTGA
ITS4-Tul2a *reverse*	*Tulasnella*	TTCTTTTCCTCCGCTGAATA
ITS4-Tul2b *reverse*	*Tulasnella*	TTCTTTTCCTCCGCTGATTA

PCR reactions were set up in 20μL total volumes in duplicate, with positive (a fungal sample with ITS region known to amplify from the primers used) and negative (sterile, distilled, autoclaved H_2O instead of DNA) controls. For reactions using ITS1F and ITS4 primers, each volume contained 10μL of Extract-N-Amp Readymix, 7μL H_2O, 1μL of each primer, and 1μL of extracted genomic DNA. For reactions using OMF- and *Tulasnella*-specific primers, each volume contained 10μL of Extract-N-Amp Readymix, 6μL of H_2O, 0.5μL of each

primer, and 1μL of extracted genomic DNA. The volume of DNA included in the reactions was reduced to 1μL from 4μL after poor initial results.

PCR reactions were performed in a Thermo Hybaid PCR Express thermocycler (Integrated Sciences, Willoughby NSW) with the following temperature protocol: 35 cycles of 95°C for 1 min (strand separating); 50°C for 1 min (primer annealing); 72°C for 1 min (enzymatic replication of target DNA) and a final elongation step of 72°C for 10 min. A 2.5μL sample of each PCR product was visualised using 30-minute gel electrophoresis (1% agarose w/v) with 0.005% Gel Red (Thermo Fisher Scientific, North Ryde NSW) as the staining agent, and exposed to UV light to ascertain whether amplification was successful.

PCR products were purified with QiaQuick spin columns (Qiagen, Chadstone VIC) or Diffinity Rapidtips (West Chester, Pennsylvania). Purified DNA was sequenced in 12μL Sanger sequencing reactions containing 11μL (~30ng) of DNA and a total of 1μL of the forward primers used in PCR. Sequencing was performed at the Australian Genome Research Facility (AGRF) in Brisbane, QLD.

DNA electropherogram files (.ab1) were viewed in the program SnapGene Viewer 4.0.2 to check sequence quality. High-quality sequences with little background noise were retained for molecular identification and phylogenetic analysis. Poor sequences showing evidence of DNA contamination were discarded and DNA re-extracted from original cultures for repeat processing to obtain high-quality amplicons.

2.1.5 Molecular identification of mycorrhizal fungi

Fungal ITS sequences were used as queries to search the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) with the standard nucleotide BLASTn algorithm. Search parameters were default except that uncultured environmental samples were excluded from the list of returned matches. The reason for this is that poor quality control over

misidentified sequences in the database has led to a high number of 'false' sequences being uploaded to GenBank, with uncultured environmental samples being frequently mislabelled (Harris 2003). Closest BLAST matches (i.e. highest e-value and % identity) were recorded and isolates identified to family, genus or species level.

2.1.6 Phylogenetic analysis of fungal ITS gene sequences

Sequences from isolates were assembled into alignments for phylogenetic analysis. Backbone alignments were built using representative ITS GenBank sequences for established clades within the family or genus to which isolates showed highest similarity in BLAST. In each case, the most recent comprehensive publication on the phylogeny of the relevant fungal group was used as a template (see sections below for references), and the type of phylogenetic analysis performed in that publication was replicated to retain cladistic resolution.

2.1.6.1 Phylogeny of *Serendipita* isolates

A Sebacinales backbone alignment was assembled based on the Maximum Likelihood analysis of Weiß et al. (2016). Representative ITS sequences from the clades Serendipitaceae, Sebacinaceae and *Helvellosebacina* were used, as well as three isolates obtained in this study that had highest sequence identity to *Serendipita* spp. One isolate with highest sequence identity to *Serendipita* spp. (from *B. elisae*) was omitted from phylogenetic analysis due to contamination of the culture by yeast, which produced an unreliable gene sequence. The chantarelle mushroom *Cantharellus cibarius* was included as an outgroup. ClustalW sequence alignment was performed in MEGA 6.0 (Tamura et al. 2013) with a gap opening penalty of 15 and gap extension penalty of 6.66 for pairwise and multiple alignments, an IUB DNA weight matrix, and transition weight of 0.5. The alignment was trimmed at both ends to the first base common to all sequences Using MEGA 6.0, a Tamura-Nei model Maximum-Likelihood

tree with 1000 bootstrapped replicates was constructed with uniform rates among sites. The resulting tree was visualised in Figtree 1.4.3 (Rambaut 2014) and edited with GIMP 2.8.

2.1.6.2 Phylogeny of *Tulasnella* isolates

A *Tulasnella* backbone alignment was assembled using 17 Australian *Tulasnella* sequences from Linde et al. (2017) and representative *Tulasnella* sequences from clades A-F from other parts of the world published by Suarez et al. (2006). *Sebacina incrustans*, which belongs to a separate clade within the Cantharellales, was included as an outgroup. A ClustalW alignment was produced in MEGA 6.0 using the settings described above for the *Serendipita* analysis, and trimmed at both ends. As the majority of backbone sequences were derived from the Suarez et al. phylogeny, which was created using a Neighbour-Joining approach, a Neighbour-Joining tree was constructed in this analysis. Using MEGA 6.0, a Tamura-Nei model Neighbour-Joining tree with 1000 bootstrapped replicates and uniform rates among sites was generated. The tree was visualised in Figtree 1.4.3 and edited with GIMP 2.8.

2.1.6.3 Phylogeny of *Ceratobasidium* isolate

A Cantharellales backbone alignment was assembled using representative sequences from the cantharelloid clades Ceratobasidiaceae, Clavulinaceae, Botryobasidiaceae, Sebacinaceae, Tulasnellaceae and Cantharellaceae as outlined by Moncalvo et al. (2006) in their Maximum-Parsimony analysis of the Cantharellales. The *Ceratobasidium* isolate was included in the alignment, and the agaricoid ectomycorrhizal fungus *Laccaria bicolor* was selected as an outgroup. Using MEGA 6.0, a Tamura-Nei model Maximum-Parsimony tree with 1000 bootstrapped replicates and uniform rates among sites was generated. The tree was visualised in Figtree 1.4.3 and edited with GIMP 2.8.

2.1.6.1 Phylogeny of Helotiales isolates

A backbone alignment was constructed using representative species from 9 Helotiales clades identified by Wang et al. (2006) using Bayesian inference. ITS sequences of two species per clade were assembled, as well as the 9 putative Helotiales sequences identified in this study. The Ascomycete *Capronia mansonii* was included as an outgroup. A ClustalW alignment was produced in MEGA 6.0 using the settings described above for the *Serendipita* analysis, and trimmed at both ends. The alignment was saved as a .MEGA file and converted to .NEXUS format using the online tool ALignment Transformation EnviRonment (ALTER) (Glez-Peña et al. 2010). The alignment was imported to MrBayes 3.2.6 (Ronquist et al. 2012) for Bayesian analysis with default parameters (Ronquist, Huelsenbeck & Teslenko 2011). The resulting phylogenetic tree was visualised in Figtree 1.4.3 and edited in GIMP 2.8.

2.2 Phylogenetic analysis of *Bulbophyllum* orchids

2.2.1 Leaf tissue collection

Entire leaves from *B. exiguum, B. bracteatum, B. shepherdii* and *B. elisae* were collected with sterilised forceps and scissors, placed in 1.5mL centrifuge tubes and transported on ice from collection sites to the USQ laboratory. In the case of *B. minutissimum*, entire individual plants (~3mm diameter; Figure 3C) were sampled due to their small size. Leaf samples for *B. elisae* were not taken from the same colonies as those from which roots were sampled, but were taken from a horticultural specimen in Mount Tully. Species collection sites and dates are presented in Table 2.

Table 2 Leaf tissue collection sites for DNA analysis of 5 *Bulbophyllum* spp.

Bulbophyllum sp.	Collection site for DNA	Co-ordinates (lat., long.)	Date
1. *B. exiguum*	Springbrook NP	-28.1327, 153.1623	23.05.2017
2. *B. minutissimum*	Yalangur	-27.2521, 151.5214	04.04.2017
3. *B. bracteatum*	Main Range NP	-28.3401, 152.3714	11.03.2017
4. *B. shepherdii*	Stanthorpe	-28.3814, 151.5549	20.06.2017
5. *B. elisae*	Mt. Tully	-28.4321, 151.5751	20.06.2017

2.2.2 Plant DNA extraction, polymerase chain reaction (PCR) and sequencing

DNA was extracted from approximately 5mm^3 of orchid leaf material using a commercial kit (Extract-N-Amp, Sigma-Aldrich, Castle Hill NSW). PCR targeting the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) large subunit (rbcL) gene coding region was carried out using the primers rbcL-1F (5' ATGTCACCACAAACAGAAAC 3') (Sulaiman, Culham & Harborne 2003) and rbcL-1360R (5' CTTCACAAGCAGCAGCTAGTTC 3') (Reeves et al. 2001). PCR reactions were set up in 20μL total volumes in duplicate, with positive and negative controls. Reaction quantities and protocols proceeded as described for fungal PCR (Section 2.1.4), with adjusted temperature settings tailored to the rbcL primer set: 35 cycles of 95°C for 1 min (strand separating); 48°C for 1 min (primer annealing); 72°C for 1 min (enzymatic replication of target DNA); and a final elongation step of 72°C for 10 min. A 2.5μL sample of each PCR product was visualised using 30-minute gel electrophoresis (1% agarose w/v) with Gel Red as the staining agent, and exposed to UV light to ascertain whether amplification was successful. PCR products were purified and sequenced at the AGRF as outlined in Section 2.1.4.

2.2.3 Phylogenetic analysis

An rbcL gene phylogeny of the entire Orchidaceae by Cameron et al. (1999) was used as a template to construct a phylogenetic tree containing the 5 *Bulbophyllum* spp. in this study. A family-level analysis was chosen because of the unresolved taxonomy of these 5 species (Jones 2006), to cater for the possibility of them falling into clades other than *Bulbophyllum*. At the plant family level, chloroplast protein-coding genes such as rbcL are the most widely used in phylogenies (Górniak, Paun & Chase 2010), and the NCBI GenBank database contains approximately 2700 rbcL gene sequences from the Orchidaceae. Within the Cameron et al. (1999) phylogeny, a sub-tree of 'higher' epidendroid orchids was used to source backbone gene

sequences from representative epidendroid clades, which were supplemented with 13 non-Australian *Bulbophyllum* sp. rbcL sequences from GenBank. The non-epidendroid orchid *Vanilla planifolia* was included as an outgroup. A search for rbcL sequences for every Australian *Bulbophyllum* spp. listed by Jones (2006) returned only one hit to the database. However, the single Australian rbcL sequence, from *Bulbophyllum gadgarrense* (Costion et al. 2015; GenBank accession KF496557.1) was excluded from the initial analysis. This was due to its small size (530 bp) and poor alignment with all other sequences, which abbreviated the alignment to 227 bp.

A 700 bp ClustalW alignment was produced in MEGA 6.0 using the settings described above for the Helotiales mycorrhizal fungi analysis, and trimmed at both ends. To create a phylogenetic tree, the Maximum Parsimony analysis by Cameron et al. (1999) was replicated using the web server version of TNT (Goloboff, Farris & Nixon 2008) at www.phylogeny.fr. Settings were as follows: New Technology sectorial search with RSS, CSS and tree fusing; amino acids stepmatrix disabled; nucleic acids transversion cost of 1; standard bootstrapping with 1000 replicates.

The resulting Maximum Parsimony tree showed very poor cladistic resolution (see Appendix B), so an alternative approach was pursued. Using MEGA 6.0, a Tamura-Nei Neighbour-Joining tree with 1000 bootstrapped replicates was constructed with uniform rates among sites. This tree showed much higher resolution of clades but had very low bootstrap support at nodes, so a Tamura-Nei Maximum-Likelihood tree was produced for comparison in MEGA 6.0. The tree had 1000 bootstrapped replicates, uniform rates among sites, a Nearest-Neighbour-Interchange heuristic method, and a very strong branch-swap filter. As the Maximum-Likelihood tree showed even lower bootstrap support, the Neighbour-Joining tree was retained for analysis.

To compare the *Bulbophyllum* sp. in this study with other sequences from the new genera proposed by Jones (2006), a smaller additional tree was constructed. The short sequence for *B. gadgarrense* was included this time, as that species has been assigned to the genus *Oxysepala*, to which Jones proposed that *B. shepherdii* also belongs. An rbcL sequence for *B. tuberculatum* (Millar et al. 2017; GenBank accession KT007193.1), assigned to the new genus *Adelopetalum*, was also included, as *B. exiguum, B. elisae* and *B. bracteatum* have been proposed to belong to *Adelopetalum* (Jones 2006). No sequences were available to represent *Oncophyllum*, the proposed new genus of *B. minutissimum*. The non-epidendroid orchid *Vanilla planifolia* was included as an outgroup. A 227 bp alignment was produced in MEGA 6.0 using the settings described above for the *Serendipita* analysis, and trimmed at both ends. A Tamura-Nei model Neighbour-Joining tree with 1000 bootstrapped replicates and uniform rates among sites was generated.

2.3 *Bulbophyllum exiguum* mycorrhizal seed germination

2.3.1 Collection of seed pods

Sites at which *B. exiguum* were observed flowering in February/March (Main Range NP & Stanthorpe) were returned to approximately 2 months later for collection of seed pods. Ten pods were collected from each site using sterilised forceps and scissors, with care taken to ensure that this amount represented only a small fraction of the pods that remained on orchid colonies after collection. Pods were desiccated at room temperature using a silica gel desiccator and observed until they began to dehisce.

2.3.2 Mycorrhizal seed germination experiments

To kill seed coat- and pod-dwelling micro-organisms, dried seed pods were finely chopped with a scalpel and surface sterilised for 15 minutes in a 25% bleach solution (NaClO), with 1µL of

the detergent Tween to ensure that bleach had thorough contact with plant tissue. Chopped pods were rinsed 3 times with sterile, distilled H_2O through sterile filter paper and then spread over the surface of the paper using sterile forceps. The filter paper was cut into wedge-shaped pieces and the pieces laid on individual 90mm plates (Figure 6) containing oatmeal agar (30g oatmeal, 15g agar, 1000 mL water) (Pereira et al. 2003).

Each plate was inoculated with a $5mm^3$ mycelial/agar plug from a putatively mycorrhizal fungal culture isolated in the first phase of the study (see Section 2.1.3). Collection sites and host orchid information for the isolates that were used are listed in Table 3. Plates were sealed with parafilm, incubated in the dark at 21°C, and observed every 5 days under a light microscope to record whether seed germination had occured. A growth scale was used to categorise seed developmental stages (Table 4) (Stewart & Kane 2007; Khamchatra et al. 2016). Calculations for a germination rate index (GRI) and developmental rate index (DRI) were based on analyses by Papenfus et al. (2016) and Khamchatra et al. (2016). Indices were used to condense multiple seed counts over time into a standardised figure for each treatment that could be statistically compared. To obtain percentages for seed germination and developmental stages, calculations were to divide numbers of germinated seeds and seeds at each developmental stage by the total number of seeds on each plate. Calculations for GRI and DRI were as follows:

$$GRI = \frac{G1}{1} + \frac{G2}{2} + \ldots + \frac{Gx}{x.}$$

(G1 = percentage of germinated seeds × 100 counted at the first five-day interval. G2 = percentage of germinated seeds × 100 counted at the second five-day interval, etc.)

$$DRI = \frac{D1}{1} + \frac{D2}{2} + \ldots + \frac{Dx}{x}$$

(D1 = percentage of protocorms at a given developmental stage (2 to 5) × 100 counted at the

first five-day interval. D2 = percentage at a given stage (2 to 5) × 100 at the second five-day interval, etc.)

GRIs for each treatment (3 replicates per treatment) were to be compared using one-way (single-factor) ANOVA in LibreOffice Calc to determine whether the source of variation in GRI for each treatment was likely to be the fungal species used as inoculum. ANOVA was deemed appropriate due to the high likelihood, based on prior studies, of each treatment containing at least one germinated seed (stage 1) even on uninoculated control plates (Swangmaneecharern, Serivichyaswat & Nontachaiyapoom 2012; Tan et al. 2014; Mala et al. 2017), which would provide a suitable ANOVA dataset with few zero figures. Statistical analysis of DRI at each developmental stage across treatments was not deemed suitable for two-way (dual-factor) ANOVA due to the likelihood of there being a high number of zero figures for some treatments and developmental stages (Stewart & Zettler 2002; Khamchatra et al. 2016), which would violate the ANOVA assumption of normal distribution of residuals (Glass, Peckham & Sanders 1972). Instead, a non-parametric Fisher's exact test, suitable for small sample sizes with frequent zero values (Routledge 2005) was to be applied separately to data from each treatment to establish if DRI at each developmental stage differed significantly. Data was then to be presented in a matrix (see Appendix C) to contrast DRI between treatments.

Table 3 Orchid hosts and collection sites for fungal isolates used as inocula in seed germination experiments.

Fungal isolate	Orchid host	Site of collection
P5 1.1 (***Serendipita* sp.**)	*B. exiguum*	D'Aguilar NP
P1 1.2 (***Tulasnella* sp.**)	*B. exiguum*	D'Aguilar NP
P5 1.7 (**Helotiales sp.**)	*B. exiguum*	D'Aguilar NP
P3 1.12 (***Phoma* sp.**)	*B. minutissimum*	Yalangur

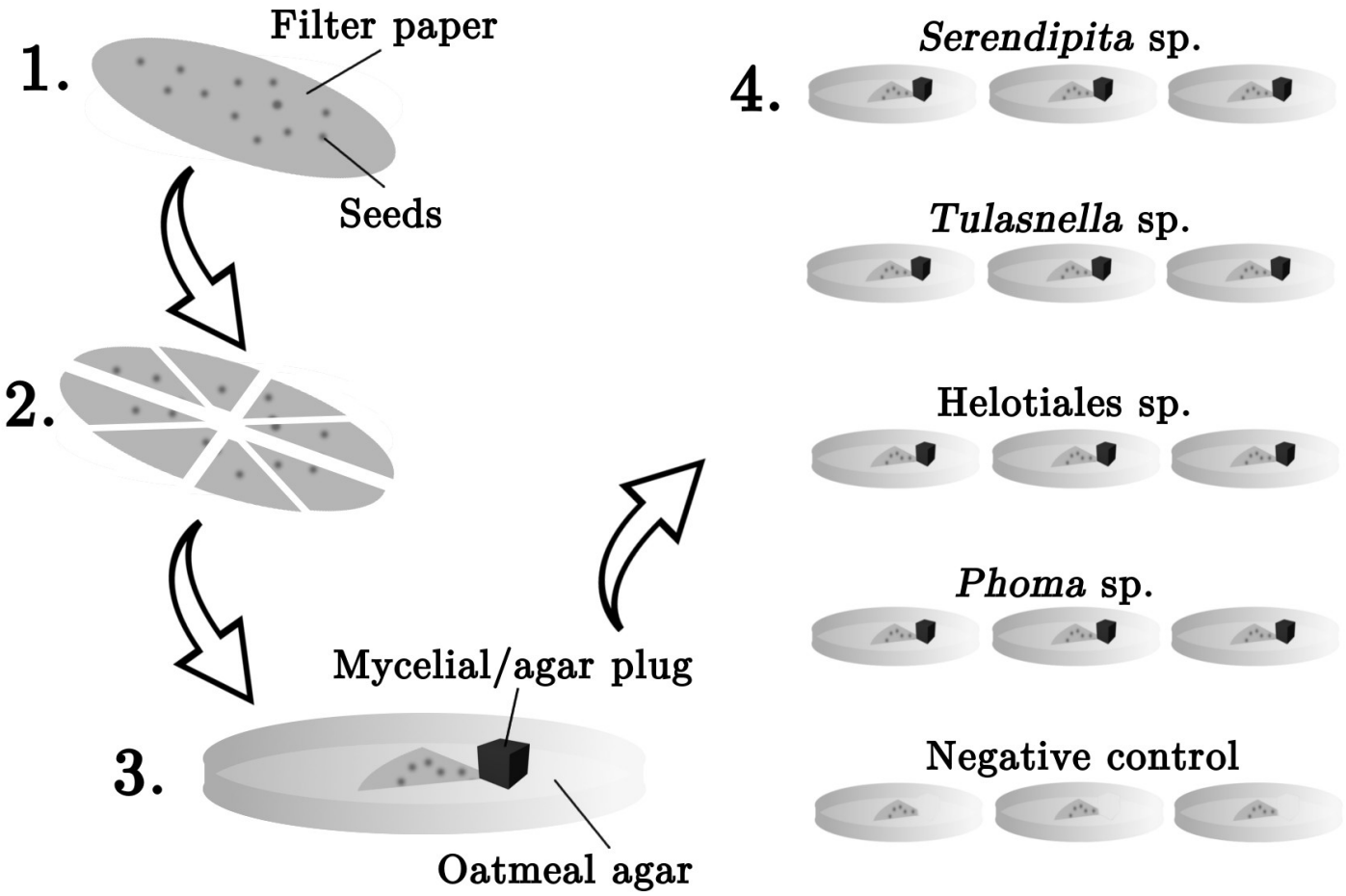

Figure 6 Mycorrhizal seed germination experiment for seeds of *B. exiguum*. (1) Sterilised seeds were spread over sterile filter paper. (2) Filter paper was cut into wedges, (3) laid over oatmeal agar plates and inoculated with mycelial/agar plugs from different fungal isolates. (4) Three replicates were made of each inoculum: *Sebacina* sp. (putative OMF), *Tulasnella* sp. (putative OMF), Helotiales sp. (putative DSE/ericoid mycorrhizal fungus), *Phoma* sp. (plant pathogen), and a negative control with seeds but no fungal inoculum.

Table 4 Growth scale for analysis of symbiotic seed germination experiments. Adapted from Stewart & Kane (2007) and Khamchatra et al. (2016).

Stage	Description
0	No germination, seed coat intact
1	Embryo swollen (germination)
2	Continued embryo enlargement, seed coat ruptured, rhizoids present
3	Appearance of protomeristem
4	Emergence of first leaf
5	Elongation of first leaf and further development

3. Results

3.1 Molecular identification of mycorrhizal fungi

3.1.1 Isolation and culturing of fungi

A total of 90 individual *Rhizoctonia*-like isolates were cultured from the roots of 39 individual orchid plants representing the 5 orchid species in this study (Table 5). The orchid species and site from which the highest number of isolates were obtained per root sampled was *B. exiguum* at Mount Tully, with 19 isolates from 5 root samples. The lowest was *B. shepherdii* at Stanthorpe, with 4 isolates from 3 root samples. All orchid roots contained high numbers of fungal species based on the wide morphological variation of fungal growths.

Table 5 Collection data for orchid roots sampled between February and June 2017.

Location	Date	*Bulbophyllum* species	# of roots sampled	# of fungal isolates	Host	Latitude	Longitude
D'Aguilar NP	14.02.2017.	***B. exiguum***	6	18	*Rhodamnia* sp.	-27.4016	152.7996
Main Range NP	10.03.2017.	***B. exiguum***	5	7	Rock	-28.3394	152.3698
Main Range NP	11.03.2017.	***B. bracteatum***	5	9	Rock	-28.3401	152.3714
Yalangur	04.04.2017.	***B. minutissimum***	5	11	Rock	-27.2521	151.5214
Girraween NP	02.05.2017.	***B. elisae***	5	16	Rock	-28.5211	151.5957
Mt Tully	02.05.2017.	***B. exiguum***	5	19	Rock	-28.4321	151.5751
Springbrook	23.05.2017.	***B. exiguum***	5	6	*Acacia melanoxylon*	-28.1327	153.1623
Stanthorpe	20.06.2017.	***B. shepherdii***	3	4	Rock	-28.3814	151.5549
Total			**39**	**90**			

Fungal growths became visible under light microscopy after 24-48 hours. Many were rapidly-growing species that were identified as *Fusarium* or *Penicillium* spp. based on hypha and spore morphology. Intracellular, free and germinated fungal pelotons were visualised microscopically during the isolation process (Figures 7 & 8). The majority of fungal isolates were obtained from *B. exiguum* (50 isolates or 55.5% of total isolates), with the minority isolated from *B. shepherdii* (4 isolates or 4.4% of total isolates).

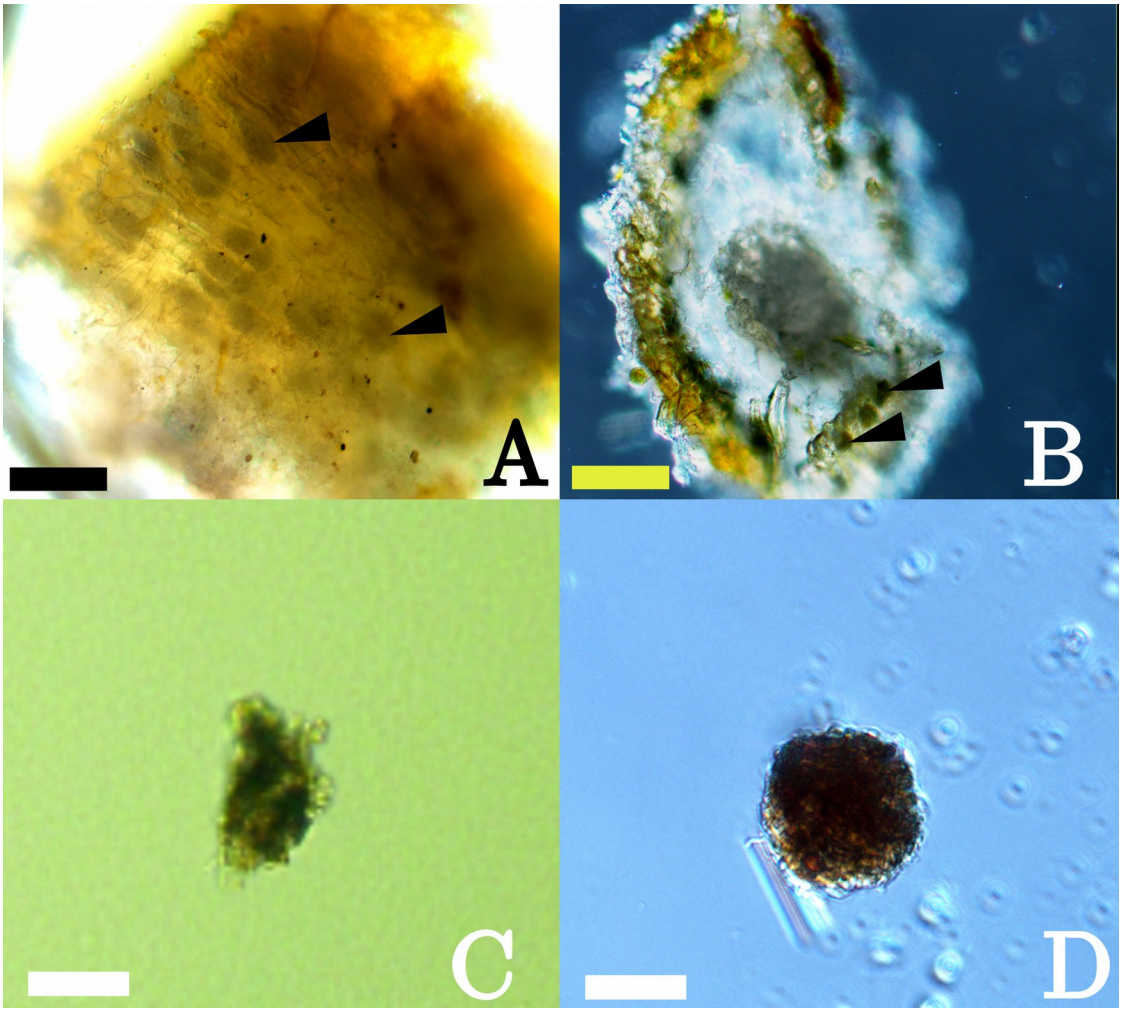

Figure 7 Pelotons isolated from *B. exiguum* roots. (A) Root fragment showing intracellular pelotons (arrowheads). Bar is 250µm. (B) Transverse section of root showing pelotons inside the cortical cell layer (arrowheads). Bar is 500µm. (C) and (D) Free pelotons suspended in potato dextrose agar. Bars are 60µm.

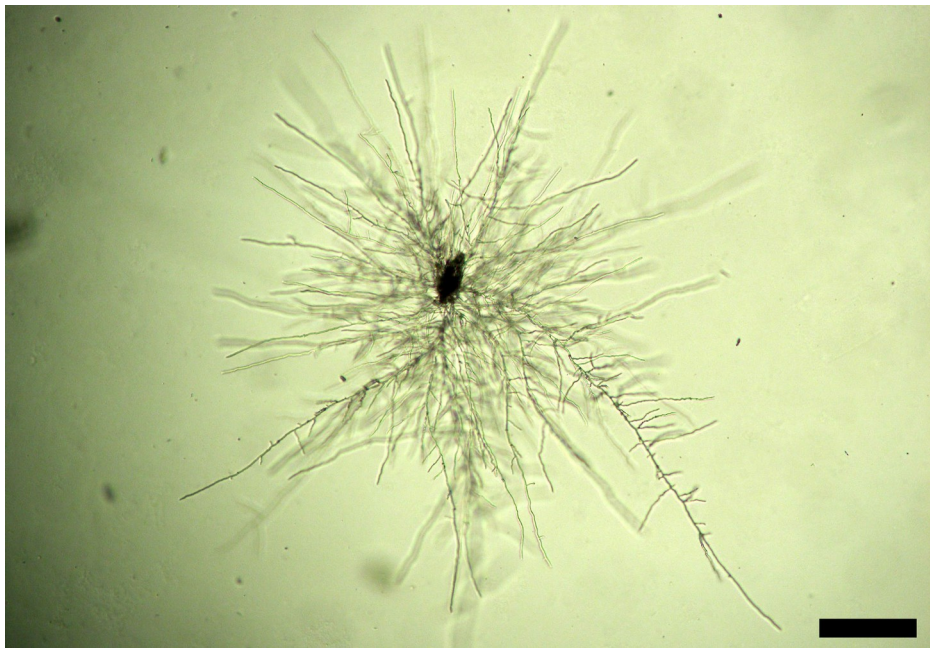

Figure 8 Germinated peloton isolated from a *B. exiguum* root, growing in potato dextrose agar. Bar is 180µm.

3.1.2 Fungal PCR of ITS gene region

Initial PCR using 4μL of extracted fungal DNA and the universal fungal primers ITS1F and ITS4 resulted in no amplification. After reducing the amount of DNA in PCR reactions to 1μL, some electrophoretic banding was observed (Figure 9), suggesting that 4μL of sample DNA may have been too large an amount for the polymerase to effectively replicate the target gene region. However, amplification success using 1μL of sample DNA was low, with only approximately 45% of isolates showing sufficient band intensity (DNA concentration) for sequencing. PCR using a set of OMF- or *Tulasnella*-specific primers (ITS1OFa, ITS1OFb, ITS4-OF, ITS4-Tul, ITS4-Tul2a, ITS4-Tul2b; Taylor & McCormick 2008) resulted in much higher amplification rates (success in ~85% of isolates) (Figure 10), and these primers were used in all subsequent PCR reactions. The initial poor amplification of ITS using the universal primers ITS1F and ITS4 may have been due to their incompatibility with rapidly-evolving ITS sequences in some OMF groups, particularly *Tulasnella* (Jacquemyn et al. 2012).

PCR products were approximately 600 bp in length, with DNA concentrations between 20ng/10μL and 150ng/10μL.

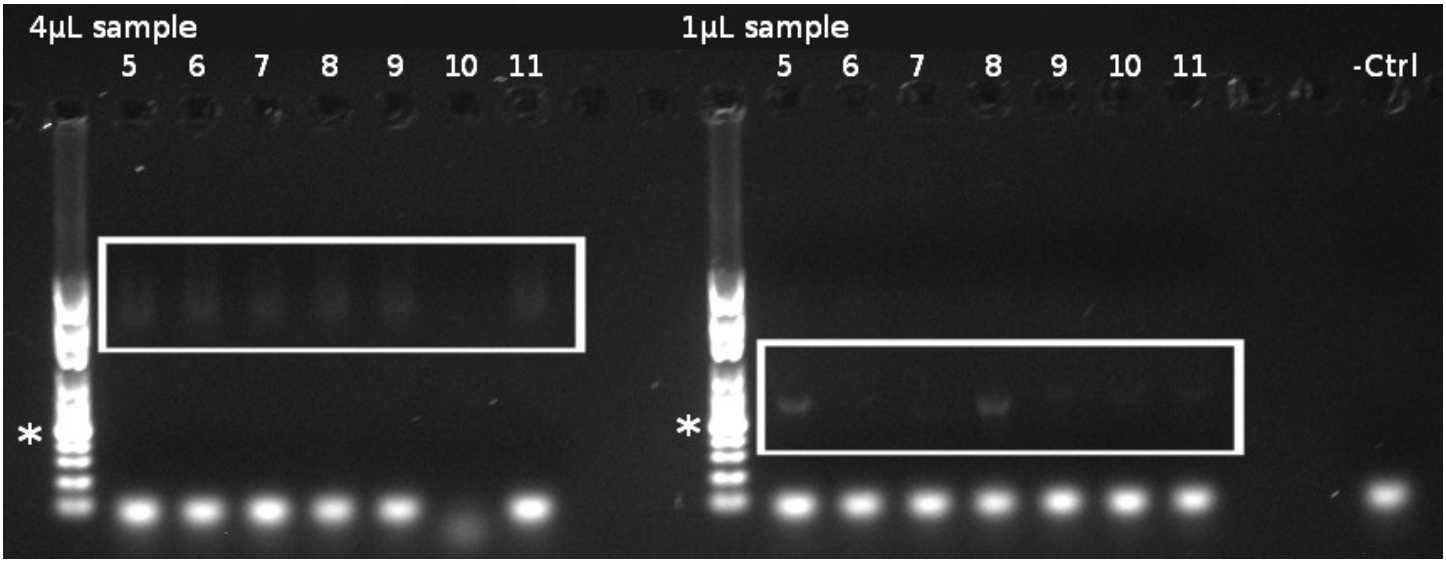

Figure 9 Gel electrophoresis of PCR-amplified fungal ITS regions using the primers ITS1F and ITS4 and different amounts of total extracted DNA. Left panel shows results from 4μL of DNA in the PCR reaction: genomic DNA (box) appears in banding around the 3000 bp region, with no DNA bands appearing in the 650-700 bp region, which is the length of the ITS (500 bp indicated by *). Right panel shows results from reducing sample DNA volume to 1μL.

No genomic DNA can be observed, and faint banding (box) is evident in the 650-700 bp region, indicating that amplification of ITS DNA with these primers has been somewhat successful. Bright bands in lower sections of the panels are primers.

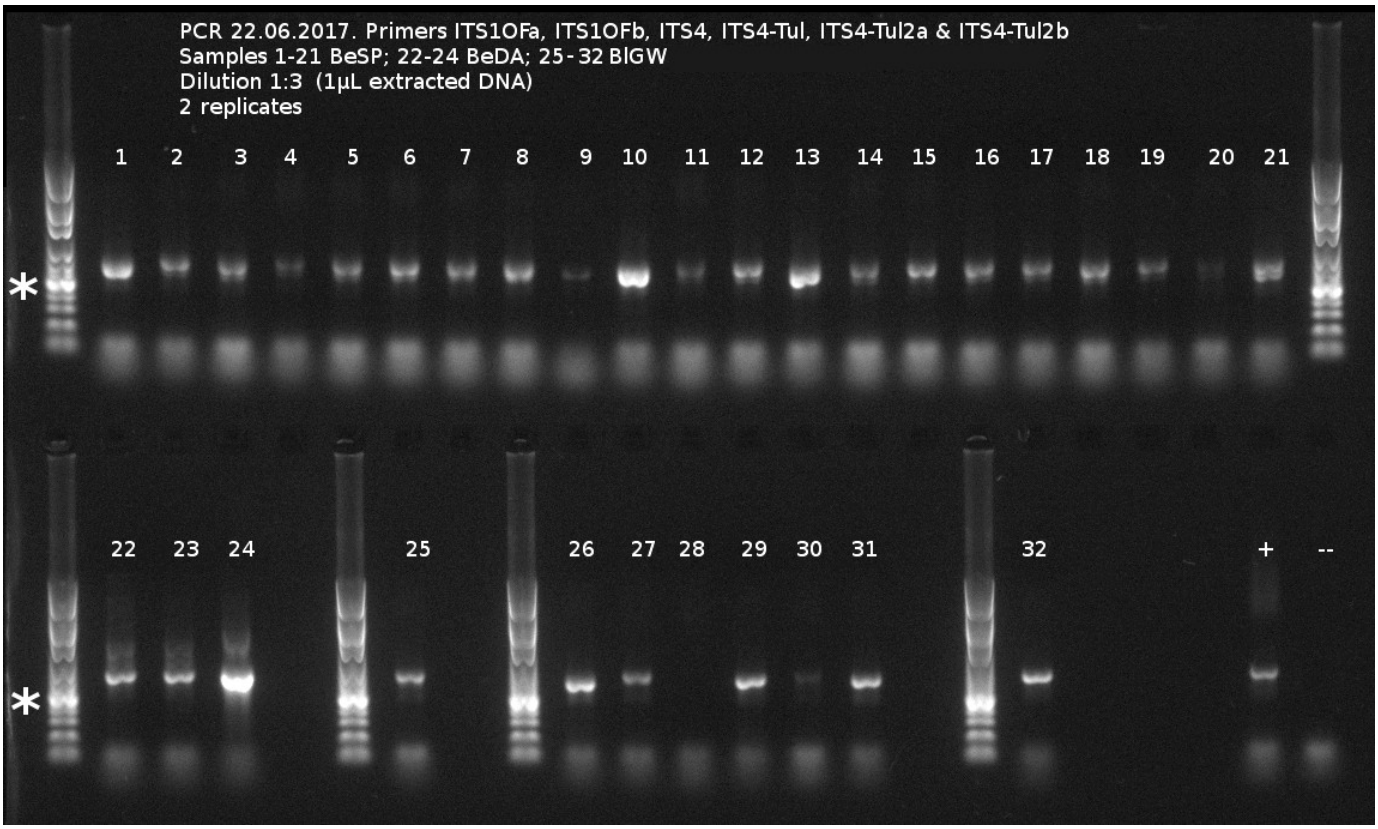

Figure 10 Gel electrophoresis of PCR-amplified fungal ITS regions using the OMF- and *Tulasnella*-specific primers ITS1OFa, ITS1OFb, ITS4-OF, ITS4-Tul, ITS4-Tul2a, and ITS4-Tul2b (Taylor & McCormick 2008). Twenty-eight of 32 samples (87.5%) amplified with sufficient concentration to be sequenced, indicating that this primer set was more effective for use with *Bulbophyllum* OMF than the ITS1F and ITS4 set. 500 bp is indicated by *.

3.1.3 DNA sequencing and identification using the basic local alignment search tool (BLAST)

DNA electropherograms returned from sequencing exhibited variable sequence quality. High-quality sequences (Figures 11A-E) were retained for molecular identification and phylogenetic analysis. Poor sequences showing evidence of DNA contamination (Figure 11F) were discarded and isolates sub-cultured to remove extraneous yeast and bacterial contaminants.

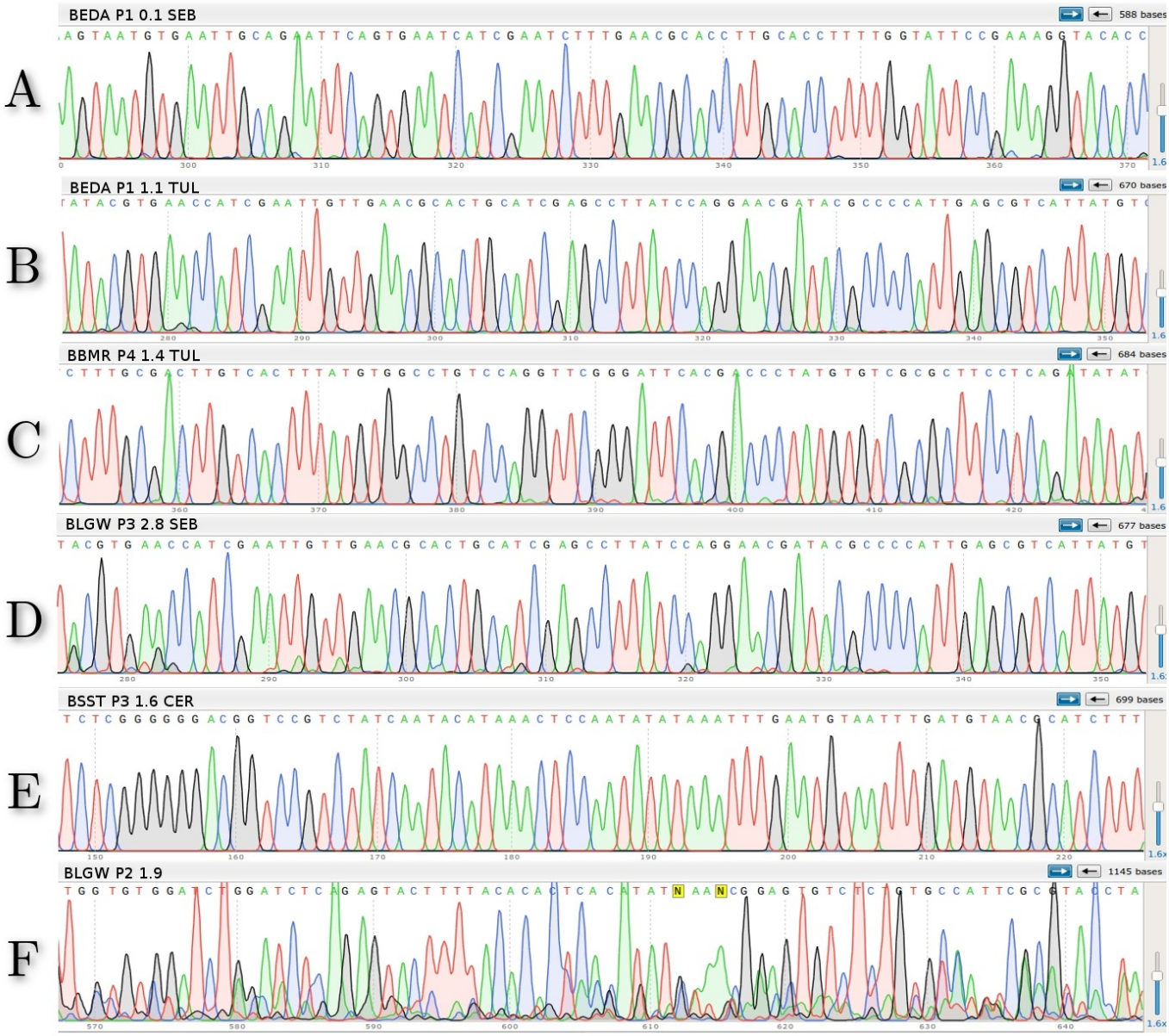

Figure 11 Examples of fungal DNA electropherograms returned by Sanger sequencing reactions at the AGRF. (A)-(E) show clear peaks and little to no background noise, indicating that each nucleotide has been identified with high confidence. (F) contains a high level of background noise, indicating that the sequence was unsuitable for further bioinformatic analysis and that DNA needed to be re-extracted and re-amplified from a subcultured isolate. (Screenshots from SnapGene Viewer 4.0.2.)

3.1.4 Molecular identification of fungal isolates

Of the 90 *Rhizoctonia*-like isolates cultured *in vitro*, 11 (12.2% of total isolates) had highest identity with archived cantharelloid OMF sequences: 6 from the orchid *B. exiguum*, 2 from *B. bracteatum*, 2 from *B. elisae*, 1 from *B. shepherdii* and 0 from *B. minutissimum*. Of these, only one (from *B. shepherdii* in Stanthorpe) had >97% identity to its highest BLAST match.

Based on the rule-of-thumb <3% species delineation for fungal ITS sequences (Nilsson et al. 2008), all but the *B. shepherdii* cantharelloid isolates are species likely to be new to science.

Additionally, 11 isolates (12.2% of total isolates) from *B. exiguum* returned highest sequence identity to orchid-associated ascomycete endophytes in the fungal orders Helotiales, Xylariales and Chaetothyriales. Seven isolates (7.8% of total isolates), 3 from *B. exiguum*, 3 from *B. elisae* and 1 from *B. bracteatum,* shared highest sequence identity with ericoid mycorrhizal fungi in the order Helotiales. Eight isolates (8.9% of total isolates), 5 from *B. exiguum* and 3 from *B. elisae,* had top matches to lichen-associated fungal sequences. One isolate (1.1% of total isolates) from *B. bracteatum* had highest identity to a fern-associated endophyte. Fifty-two additional sequences matched non-mycorrhizal fungal ITS regions of ascomycetes from the orders Xylariales, Hypocreales, Diaporthales, Pleosporales, Helotiales and Coniochaetales, and a basidiomycete from the order Polyporales. All of these orders contain known saprotrophs or plant pathogens (Cannon & Kirk 2007). Figure 12 presents all categories of isolate obtained from orchid roots in this study.

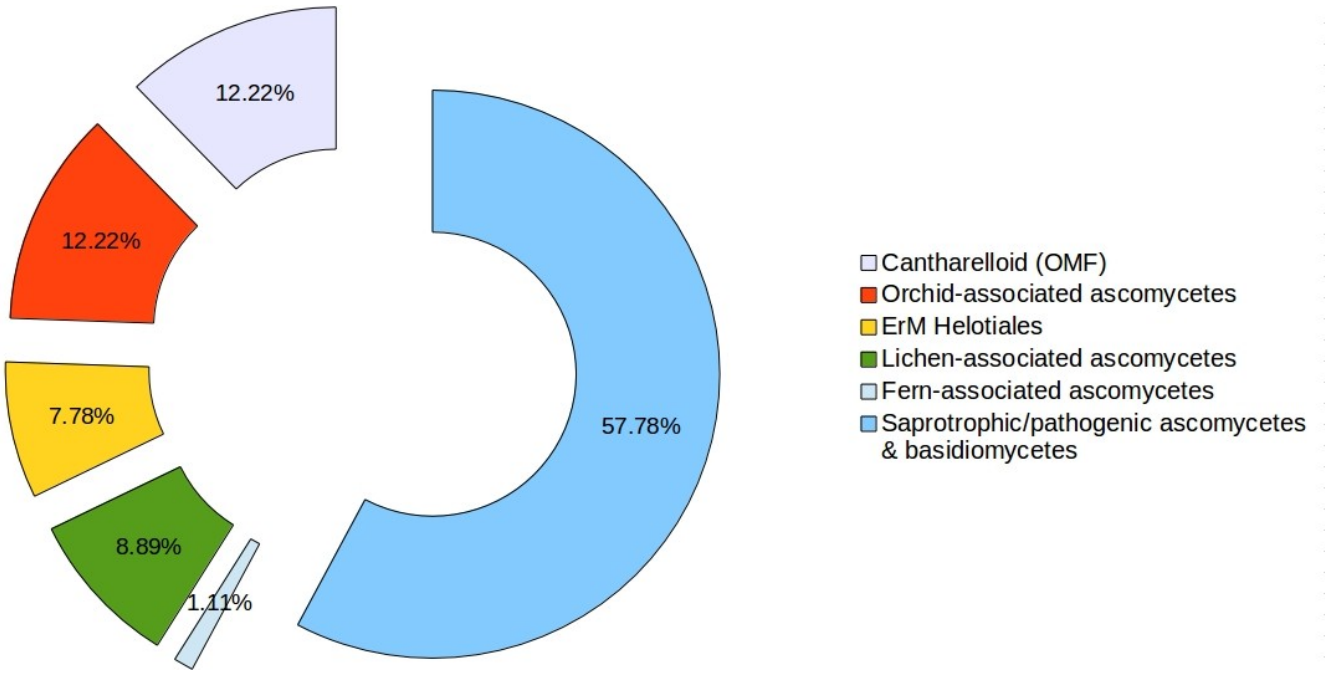

Figure 12 Categories of fungal isolate expressed as percentages of the total number of cultures obtained from orchid roots. Saprotrophic or pathogenic ascomycetes and

basidiomycetes predominated, with OMF and orchid-associated ascomycetes together comprising ~25% of isolates. Ericaceae- (ErM) and lichen-associated fungi each formed ~8% of total isolates, with only a single fern-associated sequence identified. Categories were assigned based on the host plant of each isolate's closest BLAST match when searched in GenBank.

Analysis of all sequenced ITS regions using BLAST searches of the NCBI GenBank database returned archived sequences with moderate to high (80-100%) identity to each fungal isolate. A single isolate returned lower (74%) sequence identity to an archived ITS region (isolate P2 1.9 from *B. elisae* in Girraween NP), and this was due to yeast or bacterial DNA contamination of the isolate that could not be removed through sub-culturing.

Complete data for BLAST results from isolates obtained from each orchid species are presented in Tables 6-10. Photographic examples of putative OMF and ericoid mycorrhizal (ErM) fungal isolates are presented in Figures 13 and 14.

Table 6 BLAST search results for ITS regions from *Rhizoctonia*-like fungi isolated from *B. exiguum* at 4 field sites in south-east Queensland. Yellow=orchid mycorrhizal fungi; orange=orchid-associated endophytes; blue=ericoid mycorrhizal fungi; pink=lichen-associated fungi. Grey shading of fungal isolates indicates cultures used as inocula in seed germination experiments. 'Not listed' denotes GenBank accessions for which no identifying family or order were provided.

Orchid host	Site	Fungal isolate	Closest BLAST match	Query cover (%)	E value	Identity (%)	Genbank accession	Family of closest BLAST match/es	Order of closest BLAST match/es
B. exiguum	D'Aguilar NP	P5 1.1	Sebacina vermifera isolate K225	100	2E-172	89	EU625992.1	Serendipitaceae	Sebacinales
		P1 0.1	Sebacina vermifera	83	6E-143	91	FN663142.1	Serendipitaceae	Sebacinales
		P1 3.1	Sebacinales sp. 2 CC 12-26	99	2E-86	89	KF359622.1	Serendipitaceae	Sebacinales
		P1 1.2	Tulasnella sp. HJ24T	100	1E-144	86	KC291648.1	Tulasnellaceae	Cantharellales
		P1 1.2a	Tulasnella sp. HJ24T	97	1E-150	86	KC291648.1	Tulasnellaceae	Cantharellales
		P1 1.1	Tulasnella sp. C2-DT-TC-1	96	2E-153	88	GU166427.1	Tulasnellaceae	Cantharellales
		P2 1.5, P2 2.4	Sordariomycetes sp. WF149	100	0.0	99	HQ130705.1	Herpotrichiellaceae	Chaetothyriales
		(BE 1.3)	Daldinia eschscholtzii isolate F5	100	0.0	99	MF185103.1	Xylariaceae	Xylariales
		P1 1.3	Fungal sp. voucher Robert L. Gilbertson Mycological Herbarium 1363	100	0.0	99	KT289540.1	*not listed*	Microascales
		P3 1.2	Nemania sp. genotype 407 isolate FL0916	100	0.0	99	JQ760549.1	Xylariaceae	Xylariales
		(BE 1.2)	Biscogniauxia sp. UFMGCB 3834	100	0.0	99	JQ327868.1	Xylariaceae	Xylariales
		(BE 1.4)	Pestalotiopsis sp. UMAS P4	100	0.0	97	KT337387.2	Pestalotiopsidaceae	Xylariales
		P2 1.10	Tolypocladium inflatum isolate PANM200T9ZM1D	100	0.0	99	JF796050.1	Ophiocordycipitaceae	Hypocreales
		P4 1.6	Muscodor sp. M112	100	0.0	95	HM595541.1	Xylariaceae	Xylariales
		P2 1.3	Tolypocladium inflatum isolate PANM200T9ZM1D	100	0.0	99	JF796050.1	Ophiocordycipitaceae	Hypocreales
		P1 1.4, P1 2.2	Muscodor sp. M112	99	0.0	95	HM595541.1	Xylariaceae	Xylariales
		P4 1.5, P4 2.2	Muscodor sp. M112	100	0.0	94	HM595541.1	Xylariaceae	Xylariales
		P6 2.1, P6 2.3	Muscodor sp. M112	100	0.0	94	HM595541.1	Xylariaceae	Xylariales
	Mt Tully	P1 1.1	Cryptosporiopsis radicicola strain W4-1	95	0.0	99	HQ889715.1	Dermateaceae	Helotiales
		P1 1.2	Cryptosporiopsis radicicola strain W4-1	96	0.0	99	HQ889715.1	Dermateaceae	Helotiales
		P5 1.7	Cryptosporiopsis radicicola strain W4-1	96	0.0	99	HQ889715.1	Dermateaceae	Helotiales
		P5 1.8	Cryptosporiopsis radicicola strain W4-1	97	0.0	99	HQ889715.1	Dermateaceae	Helotiales
		P5 1.9	Cryptosporiopsis radicicola strain W4-1	94	0.0	99	HQ889715.1	Dermateaceae	Helotiales
		P4 1.4	Cryptosporiopsis radicicola strain W4-1	94	0.0	99	HQ889715.1	Dermateaceae	Helotiales
		P4 1.5	Cryptosporiopsis radicicola strain W4-1	95	0.0	99	HQ889715.1	Dermateaceae	Helotiales
		P3 1.1	Cryptosporiopsis radicicola strain W4-1	95	0.0	99	HQ889715.1	Dermateaceae	Helotiales
		P3 1.2	Cryptosporiopsis radicicola strain W4-1	96	0.0	99	HQ889715.1	Dermateaceae	Helotiales
		P5 1.6	Sarcosomataceae sp. GS2_1_3	96	0.0	97	KF128771.1	Sarcosomataceae	Pezizales
		P1 1.3	Cytospora eucalypticola culture-collection	100	0.0	99	EU552120.1	Valsaceae	Diaporthales
		P1 1.4	Arthrinium sp. UFMGCB_908	100	0.0	97	FJ466728.1	Apiosporaceae	Xylariales
		P1 1.5	Holocryphia sp. SFC-2012b strain CMW37339	91	0.0	99	JQ862863.1	Cryphonectriaceae	Diaporthales
		P2 1.1	Trichoderma viride isolate 762F1a	100	0.0	99	KU202217.1	Hypocreaceae	Hypocreales
		P2 1.2	Trichoderma viride strain LESF115	100	0.0	99	KT278861.1	Hypocreaceae	Hypocreales
		P4 1.12	Dothideomycetes sp. genotype 800 JMUR-2016 voucher ARIZ:NC0825	98	0.0	99	KX908469.1	Lophiostomataceae	Pleosporales
		P4 1.6	Dothideomycetes sp. genotype 800 JMUR-2016 voucher ARIZ:NC0825	99	0.0	100	KX908469.1	Lophiostomataceae	Pleosporales
		P4 1.7	Proliferodiscus sp. CJL-2014 strain Rs-L-1	100	0	99	KJ542332.1	Hyaloscyphaceae	Helotiales
		P4 1.8	Proliferodiscus sp. KUS-F52660	97	0	99	JN033427.1	Hyaloscyphaceae	Helotiales
	Main Range NP	P5 1.7	Fungal sp. R30	100	0.0	99	AY699688.1	Incertae sedis	Helotiales
		P5 1.14	Fungal sp. R30	100	2E-172	99	AY699688.1	Incertae sedis	Helotiales
		P5 1.5	Fungal sp. R30	100	0.0	99	AY699688.1	Incertae sedis	Helotiales
		P3 1.2	Nemania diffusa isolate DOF-42	100	0.0	100	KX611665.1	Xylariaceae	Xylariales
		P3 1.6	Nemania sp. isolate DOF-21	100	0.0	98	KX611644.1	Xylariaceae	Xylariales
		P1 1.3	Xylaria sp. ICMP 20643	100	3E-139	99	KP689111.1	Xylariaceae	Xylariales
		P3 1.7	Xylariaceae sp. IZ-1249	100	4E-103	93	AM921731.1	Xylariaceae	Xylariales
	Springbrook NP	P1 1.9	Sordariomycetidae sp. N133	89	8E-42	93	KP689127.1	*not listed*	*not listed*
		P4 1.1	Anthostomella sp. N129	95	0	99	KP689108.1	Xylariaceae	Xylariales
		P5 1.8	Sordariomycetes sp. genotype 784	98	0	99	KX908577.1	*not listed*	*not listed*
		P1 1.13b	Fungal endophyte strain HM-10-A	100	0	99	KT291034.1	*not listed*	*not listed*
		P1 1.5	Sordariomycetes sp. genotype css033	98	0	99	KM519274.1	*not listed*	*not listed*
		P1 1.4	Fungal endophyte strain HM-10-A	100	0	99	KT291034.1	*not listed*	*not listed*

Table 7 BLAST search results for ITS regions from *Rhizoctonia*-like fungi isolated from *B. bracteatum* at Main Range NP, south-east Queensland. Yellow=orchid mycorrhizal fungi; blue=ericoid mycorrhizal fungi; brown=fern-associated fungi.

Orchid host	Site	Fungal isolate	Closest BLAST match	Query cover (%)	E value	Identity (%)	Genbank accession	Family of closest BLAST match/es	Order of closest BLAST match/es
B. bracteatum	**Main Range NP**	P5 1.11	Tulasnella sp. C2-DT-TC-1	92	2.00E-164	86	GU166427.1	Tulasnellaceae	Cantharellales
		P4 1.4	Tulasnella sp. C2-DT-TC-1	97	4.00E-161	86	GU166427.1	Tulasnellaceae	Cantharellales
		P5 1.1	Fungal sp. R30	95	0.0	99	AY699688.1	Incertae sedis	Helotiales
		P2 1.0	Fungal sp. F481 (Acremoniopsis suttonii)	96	1.00E-104	88	JQ747718.1	Incertae sedis	Hypocreales
		P3 2.1.2	Apiognomonia lasiopetali strain CPC 29158	100	0.0	97	KY173386.1	Gnomoniaceae	Diaporthales
		P5 1.8	Fungal sp. isolate E3430A (Nemania sp.)	100	0.0	99	KT996069.1	Xylariaceae	Xylariales
		P3 1.0	Apiognomonia lasiopetali strain CPC 29158	99	0.0	99	KY173386.1	Gnomoniaceae	Diaporthales
		P3 2.1.1	Apiognomonia lasiopetali strain CPC 29158	97	0.0	99	KY173386.1	Gnomoniaceae	Diaporthales
		P5 2.12	Lecythophora sp. Sib2-1-11	95	0	98	KX100366.1	Coniochaetaceae	Coniochaetales

Table 8 BLAST search results for ITS regions from *Rhizoctonia*-like fungi isolated from *B. elisae* at Girraween NP, south-east Queensland. Yellow=orchid mycorrhizal fungi; blue=ericoid mycorrhizal fungi; pink=lichen-associated fungi. Grey shading of fungal isolates indicates cultures used as inocula in seed germination experiments. 'Not listed' denotes GenBank accessions for which no identifying family or order were provided.

Orchid host	Site	Fungal isolate	Closest BLAST match	Query cover (%)	E value	Identity (%)	Genbank accession	Family of closest BLAST match/es	Order of closest BLAST match/es
B. elisae	**Girraween NP**	P2 1.9	Sebacina vermifera	41	1E-39	74	FN663143.1	Serendipitaceae	Sebacinales
		P3 2.8	Tulasnella sp. mara13	100	2E-147	86	KC928366.1	Tulasnellaceae	Cantharellales
		P1 1.15	Helotiales sp. 1 RB-2011	100	0.0	94	JQ272327.1	*not listed*	Helotiales
		P5 1.3	Hyaloscyphaceae sp. IV GK-2010	88	0.0	99	HQ157957.1	Hyaloscyphaceae	Helotiales
		P1 1.9	Calluna vulgaris root associated fungus	99	0.0	99	FM172863.1	Dermateaceae	Helotiales
		P5 1.2	Elaphocordyceps sp. 20124256c	98	0.0	95	KC237381.1	Ophiocordycipitaceae	Hypocreales
		P3 1.6	Hypoxylon sp. ARIZ NC1404	99	0.0	94	KU684005.1	Xylariaceae	Xylariales
		P3 1.7	Hypoxylon sp. ARIZ NC1404	99	0.0	94	KU684005.1	Xylariaceae	Xylariales
		P4 1.14	Scleropezicula sp. IP-2014 strain CL337	96	2E-63	100	KM216323.1	Dermateaceae	Helotiales
		P2 1.5	Fungal sp. 1 RJ-2015 isolate 83Jc14	35	8E-84	91	KU516620.1	Rutstroemiaceae	Helotiales
		P1 4.12	Acremoniopsis suttonii FMR 11780	100	0.0	98	NR_145059.1	Incertae sedis	Hypocreales
		P1 1.11	Acremoniopsis suttonii FMR 11780	99	0.0	95	NR_145059.1	Incertae sedis	Hypocreales
		P1 1.12	Tolypocladium RG-2013d strain MX338	99	0.0	99	KF747259.1	Ophiocordycipitaceae	Hypocreales
		P2 1.6	Fungal endophyte isolate 5793	99	0.0	99	KR016096.1	Incertae sedis	Pleosporales
		P2 1.8	Xylariaceae sp. 4Y-Dr2-3	97	0.0	87	AB741597.1	Xylariaceae	Xylariales
		P2 1.7	Polyporales sp. 4 SR-2012 strain 104	99	0.0	99	JQ312162.1	Meripilaceae	Polyporales

Table 9 BLAST search results for ITS regions from *Rhizoctonia*-like fungi isolated from *B. minutissimum* at a private property in Yalangur, south-east Queensland. Grey shading of fungal isolates indicates cultures used as inocula in seed germination experiments.

Orchid host	Site	Fungal isolate	Closest BLAST match	Query cover (%)	E value	Identity (%)	Genbank accession	Family of closest BLAST match/es	Order of closest BLAST match/es
B. minutissimum	**Yalangur**	P2 1.13	Fusarium sp. 5 SO-2015	100	1E-158	97	KJ817322.1	Nectriaceae	Hypocreales
		P2 1.7	Fusarium sp. 5 SO-2015	100	8E-105	91	KJ817322.1	Nectriaceae	Hypocreales
		P3 1.2	Fusarium sp. 6	100	5.00E-90	99	KP195156.1	Nectriaceae	Hypocreales
		P2 1.6	Fusarium sp. 5 SO-2015	100	4.00E-174	98	KJ817322.1	Nectriaceae	Hypocreales
		P2 1.10	Fusarium sp. 5 SO-2015	98	0.0	98	KJ817322.1	Nectriaceae	Hypocreales
		P2 1.2	Aschersonia sp. Ag-11	100	0.0	97	AY225332.1	Clavicipitaceae	Hypocreales
		P2 1.14	Aschersonia sp. Ag-11	100	0.0	98	AY225332.1	Clavicipitaceae	Hypocreales
		P3 1.12	Phoma sp. JF-2013 isolate NLF02	100	0.0	99	KC005682.1	Didymellaceae	Pleosporales
		P3 1.9	Fusarium sp. 5 SO-2015	99	0.0	97	KJ817322.1	Nectriaceae	Hypocreales
		P1 1.2	Fusarium tricinctum strain XS61m1	99	0.0	99	KJ188669.1	Nectriaceae	Hypocreales
		P2 1.8	Fusarium lateritium strain BBA 63665	100	0.0	97	AF310982.1	Nectriaceae	Hypocreales

Table 10 BLAST search results for ITS regions from *Rhizoctonia*-like fungi isolated from *B. shepherdii* at a private property in Stanthorpe, south-east Queensland. Yellow=orchid mycorrhizal fungi. 'Not listed' denotes GenBank accessions for which no identifying family or order were provided.

Orchid host	Site	Fungal isolate	Closest BLAST match	Query cover (%)	E value	Identity (%)	Genbank accession	Family of closest BLAST match/es	Order of closest BLAST match/es
B. shepherdii	**Stanthorpe**	P3 1.6	Ceratobasidium sp. MB-2014a	95.0	0	99	KP056301.1	Ceratobasidiaceae	Cantharellales
		P2 1.11	Fungal endophyte isolate SNP291	95.0	0	95	KP335478.1	*not listed*	*not listed*
		P3 1.7	Fungal sp. voucher ARIZ:PS0310	98	0	99	KU977719.1	*not listed*	*not listed*
		P3 1.8	Virgaria nigra	95	0	99	AB670713.1	Xylariaceae	Xylariales

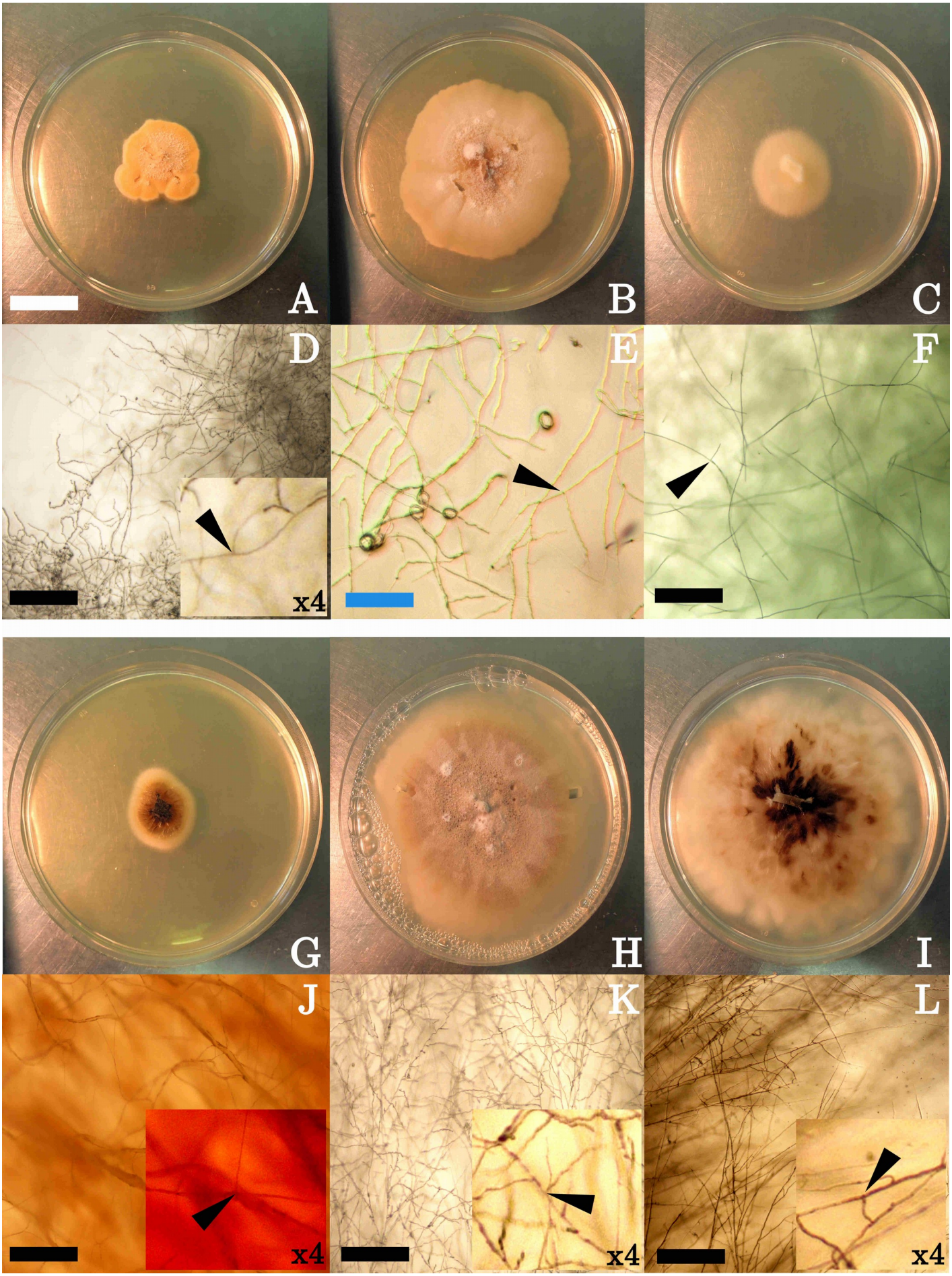
A
B
C
D
x4
E
F
G
H
I
J
x4
K
x4
L
x4

Figure 13 (previous page) Macro- (A-C, G-I) and microscopy (D-F, J-L) of selected putatively mycorrhizal fungal partners of *Bulbophyllum* sp. orchid roots. Right-angled hyphal branches are marked with arrowheads. (A & D) *Serendipita* sp. (isolate BEDA P5 2.2) from *B. exiguum* in D'Aguilar NP. Plate is 10 weeks post-sub-culture. (B & E) *Serendipita* sp. (isolate BLGW P1 2.9) from *B. elisae* in Girraween NP. Plate is 5 weeks post-sub-culture. (C & F) *Tulasnella* sp. (isolate BEDA P1 2.2) from *B. exiguum* in D'Aguilar NP. Plate is 10 weeks post-sub-culture. (G & J) *Tulasnella* sp. (isolate BBMR P4 4.4) from *B. bracteatum* in Main Range NP. Plate is 2 weeks post-sub-culture. (H & K) *Tulasnella* sp. (isolate BBMR P5 2.11) from *B. bracteatum* in Main Range NP. Plate is 10 weeks post-sub-culture. (I & L) Helotiales sp. (isolate BEMR P5 2.7) from *B. exiguum* from Main Range NP. Plate is 12 weeks post-sub-culture. White bar is 1.5cm, blue is 250µm, black are 450µm. Insets are x4 zoom.

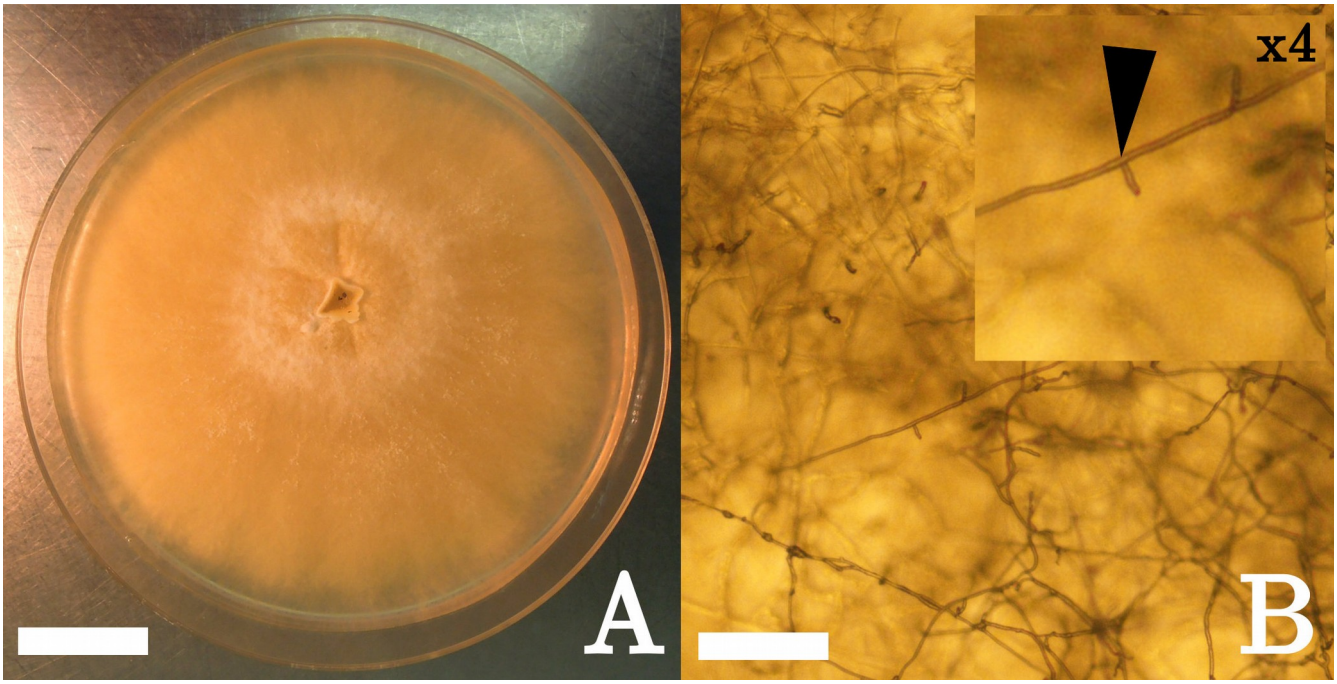

Figure 14 Macro- (A) and microscopy (B) of *Ceratobasidium* sp. (isolate BSST P3 1.6) obtained from *B. shepherdii* at Stanthorpe. Plate is 6 weeks post-sub-culture. Right-angled hyphal branching is marked with an arrowhead. Inset is x4 zoom. Bar in (A) is 1.5cm; bar in (B) is 450µm.

3.1.5 Phylogenetic analysis of fungal isolates

3.1.5.1 Phylogeny of *Serendipita* isolates

Three ITS sequences from fungal isolates that showed highest identity to sebacinoid sequences in the GenBank archive were included in a phylogenetic analysis of the Sebacinales to determine their phylogenetic relationships (Figure 15). All 3 isolates clustered together in one of two distinct Serendipitaceae clades (designated here as Clade 1), along with 1 OMF sequence from Réunion Island and 1 ErM *Serendipita* spp. sequence from Sweden. Isolates BEDA P1 0.1 and BEDA P1 3.1 were most closely related, with isolate BEDA P5 1.1 positioned on a separate branch of the same clade. Within-clade bootstrap support for isolates BEDA P5 1.1, BEDA P1 0.1 and BEDA P1 3.1 were 45%, 94% and 89%, respectively. Between-clade bootstrap support values throughout the tree ranged from 2% to 99%, with an average value of 80.6% based on bootstrap figures at the nodes from which the clades Sebacinaceae, *Helvellosebacina*, and Serendipitaceae 1 and 2 diverged. Given that joint confidence (overall confidence in the combined bootstrap values of all nodes) in large trees is inescapably low (Soltis & Soltis 2003), an average of >70% may be considered adequate for the analyses performed here.

Visual inspection of the alignment (e.g. Figure 17) confirmed 1 nucleotide substitution between isolates BEDA P5 1.1 and BEDA P1 0.1, 2 nucleotide substitutions between isolates BEDA P1 3.1 and BEDA P1 0.1, and 3 nucleotide substitutions between isolates BEDA P5 1.1 and BEDA P1 0.1. In the 220 bp alignment, a single substitution represented 99.45% identity, 2 substitutions represented 99%, and 3 substitutions represented 98.64%, all of which fell within the commonly-used 3% fungal species threshold (Nilsson et al. 2008). By this measure, all 3 isolates appeared to be individuals of the same species.

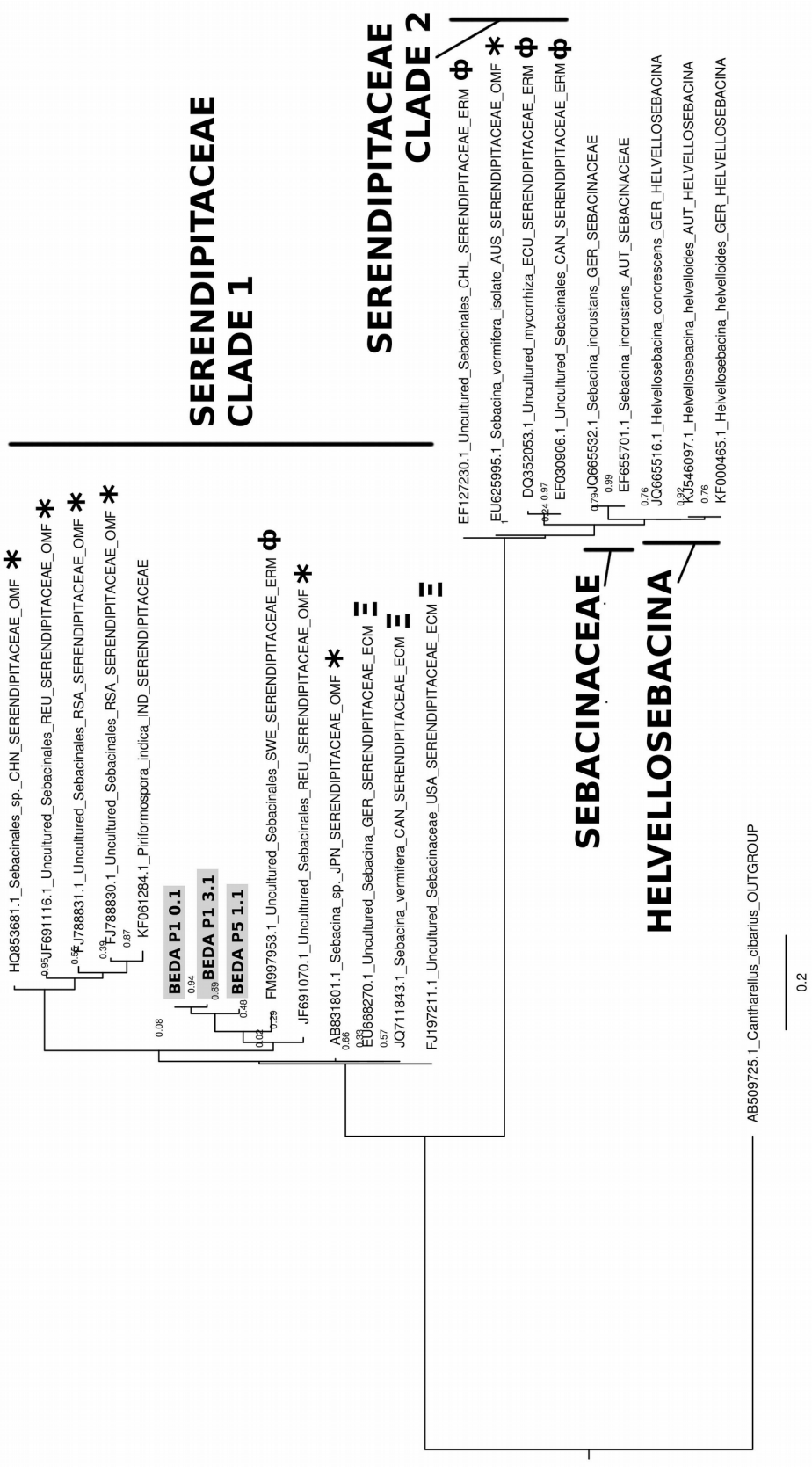

Figure 15 Phylogeny of *Serendipita* spp. isolates (shaded in grey). *=orchid mycorrhizal sequence; ϕ=ericoid mycorrhizal sequence; Ξ=ectomycorrhizal sequence. Tree is a Tamura-Nei model Maximum-Likelihood analysis based on a ClustalW alignment with 1000 bootstrapped replicates. Country codes follow isolate name: CHN=China; REU=Réunion Island; RSA=South Africa; IND=India; SWE=Sweden; JPN=Japan; GER=Germany; CAN=Canada; USA=America; CHL=Chile; AUS=Australia; ECU=Ecuador; AUT=Austria; GUY=Guyana; NOR=Norway; FIN=Finland; KOR=Korea; MDG=Madagascar. Nodes

within tree represent putative common ancestors. Scale bar represents average number of nucleotide substitutions per site (number of substitutions divided by length (bp) of sequence).

3.1.5.2 Phylogeny of *Tulasnella* isolates

Six ITS sequences from fungal isolates that showed highest identity to tulasnelloid sequences in the GenBank archive were included in a phylogenetic analysis of the Tulasnellaceae to determine their phylogenetic relationships (Figure 16). All 6 isolates clustered together in a distinct clade with sequences from 2 uncultured *Tulasnella* spp. from Tulasnellaceae Clade E. Resolution of representative sequences from other clades was high, with all backbone sequences from each clade clustering together. Within-clade bootstrap support for the sequences obtained in this study was low (1%, 3%, 3%, 8%, 45% and 45% for isolates BBMR P5 1.11, BBMR P4 1.4, BEDA P1 1.1, BLGW P3 2.8, BEDA P1 1.2 and BEDA P1 1.2a, respectively), indicating a high degree of uncertainty in the within-clade placement of these sequences relative to each other. Between-clade bootstrap support values throughout the tree ranged from 1% to 100%, with an average value of 74.8% based on bootstrap figures at the nodes from which the major clades diverged.

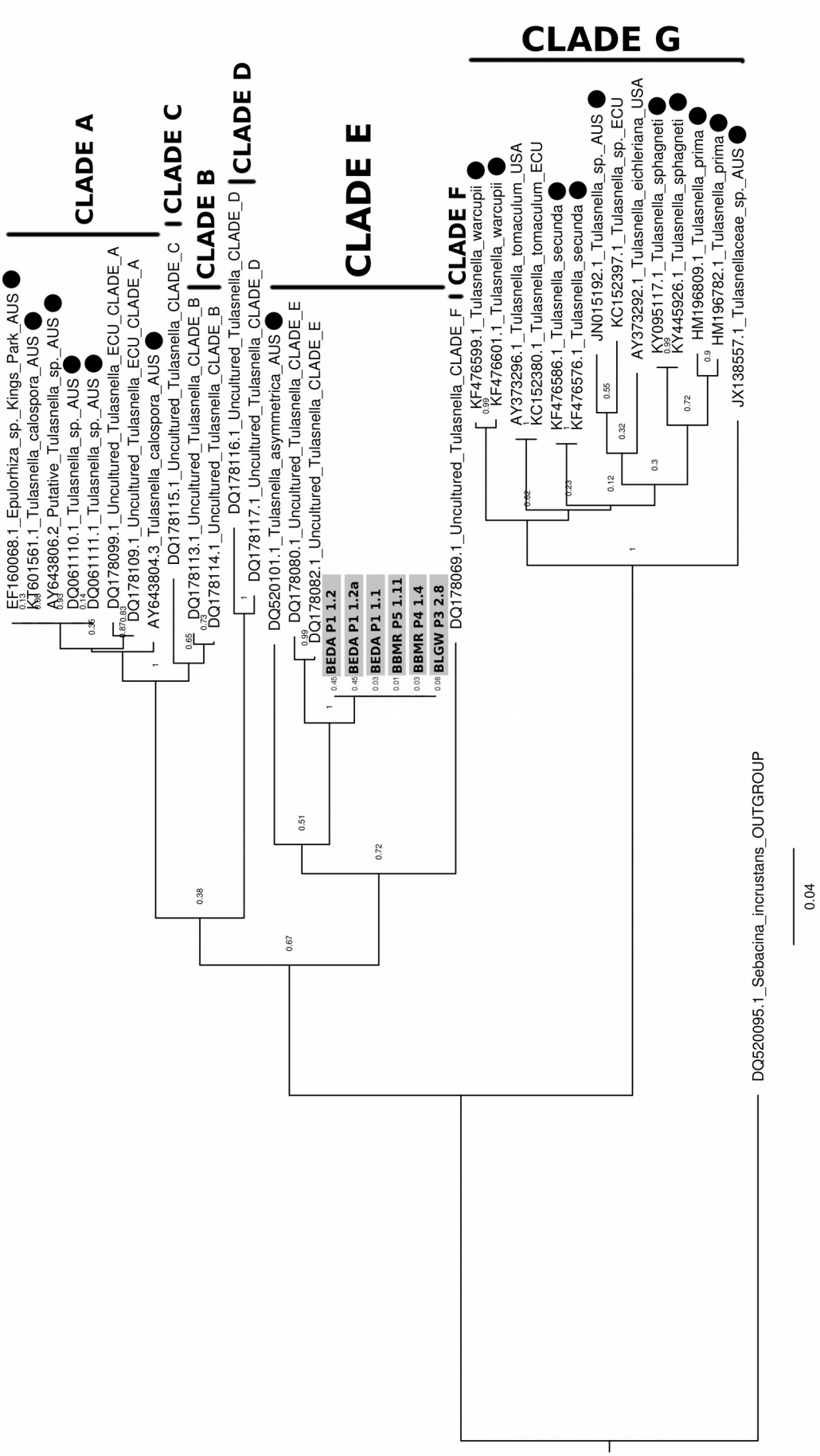

Figure 16 Phylogeny of *Tulasnella* spp. isolates (shaded in grey). Tree is a Tamura-Nei model Neighbour-Joining analysis with 1000 bootstrapped replicates and based on a ClustalW alignment. Country codes follow isolate names and are outlined in Figure 15. Other Australian sequences are marked with a ●. Scale bar represents average number of nucleotide substitutions per site.

Given the lack of branch separation between the 6 isolates, which indicates very similar or identical sequences, the alignment was visually inspected. This confirmed that sequences were all identical with the exception of a single-nucleotide substitution in 2 isolates (Figure 17). BEDA P1 1.1 had a substitution of thymine and BLGW P3 2.8 a substitution of adenine at a single site occupied in all other isolates by a guanine residue. In the 414 bp alignment, a single substitution represented 99.2% identity, well inside the 3% species threshold. This suggested that these 6 isolates, from 3 orchid species over 3 sites, were the same species of *Tulasnella*.

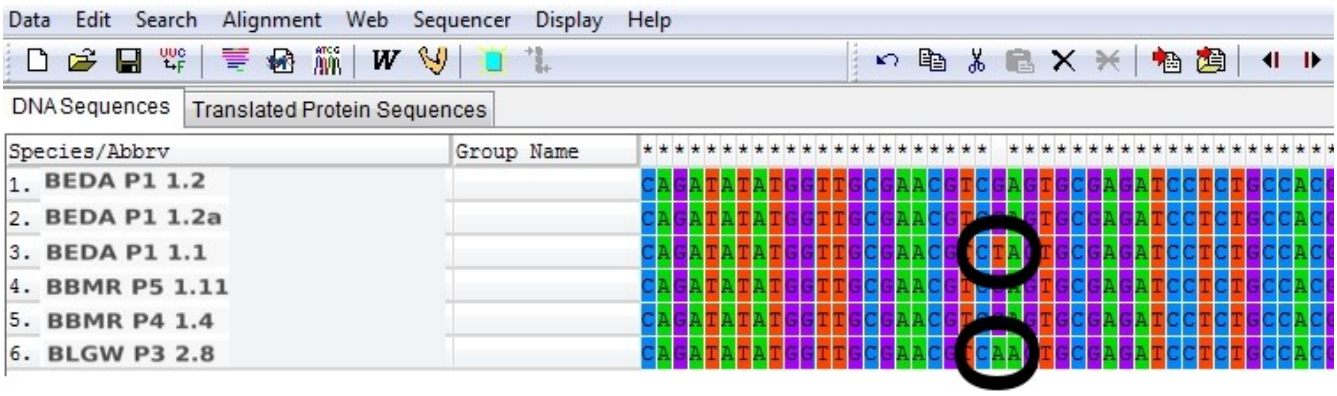

Figure 17 Section of MEGA alignment of 6 *Tulasnella* sp. isolates obtained in this study. Black circles indicate the single locus at which BEDA P1 1.1 had a substitution of thymine and BLGW P3 2.8 a substitution of adenine. All other loci in the alignment were identical.

3.1.5.3 Phylogeny of *Ceratobasidium* isolate

The ITS sequence from a single fungal isolate that showed highest identity to ceratobasidioid sequences in the GenBank archive was included in a phylogenetic analysis of the broader Cantharellales to determine its phylogenetic relationships (Figure 18). The isolate, BSST P3 1.6, clustered with Ceratobasidiaceae sequences from Australia, Norway, Finland, China and the USA with high (88%) bootstrap support. The sub-clade (A) into which the isolate fell was shared solely with OMF sequences. Within-clade bootstrap support in the Ceratobasidiaceae ranged from 40% to 97% and averaged 70.5%, indicating a moderate degree of certainty for the structure of the clade. Between-clade bootstrap support values throughout the tree ranged from 33% to 100%, with an average value of 83% based on bootstrap figures at the nodes from

which the major clades diverged. BSST P3 1.6, isolated from *B. shepherdii* in Stanthorpe, had 99% identity to a fungal *Ceratobasidium* sp. sequence obtained by Liebel, Bidartondo & Gebauer (2014) from roots of the terrestrial orchid *Goodyera repens* in Norway. This >97% identity suggests the two sequences may be from the same species of OMF.

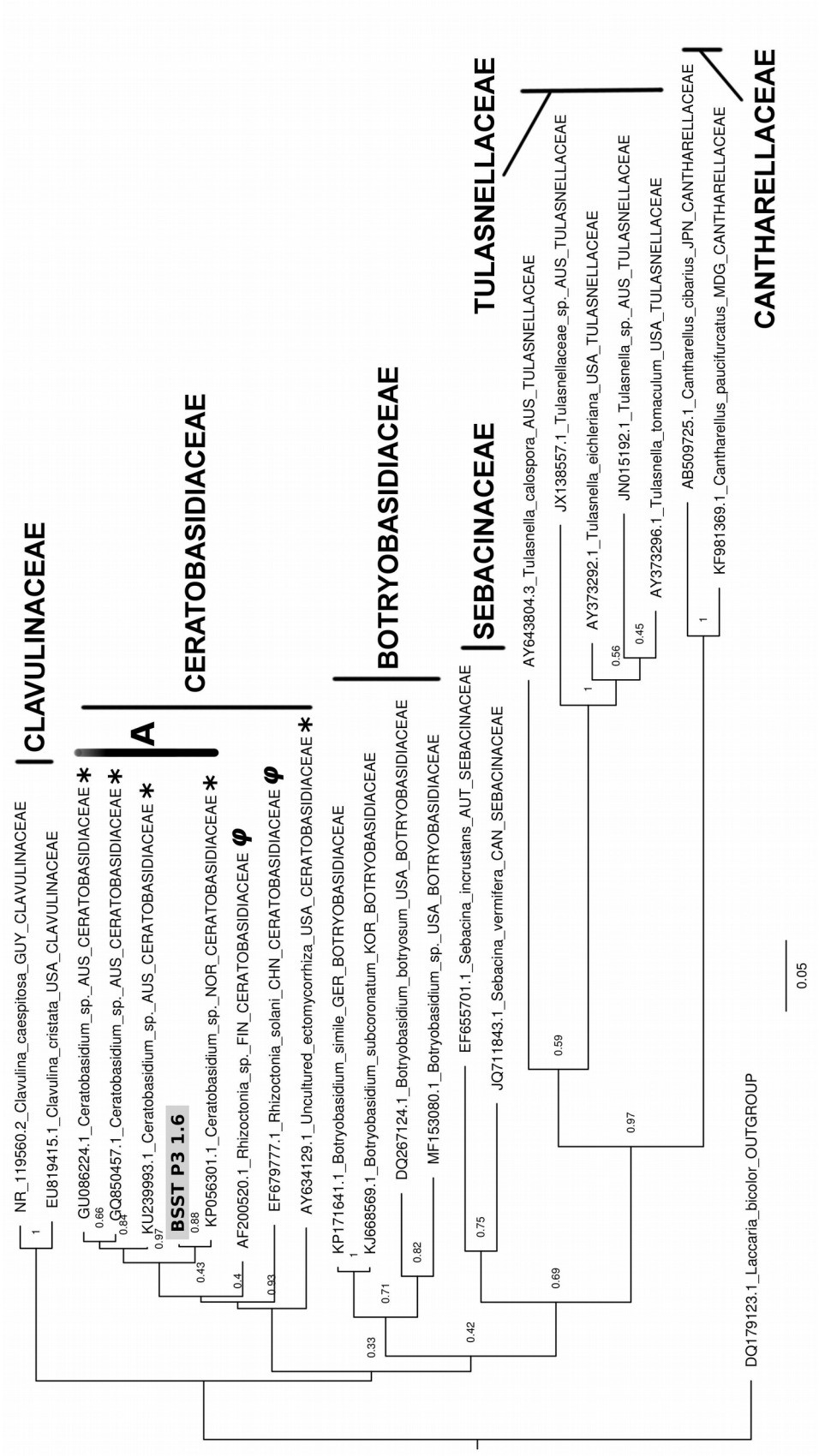

Figure 18 Phylogeny of *Ceratobasidium* sp. isolate (shaded in grey) from the relocated *B. shepherdii* growing in Stanthorpe. Tree is a Tamura-Nei model Maximum-Parsimony analysis with 1000 bootstrapped replicates and based on a ClustalW alignment. Country codes follow isolate names and are outlined in Figure 15. OMF sequences in the Ceratobasidiaceae are marked with a *****; pathogenic Ceratobasidiaceae are marked with a **φ**. The clade marked **A** identifies the group of OMF with which the isolate's sequence clustered. Scale bar represents average number of nucleotide substitutions per site.

3.1.5.4 Phylogeny of Helotiales isolates

Nine ITS sequences from fungal isolates that showed highest identity to Helotiales sequences in the GenBank archive were included in a phylogenetic analysis of the Helotiales to determine their phylogenetic relationships (Figure 19). Three of these sequences had highest identity to orchid-associated Helotiales in the GenBank archive, and 6 had highest identity to ericoid mycorrhizal Helotiales sequences. Cladistic resolution as outlined by Wang et al. (2006) showed a moderate degree of consistency, with 6 of 9 representative pairs from 9 Helotiales clades clustering together in the final tree. The 9 isolates obtained in this study did not cluster together but fell into 2 main clades, designated here as clades A & B. Between-clade bootstrap support values throughout the tree ranged from 1% to 100% with an average value of 72%.

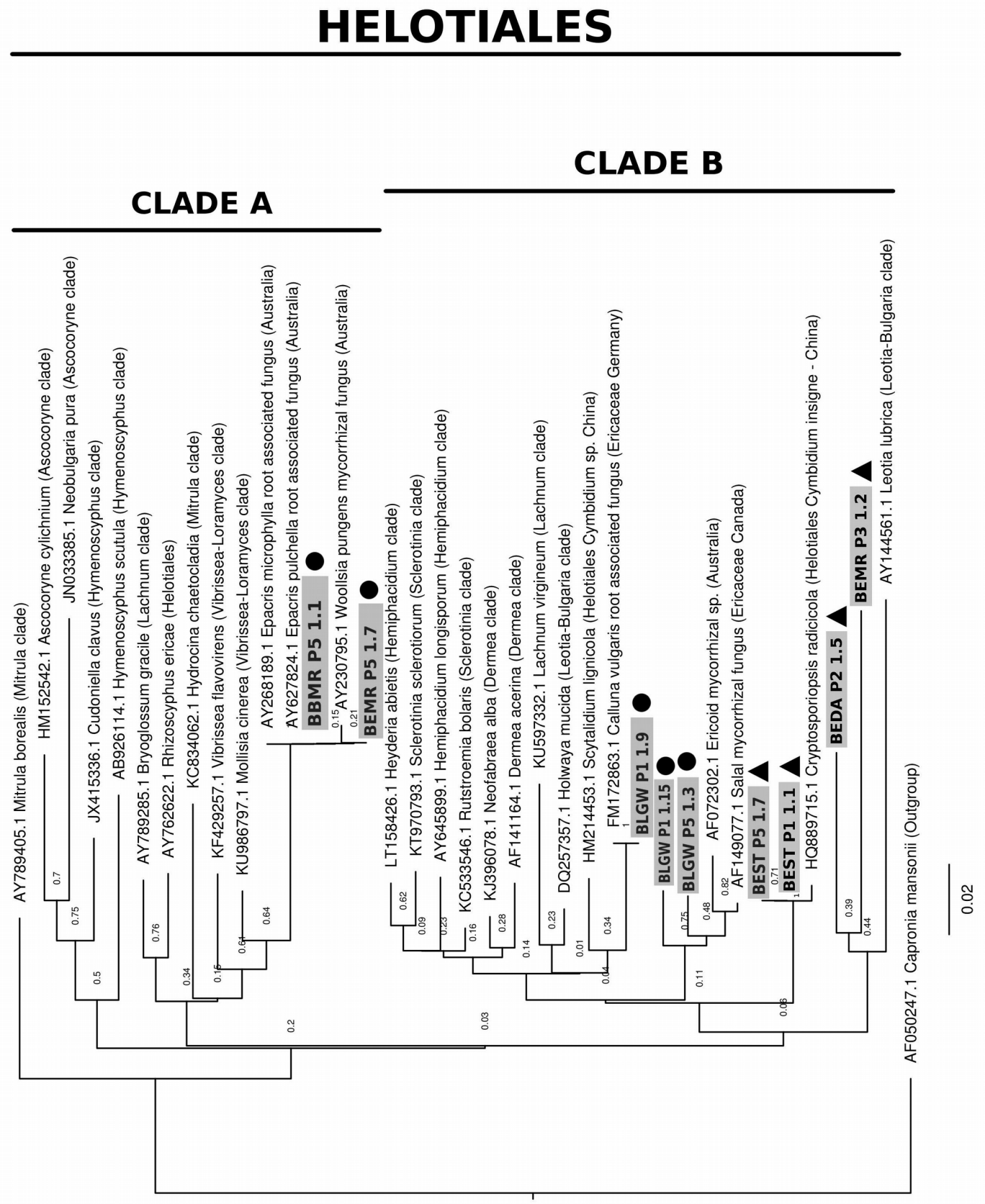

Figure 19 Bayesian phylogeny of Helotiales spp. isolates (shaded in grey) from *B. exiguum, B. elisae* and *B. bracteatum*. Clades A & B were designated based on the output of this analysis. Helotiales clades outlined by Wang et al. (2006) are marked after GenBank sequence names. Isolate sequences that were closest BLAST matches to orchid-associated Helotiales are marked with a ▲; those closest to Ericaceae-associated sequences are marked with a ●. Scale bar represents average number of nucleotide substitutions per site.

The alignment was visually inspected to establish whether any isolates that clustered together represented individuals of the same species. A single nucleotide deletion in isolate BEMR P5 1.7 compared to BBMR P5 1.1 in the 361 bp alignment represented 99.3% identity, suggesting that these isolates, which are from two orchid spp. at the same site (Main Range NP), are the same species (<3% difference). In the tree the two isolates fell into a clade with mycobionts of the Australian ericoid mycorrhizal plants *Woolsia pungens, Epacris pulchella* and *E. microphylla.* Comparison of the 5 sequences using the online sequence comparison tool GEvo (https://genomevolution.org/coge/Gevo.pl; Lyons & Freeling 2008) revealed an average of 98.9% sequence identity, with lowest identity of 97.5%. This indicates that the Helotiales sp. isolated from *B. exiguum* and *B. bracteatum* is likely the same species as those found associating with three species of ericaceous plant.

Of the isolates from *B. elisae* in Girraween NP, BLGW P1 1.15 had 94% identity with its highest match to the database and, based on this >3% difference, is likely a new helotialean species. BLGW P5 1.3 had 99% identity to a fungus in the family Hyaloscyphaceae (order Helotiales) isolated from roots of white spruce (*Picea glauca*) in Canada, and is therefore likely to be the same species as this Canadian fungus. Isolate BLGW P1 1.9 had 99% identity with an unnamed sequence obtained in Germany from the roots of the ericaceous shrub *Calluna vulgaris.*

The isolates BEST P5 1.7 and BEST P1 1.1 had 100% identity, indicating that these isolates are the same fungal species. BLAST searches using the shared sequence from these isolates returned 99% identity to *Cryptosporiopsis radicicola* (order Helotiales) isolated from the terrestrial orchid *Cymbidium insigne* in China.

Isolates BEDA P2 1.5 and BEMR P3 1.2, which had 99% and 100% identity to two orchid-associated GenBank sequences, clustered with an outlying Helotiales sequence, *Leotia lubrica.*

3.1.5.5 Fungal phylogeny summary: taxonomic assignments

Based on combined BLAST results and phylogenetic analyses, isolates were tentatively assigned to an order, family, genus or species (Table 11). Such assignments will need to be verified through morphological assessments of teleomorphic states if they are successfully induced (see Section 1.1) or analysis using other DNA regions such as the protein-coding RNA polymerase II subunit A gene (Schoch et al. 2012).

Table 11 Summary of taxonomic assignments for the putatively orchid mycorrhizal *Serendipita, Tulasnella, Ceratobasidium* and Helotiales isolates identified in this study. Shading in right-hand column indicates isolates likely to be of the same species. * indicates isolates representing species likely to be new to science.

Fungal isolate	*Genus species*/(Family)/**Order**	
BEDA P5 1.1	*Serendipita* *	
BEDA P1 0.1	*Serendipita* *	
BEDA P1 3.1	*Serendipita* *	
BEDA P1 1.2	*Tulasnella* *	
BEDA P1 1.2a	*Tulasnella* *	
BEDA P1 1.1	*Tulasnella* *	
BBMR P5 1.11	*Tulasnella* *	
BBMR P4 1.4	*Tulasnella* *	
BLGW P3 2.8	*Tulasnella* *	
BSST P3 1.6	*Ceratobasidium*	
BEDA P2 1.5	**Helotiales**	
BEMR P3 1.2	**Helotiales**	
BLGW P5 1.3	**Helotiales** (Hyaloscyphaceae)	
BLGW P1 1.15	**Helotiales** *	
BLGW P1 1.9	**Helotiales** (Dermateaceae)	
BEST P1 1.1	*Cryptosporiopsis radicicola*	
BEST P5 1.7	*Cryptosporiopsis radicicola*	
BEMR P5 1.7	**Helotiales**	
BBMR P5 1.1	**Helotiales**	

3.2 Phylogenetic analysis of *Bulbophyllum* orchids

3.2.1 Orchid PCR and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) large subunit (rbcL) gene sequencing

Successful PCR amplification of rbcL gene regions from extracted DNA of *B. exiguum*, *B. bracteatum, B. elisae, B. minutissimum* and *B. shepherdii* using the primers rbcL-1F and

rbcL-1360R was carried out. PCR products were approximately 1200 bp in length. A check of GenBank using the search query "(rbcl[Gene Name]) AND (orchid OR orchidaceae)" confirmed that 1200 bp is the approximate length of the rbcL gene in orchids. Amplified DNA concentrations were between 30ng/10µL and 150ng/10µL.

DNA electropherograms returned from sequencing at the AGRF (Figure 20) were of high quality with minimal background noise, indicating that each base had been identified with a high degree of certainty.

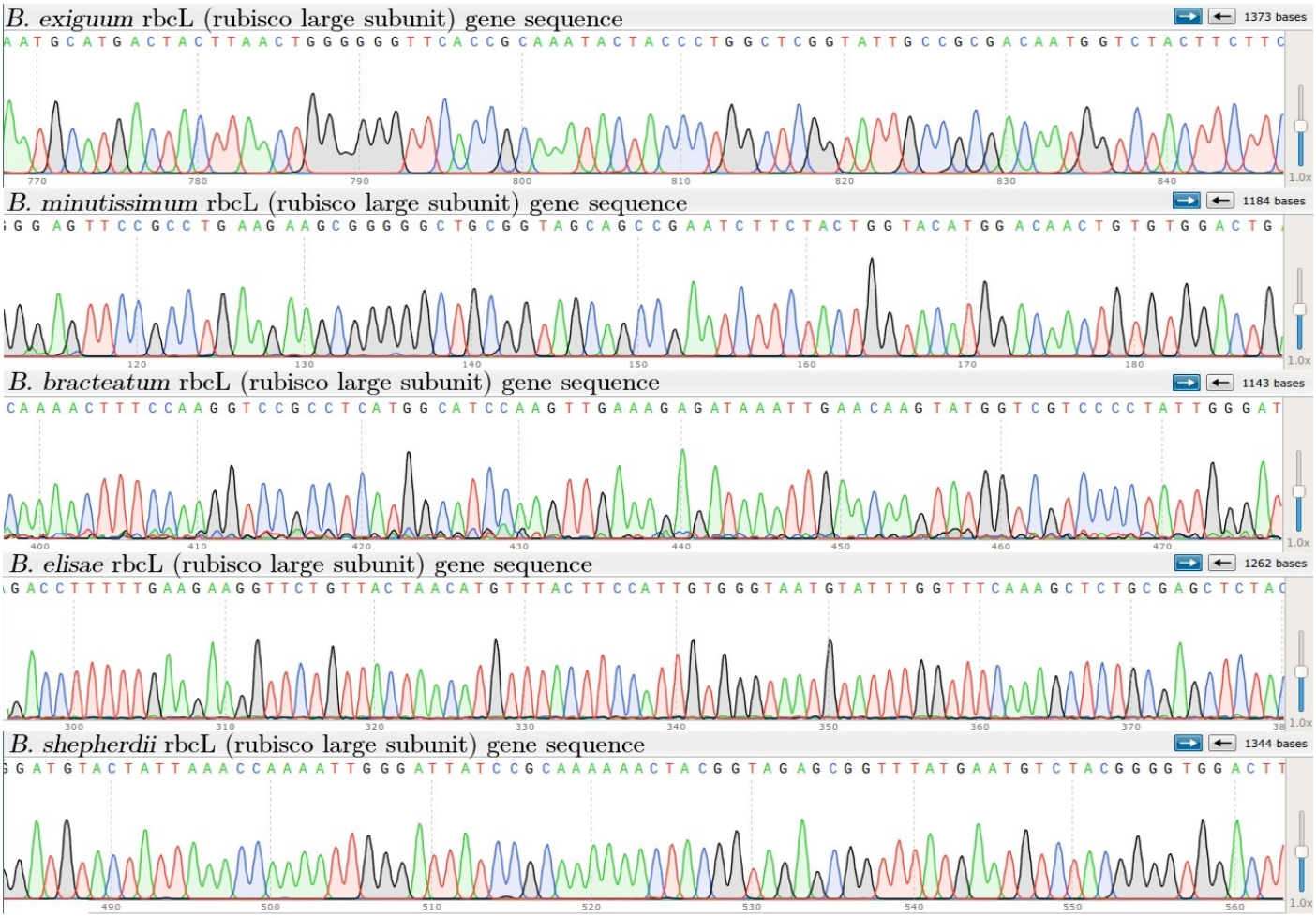

Figure 20 Orchid DNA electropherograms returned by Sanger sequencing reactions at the AGRF. All exhibit clearly-defined peaks, indicating that the sequences are of a quality suitable for phylogenetic analysis. (Screenshots from SnapGene Viewer 4.0.2.)

3.2.2 Phylogenetic analysis

DNA sequences coding for the rbcL gene from 5 *Bulbophyllum* sp. were included in a phylogenetic analysis of the orchid subfamily Epidendroideae to determine their phylogenetic

relationships (Figure 21). In all phylogenetic methods tested (Maximum-Parsimony, Maximum-Likelihood and Neighbour-Joining), bootstrap values were very low, indicating a high degree of uncertainty in the placement of clades (see Appendix B for the initial low-resolution Maximum-Parsimony tree). However, there was consistency in all 3 methods in the placement of the 5 *Bulbophyllum* sp. Four sequences, *B. exiguum*, *B. bracteatum, B. elisae,* and *B. minutissimum*, clustered together in one of two *Bulbophyllum* clades (designated here as Clade B) with *B. pygmaeum* and *B. tuberculatum* (syn. *Adelopetalum tuberculatum*) sequences from New Zealand. The other sequence, from *B. shepherdii*, fell into Clade A with the majority of other backbone sequences, which were from *Bulbophyllum* in Thailand, the Himalayas, Myanmar, Laos, Vietnam, Cambodia, India, Bangladesh, Indonesia, Papua New Guinea and the Comoros Islands near Madagascar. Between-clade bootstrap support values throughout the tree were very low, at 1% to 3%.

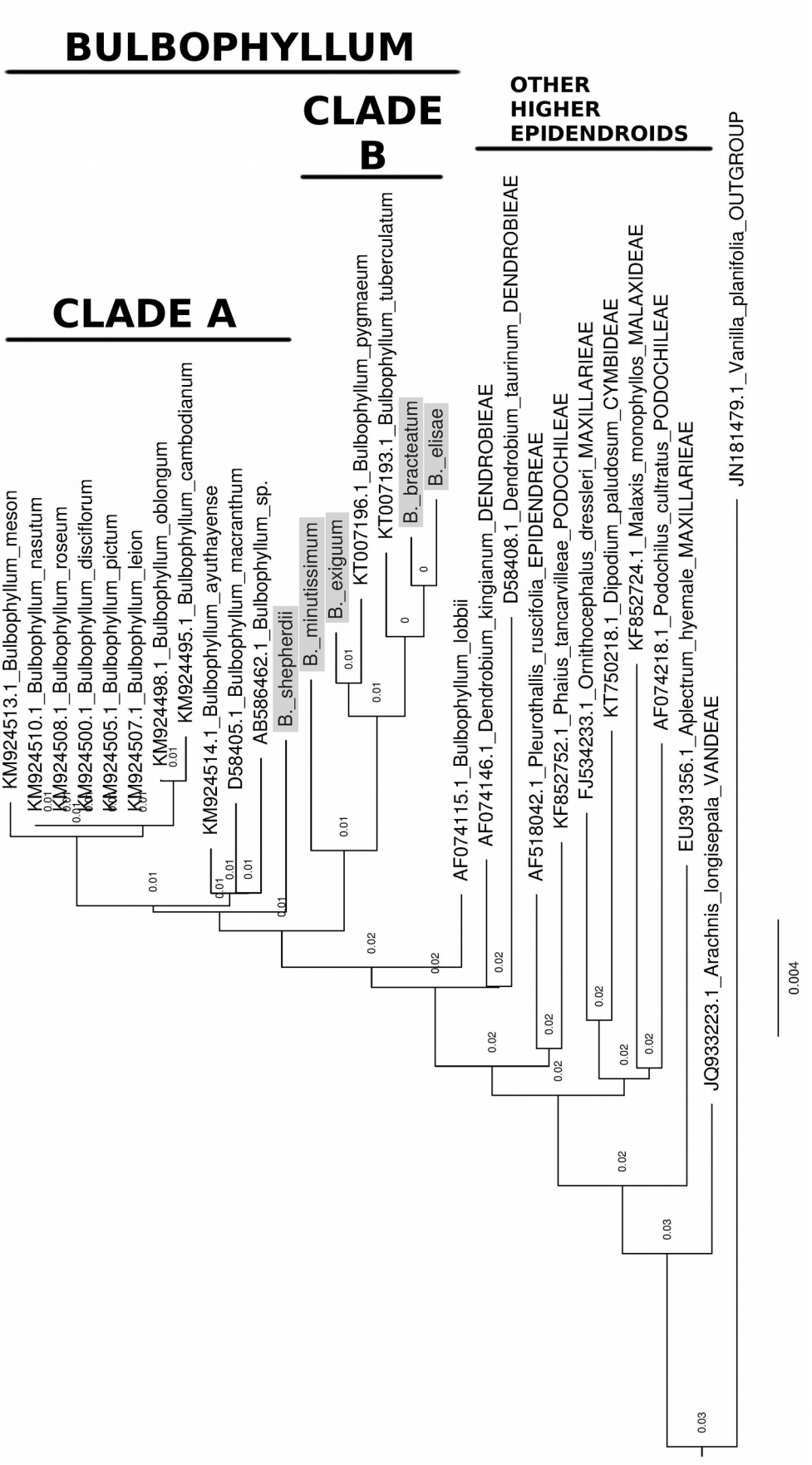

Figure 21 Phylogeny of 5 *Bulbophyllum* spp. rbcL genes (shaded in grey). Tree is a Tamura-Nei model Neighbour-Joining analysis with 1000 bootstrapped replicates and based on a ClustalW alignment. *Bulbophyllum* clades A and B were determined based on the output of this analysis. Orchid tribes within the 'other higher epidendroids' clade are listed in capitals after sequence names. Scale bar represents average number of nucleotide substitutions per site.

A smaller phylogenetic tree was constructed (Figure 22) to check the accuracy of Jones' (2006) morphology-based reassignment of *Bulbophyllum* spp. to new genera. Although clades were not well-resolved, two basic groupings appeared to confirm Jones' proposals. *B. exiguum, B. bracteatum* and *B. elisae* formed a clade with a sequence from *B. tuberculatum*, all of which have been proposed to be moved to the new genus *Adelopetalum. B. shepherdii* and *B. minutissimum* formed a clade with a sequence from *B. gadgarrense*, which has been proposed, along with *B. shepherdii,* to belong the genus *Oxysepala.* As no *Oncophyllum* spp. rbcL sequences were available to include in the analysis, the accuracy of Jones' proposal to move *B. minutissimum* to *Oncophyllum* could not be checked. Inclusion of the *B. gadgarrense* sequence reduced the alignment length from 700 to 227 bp, reducing the number of available loci for algorithmic comparison by 473 bp.

Between-clade bootstrap support values throughout the tree ranged from 0% to 51%, with an average value of 37%.

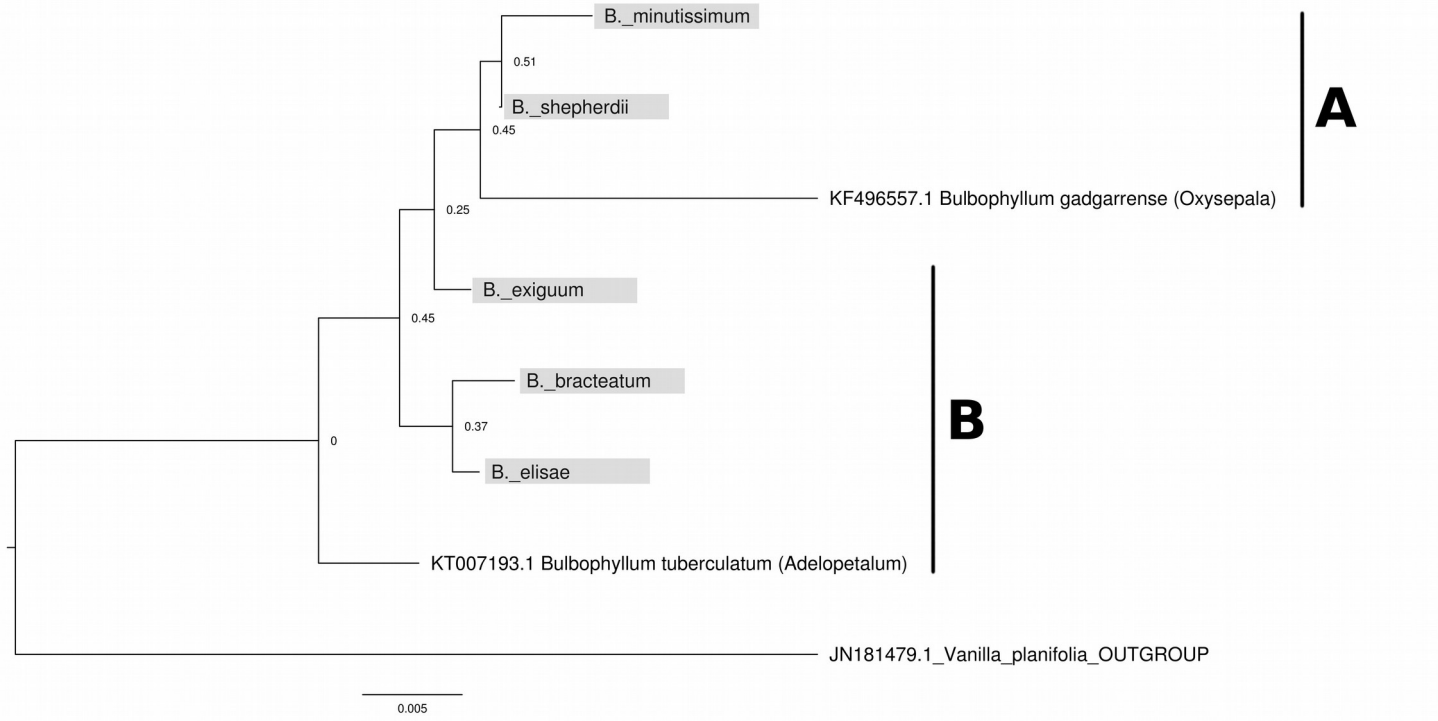

Figure 22 Reduced phylogeny of 5 *Bulbophyllum* spp. (shaded in grey). Tree is a Tamura-Nei model Neighbour-Joining analysis with 1000 bootstrapped replicates and based on a ClustalW alignment. Clade A designates sequences clustering with *B. gadgarrense* (proposed new genus: *Oxysepala*); clade B designates sequences clustering with *B. tuberculatum* (proposed new genus: *Adelopetalum*). Scale bar represents average number of nucleotide substitutions per site.

3.3 *Bulbophyllum exiguum* mycorrhizal seed germination

3.3.1 Collection and desiccation of seed pods

A total of 20 *B. exiguum* seed pods collected from Main Range NP & Mount Tully were desiccated at room temperature, using a silica gel desiccator, until they began to dehisce (Figure 23A & B). Seeds were visible but did not disassociate from pod tissue. Some pods contained no visible seeds at all and may have been immature. Total isolation of seeds proved challenging due to the small size of pods, and as a result some pod tissue remained on filter paper when it was laid over oatmeal agar plates.

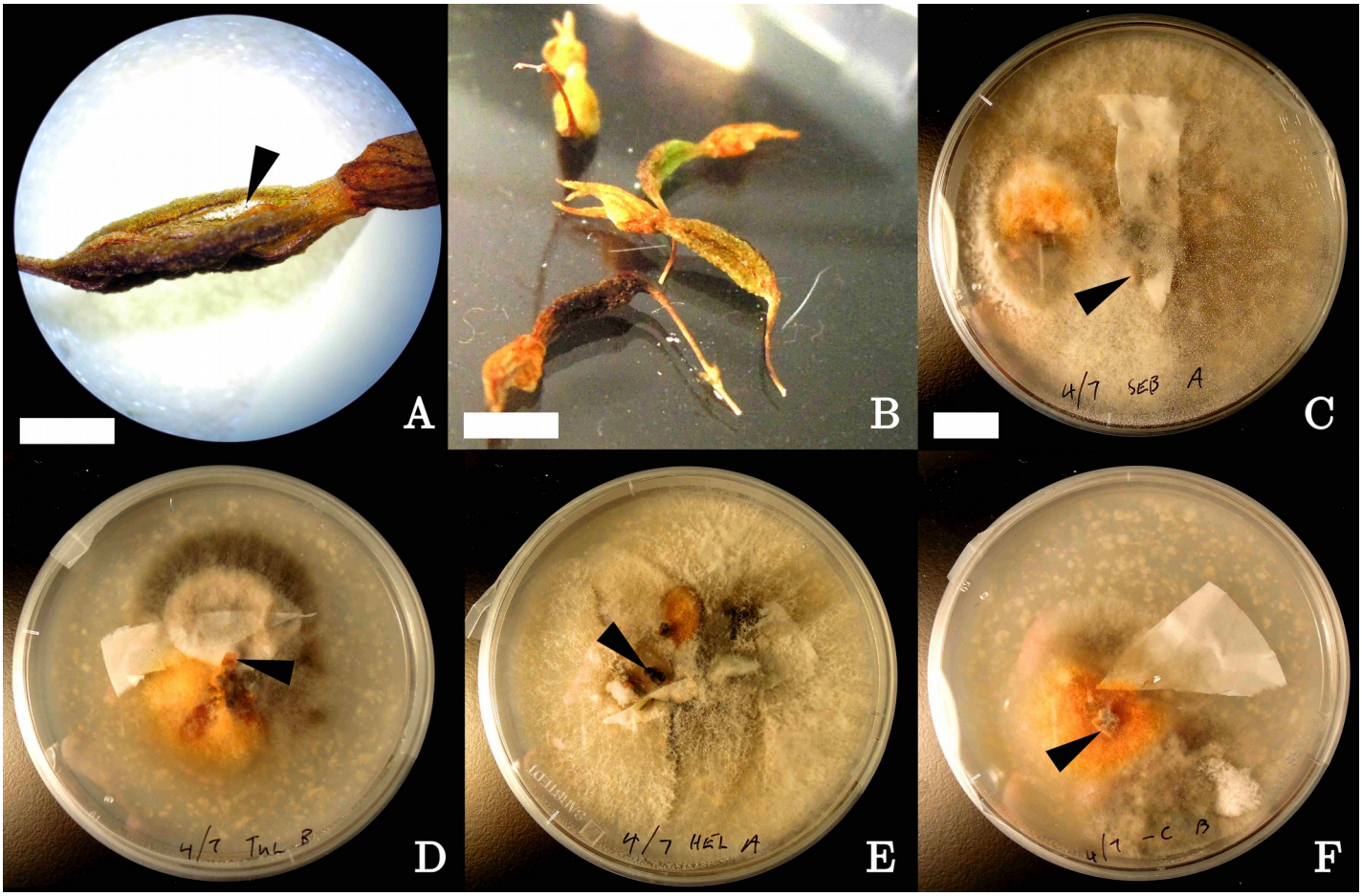

Figure 23 *B. exiguum* seed germination experiment. (A) & (B) Desiccated seed pods that have begun to dehisce. Exposed seeds in (A) are indicated with an arrowhead. (C)-(F) Oatmeal agar plates set up with seeds on filter paper and inoculated with a fungal mycelial plug (indicated with an arrowhead) as per Figure 6. (C) Plate inoculated with *Serendipita/Sebacina* isolate BEDA P5 1.1. (D) Plate inoculated with *Tulasnella* isolate BEDA P1 1.2. (E) Plate inoculated with Helotiales isolate BEMR P5 1.7. (F) Negative control plate with seeds but no inoculum. Plate inoculated with *Phoma* sp. isolate BMYA P3 1.12 is not shown. Scale bars: (A) 2mm, (B) 10mm, (C) 15mm.

3.3.2 Mycorrhizal seed germination experiments

All 15 plates (4 treatments, 1 negative control, 3 replicates) exhibited rapid growth of non-inoculum fungal contaminants (Figure 23C-F), which spread across all plates within 72 hours. These fungi out-competed the slow-growing inocula, preventing their hyphae from reaching any *B. exiguum* seeds. All visible seeds remained at developmental stage 0. No seeds germinated in response to fungal contaminants, which originated from seed pod tissue fragments. As all seed and tissue was surface sterilised, these contaminants were likely to be intra- or extracellular endophytes dwelling beneath the epidermis.

To repeat and refine the experiment, ten more pods were collected from Mount Tully and desiccated as described in Section 3.3.1. This time, some seeds disassociated from pod tissue. Protocols outlined in Section 2.3.2 were followed, except that surface sterilisation time was increased to 25 minutes and all visible seed pod fragments were removed with sterile forceps prior to the application of seeds to filter paper. Again, after 72 hours contaminating fast-growing endophytes had grown across all plates. No seed germination was evident. All visible seeds remained at developmental stage 0.

Seeds from the second round of pod collection (from Mount Tully only) were visually assessed, using mature seeds from the native Australian orchid *Eriochilus cucullatus* for morphological comparison (Figure 24). *B. exiguum* seeds exhibited irregular form, poor resolution of seed coat and no evidence of a developed embryo.

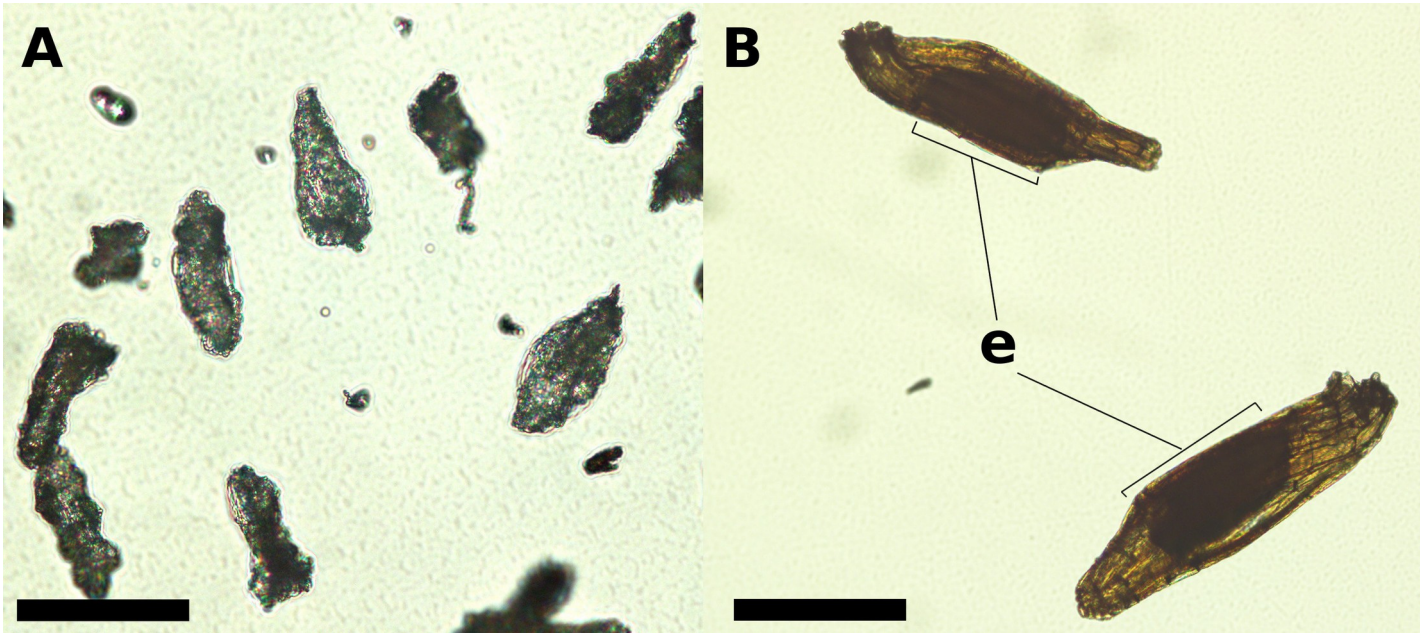

Figure 24 (A) *Bulbophyllum exiguum* seeds from pods collected for use in symbiotic seed germination experiments, and (B) mature *Eriochilus cucullatus* seeds. In contrast to *E. cucullatus* seeds, the seed coats of which had developed into a dry cellular sheath and embryos of which (e) were clearly visible, *B. exiguum* seeds exhibited irregular form, poor resolution of seed coat and no evidence of developed embryos. Scale bars: 0.5mm.

4. Discussion

4.1 Roots of SE QLD *Bulbophyllum* spp. harbour a diverse range of putatively mycorrhizal fungi from three OMF genera and one ascomycete order

This study revealed a diversity of putatively mycorrhizal fungi to be present in the roots of 5 *Bulbophyllum* orchids native to south-east Queensland. Ninety isolates from 12 fungal orders were cultured and identified based on ITS sequence homology with archived sequences. These isolates represented only a subset of the actual endophytic fungal diversity of *Bulbophyllum* spp. roots, as only mycelial colonies exhibiting *Rhizoctonia*-like hyphal morphology were selected for sub-culturing and sequencing.

The most significant finding was that a single *Tulasnella* sp. was detected in the roots of 3 orchid spp. across 3 sites, suggesting that native *Bulbophyllum* spp. show narrow specificity for tulasnelloid mycobionts, and potentially supporting the proposed taxonomic revisions for *B. exiguum, B. bracteatum* and *B. elisae* outlined in Section 1.3 (Jones 2006). *Tulasnella* spp.

have previously been isolated from Australian epiphytic (*Dendrobium:* Warcup & Talbot 1967; Warcup 1973; Warcup 1981; Boddington & Dearnaley 2008; *Sarcochilus:* Irwin & Dearnaley 2012) and terrestrial (Warcup 1990; Perkins et al. 1995; Perkins & McGee 1995; Bougoure et al. 2005) orchid roots. Additionally, fungi from the genera *Serendipita* and *Ceratobasidium* were also obtained from *Bulbophyllum* roots, which is in agreeance with the well-established concept that *Tulasnella, Serendipita* and *Ceratobasidium* are the primary mycobionts of green orchids (Smith & Read 2008). Surprisingly, a large number of isolates with high sequence identity to ericoid mycorrhizal ascomycetes in the order Helotiales were also obtained. This suggests that orchids may, like plants in the Ericaceae, enter into mycorrhizal associations with ascomycetous dark septate endophytes.

Seed germination experiments designed to establish which isolates were able to trigger orchid seed germination were impeded by pod-dwelling endophyte contamination and overgrowth, as well as poorly-developed seeds which are unlikely to have germinated even if contamination could have been averted. New protocols for testing the symbiotic germination of orchids with very small pods may need to be developed, including isolating seeds from pod tissue using a dissecting microscope, longer sterilisation procedures, and methods of assessing seed viability.

4.1.1 *Tulasnella* isolates

Isolates representing a single *Tulasnella* sp. were obtained from orchids growing at 3 sites: D'Aguilar NP, Main Range NP and Girraween NP, which are approximately 140 and 80 linear km apart, respectively. The forested areas of these National Parks are separated by large expanses of deforested land due to sharply-increasing and poorly-regulated land clearing in south-east Queensland (Field, Burns & Dale 2011). As such, the occurrence of the same species of fungus over such a wide geographical area suggests a broad distribution prior to

European colonisation, long-distance spore dispersal, or ubiquity in both forested and cleared land. Previous studies have found single *Tulasnella* spp. associating with Australian orchids over landscapes of similar scale and fragmentation (Ruibal et al. 2013; Linde et al. 2017). Although orchids have been observed recolonising an island, potentially via wind-blown seeds (Mount Krakatau; Gandawijaja & Arditti 1983), most orchid seed dispersal seldom exceeds 10m (Chung, Nason & Chung 2004), so it is unlikely that the *Tulasnella* sp. identified here was widely spread along with host seeds. Soil sampling in SE Queensland farmland and forests could clarify whether this *Tulasnella* sp. occurs throughout the landscape.

Tulasnella are frequently detected in molecular studies of OMF, with multiple species often associated with a single orchid (Suárez et al. 2006; Kottke et al. 2008; Steinfort et al. 2010). That only a single species was detected in this study suggests that *B. exiguum, B. bracteatum* and *B. elisae* may exhibit narrow specificity with regard to their tulasnelloid mycobionts. Narrow mycorrhizal specificity has been recorded in epiphytic orchids and contrasted with the lower specificity of orchids of terrestrial growth habit by Martos et al. (2012), who proposed two primary hypotheses for specificity in epiphytes. First, that symbiosis in abiotically stressful circumstances, such as the low-moisture, nutrient-poor elevated positions occupied by epiphytes, may have contributed to strong positive selection for higher levels of water and nutrient sharing between orchid and fungus. This would demand a finer degree of specialisation than in the lower-stress environments inhabited by terrestrial orchids. Secondly, they argued that higher irradiation of epiphytes compared to terrestrials may allow them to provide fungal partners with more photosynthetically-fixed carbon, leading to greater fungal dependence on epiphytic partners. However, a complicating factor lies in the influence of orchids' host plants on OM partnerships. Recently, Wang et al. (2017) found that species richness and diversity of OMF from an epiphytic Chinese orchid were strongly influenced by the different tree species on which individual plants were growing, indicating that micro-

environmental aspects such as bark texture or shedding may influence which fungi are present to colonise dispersed orchid seeds. It is possible that OMF of lithophytic orchids, 5 of which were sampled in this study, are influenced by rock type and texture in a similar manner. However, such factors do not appear to have affected the ability of the single *Tulasnella* sp. to associate with *B. exiguum, B. bracteatum* and *B. elisae*, which were each found growing on the bark of a tree, a basalt rock and a granite boulder, respectively.

In Australia, Roche et al. (2010) found a group of closely-related *Tulasnalla* spp. associating with terrestrial orchids in the genus *Chiloglottis.* Similarly, Ruibal et al. (2017) recently reported a single *Tulasnella* sp. associating with several *Chiloglottis* spp. over a range of 1000km. Similar landscape-wide OM specificity for *Tulasnella* was observed in two other Australian terrestrial orchid genera, *Drakaea* and *Arthrochilus* (Linde et al. 2014). In the context of this study these observations suggest two things. One, that OMF are indeed unlikely to be drivers of orchid speciation (which is in line with the proposition of Waterman et al. (2011); see Section 1.4). If mycorrhizal partners influenced speciation we could expect to observe a diversity of OMF partners within orchid genera, which is not the case. Two, that some epiphytic orchids, as revealed here, also appear to exhibit narrow OMF specificity, but the *Tulasnella* partner is not closely related to those fungi associating with Australian terrestrial orchids. Future root sampling over more sites will be required to verify whether the single *Tulasnella* sp. isolated here is in fact the only fungus of that genus associating with *B. exiguum, B. bracteatum* and *B. elisae.*

Morphologically, *B. exiguum, B. bracteatum* and *B. elisae* share obvious similarities, such as pseudobulb structure, leaf shape and floral anatomy (Figure 3). These similarities were the basis of a proposal to move them to the genus *Adelopetalum* (Jones 2006). With evidence indicating that fungal partners are conserved in groups of closely-related orchids (Waterman et al. 2011), the finding that a single *Tulasnella* partner is common to this group of

Bulbophyllum sp. provides indirect support for their relatedness. Genetic analysis of the orchids themselves (see Sections 3.2.2 & 4.2) also appears to underscore the relatedness of these species.

Fungi in the genus *Tulasnella* are morphologically highly cryptic (Cruz et al. 2014). Efforts to induce teleomorphs and sporulation in the laboratory have been unsuccessful except for the early work of Warcup & Talbot (Warcup & Talbot 1967; Warcup 1971; Warcup 1981). As such, species delineation has been based on data other than sexual morphology, for example by combining asexual morphospecies classifications with molecular data (Cruz 2016; Linde et al. 2017). Species delineation is further complicated by the high level of intraspecific genomic variation exhibited by *Tulasnella* spp. This can be up to 4% in the ITS barcoding region (Cruz 2016), higher than the 3% threshold commonly used by fungal phylogeneticists to separate species. Nilsson et al. (2008) have cautioned against using simple sequence-centred approaches to naming new fungal species. Given the high genetic variation observed in *Tulasnella*, the fact that 4 of 6 isolates had identical ITS sequences and the remaining 2 only single-nucleotide differences appears unusual for the genus. Species description and further phylogenetic work on *Tulasnella* isolates must bear these complicating factors in mind.

4.1.2 *Serendipita* isolates

Three isolates from 3 different *B. exiguum* plants at D'Aguilar NP are the same species of *Serendipita* based on their ITS sequence identity. With no similar publicly-available sequences, this species represents an Australian *Serendipita* sp. that is hitherto undescribed.

Fungi in the order Sebacinales (containing the family Serendipitaceae) have been shown to form a broad range of mycorrhizas with over 150 non-orchid angiosperm species on four continents, colonising rhizodermal cells and suppressing host immune responses, which allows hyphae to remain embedded between cells in root tissue (Weiß et al. 2016). It has been

proposed that Sebacinales share an ancestral endophytic growth habit that has evolved many times into different types of mycorrhizal associations, including OMF (Weiß et al. 2011). Mycorrhizal fungi in the Sebacinales have been divided into Group A, which are generally unculturable and associate primarily with woody trees and obligately mycoheterotrophic orchids; and Group B (a.k.a. the Serendipitaceae), which are culturable and associate with green orchids, ericads, liverworts and some trees (Weiß et al. 2016). The best-studied *Serendipita* species, *S. indica*, has been investigated for its plant growth-promoting properties in the crop plants barley, wheat and maize, and in the model plant *Arabidopsis thaliana* (Franken 2012). Root colonisation by *S. indica* confers benefits such as plant resistance to leaf pathogens, higher agricultural yields and plant salt tolerance (Waller et al. 2005). The new *Serendipita* sp. isolated here thus has the potential to be a valuable addition to the study of fungal agricultural inocula.

In Australia, green orchids from several genera have been shown to associate with *Serendipita* OMF. Early work by Warcup & Talbot (1967) identified OMF from *Acianthus, Caladenia, Microtis* and *Glossodia* orchids as *S. vermifera*, a designation that has now been suggested to encompass a wider species complex based on intraspecific DNA sequence variation (Deshmukh et al. 2006). Several *S. vermifera* isolates stimulated seed germination in Australian terrestrial orchids from the genera *Caladenia*, *Cyrtostylis* and *Glossodia* (Warcup 1988) and their ITS regions consistently clustered with other *Serendipita* OMF from around the world (Weiß et al. 2016). Further studies have demonstrated the importance of *Serendipita* OMF for Australian terrestrial orchids, particularly those in the genus *Caladenia* (Huynh et al. 2009; Wright et al. 2010). However, although Sebacinales OMF have been detected associating with epiphytic *Stelis* and *Pleurothallis* orchids in Ecuador (Kottke et al. 2008), this study is the first to document this group of fungi in symbiosis with epiphytic orchids in Australia.

Interestingly, in the phylogenetic analysis the *Serendipita* isolates fell into a clade not with the majority of OMF Serendipitaceae sequences, but into a distinct clade that also contained an ericoid mycorrhizal fungal (ErMF) *Serendipita* sequence from Sweden. The ErMF sequence was isolated from lingonberry (*Vaccinium vitis-idaea*) roots in a sub-Arctic mire in northern Sweden (Kjøller, Olsrud & Michelsen 2010), along with a number of fungal sequences belonging to the Helotiales, a prominent ErMF order (Bougoure et al. 2007; Leopold 2016). Additionally, top BLAST matches of 2 of the 3 *Serendipita* isolates from this study were sequences from Sebacinales associating with South American Ericaceae. Both *B. exiguum* and *B. elisae* simultaneously harboured *Serendipita* and Helotiales sp. Given that both of these fungal groups contain known mycorrhizal species, and that ericaceous plants have been shown to associate with *Serendipita* and Helotiales, it is reasonable to speculate that *Bulbophyllum* orchids might also form mycorrhizas with both fungal lineages. The frequent co-occurrence of these fungal groups may also suggest that there are fungus-fungus interactions that occur in and around plant roots. Synergistic mycorrhizal dynamics have been reported involving two species of fungus performing discrete roles to benefit a single host plant (Della Monica et al. 2015). Previous studies have also documented both OMF and helotialean fungi forming cultures from pelotons isolated from an individual orchid (Stark, Babik & Durka 2009; Kohout et al. 2013). *In vitro* orchid isotope-tracer experiments using *Serendipita* and Helotiales spp. as inocula could determine whether any three-way nutritional interactions might exist. The possible mycorrhizal status of isolates belonging to the Helotiales is further discussed in Section 4.1.4.

4.1.3 *Ceratobasidium* isolate

Isolate BSST P3 1.6 was cultured from the roots of a *B. shepherdii* colony that had been translocated ~2km from its original position in an open eucalypt woodland west of

Stanthorpe. There can therefore be no certainty that BSST P3 1.6 is regularly associated with natural *B. shepherdii* populations, as a *Ceratobasidium* sp. local to the area to which the orchid was moved may have colonised roots after translocation took place. However, a study on the mycorrhizal associations of translocated orchid populations in China found that translocated populations of the epiphytic slipper orchid *Paphiopedilum hirsutissimum* were able to form mycorrhizas with their regular *Ceratobasidium* sp. partners after being moved >200km (Downing et al. 2017). Whether this was a result of fungi being carried with the translocated plants or the ubiquity of that fungal species on the rock to which it was transferred was unclear.

Interestingly, isolate BSST P3 1.6 appears to be the same species as an unnamed *Ceratobasidium* sp. isolated from roots of the terrestrial green orchid *Goodyera repens* in Norway. This species of orchid has been shown to receive C and N from, and pass C to, the fungus *Ceratobasidium cornigerum* in experiments that demonstrated for the first time bidirectional C flow in orchid mycorrhizas, indication of true mutualism (Cameron, Leake & Read 2006; Cameron et al. 2008). The finding that *B. shepherdii* appears to associate with one of the same *Ceratobasidium* sp. as *Goodyera repens* underscores the likelihood that bidirectional nutritional dynamics are present in the mycorrhizal associations of *Bulbophyllum* orchids. Further experiments using tracer isotopes must be carried out to determine quantities of nutrients passed between *B. shepherdii* and its OMF. Root sampling of wild *B. shepherdii* populations would clarify whether BSST P3 1.6 is indeed this orchid's regular mycorrhizal partner.

4.1.4 Dark septate endophyte (Helotiales) isolates: functional overlap between ericoid and orchid mycorrhizas?

Dark septate endophytes (DSE) are a little-studied group of fungi with septate (septum = wall between cells) and melanised hyphae (see Figure 13F) (Knapp, Pintye & Kovács 2012). They are mostly members of the order Helotiales (Upson et al. 2009). DSE have been found to associate with the roots of approximately 600 plant species in 144 families, including the Orchidaceae and Ericaceae (Jumpponen & Trappe 1998) and to significantly promote plant growth in terms of biomass and N and P tissue concentrations (Newsham 2011).

Nine isolates with highest BLAST identity to helotialean GenBank sequences were obtained from *B. exiguum, B. bracteatum* and *B. elisae.* The majority (6) showed highest identity to archived Helotiales sequences obtained from the roots of plants in the Ericaceae, such as *Epacris pulchella, E. microphylla, Rhododendron lochiae* (all native to Australia), and *Calluna vulgaris.* The high isolation rate of helotialean ErMF from *Bulbophyllum* roots invites speculation as to the possible role of such fungi in orchid mycorrhizal ecology.

It has been reported that DSE are "capable of forming mutualistic associations functionally similar to mycorrhizas", and that the intracellular structures that they form resemble ectendomycorrhizas, strongly suggesting a biotrophic/mycorrhizal nutritional habit (Jumpponen 2001). Ectendomycorrhizas form a thin mantle and Hartig net over *Pinus* and *Larix* sp. root tips, but unlike ectomycorrhizas they also penetrate root cells and exchange nutrients intracellularly via coiled hyphal structures (Yu, Egger & Peterson 2001). A 2005 meta-analysis predicted that DSE are as globally widespread as better-studied groups of mycorrhizal fungi and reported that DSE and mycorrhizal fungi frequently co-occur in plants (Mandyam & Jumpponen 2005). As noted in Section 4.1.2, synergistic interactions between arbuscular mycorrhizal fungi (AMF) and DSE have been reported, with DSE making inorganic and organic soil P available to AMF, which in turn pass P to host plants (Della

Monica et al. 2015). Given the evidence that DSE are widespread and likely to be mycorrhizal, that they should be present in the roots of the heavily fungus-dependent orchids is unsurprising. It is possible that the use of a 6 primer set for PCR reactions in this study, as opposed to the ITS1F/ITS4 pair used in the majority of OMF studies, has amplified the ITS of DSE species that do not readily amplify with ITS1F/ITS4. As most studies of orchid mycorrizas tend to focus on the basidiomycete mycorrhizal fungi for which OMF status is well-established (e.g. *Tulasnella, Serendipita, Ceratobasidium*), it is possible that the role of DSE from the Helotiales has been overlooked.

A search of the GenBank database for "Helotiales AND orchid" returned 29 Helotiales fungal sequences obtained in separate studies from the roots of 9 orchid genera including *Ophrys, Spiranthes, Gymnadenia, Pecteilis, Epipactis, Pleurothallis, Cephalanthera, Bletilla* and *Stelis,* the latter 5 of which, like *Bulbophyllum*, belong to the orchid subfamily Epidendroideae. One of these Helotiales sequences was from *Cryptosporiopsis ericae* isolated from the orchid *Spiranthes*. *C. ericae* is also known to associate with ericaceous plants (Sigler et al. 2005). Considering that a *Cryptosporiopsis* fungal sequence was obtained from *B. exiguum* in this study, there is appreciable overlap in the fungal communities harboured by plants in the Orchidaceae and the Ericaceae, and the putative mycorrhizal status of helotialean DSE suggests that SE Queensland *Bulbophyllum* may represent yet another plant group that harbour DSE symbionts. Seed germination and isotope-tracer studies would assist in elucidating the nature and scale of the *Bulbophyllum*-Helotiales association.

Plants in the Orchidaceae (monocots) and Ericaceae (dicots) are distantly related, but their intracellular, highly-coiled mycorrhizal nutrient-exchange structures share more similarities than do the structures of any of the other major mycorrhizal types (Smith & Read 2008). Convergent evolution in the natural world is well-documented and often arises from identical genetic mutations in independent lineages (Stern 2013). Plants in both groups may have

evolved broadly similar physiological and genetic strategies for entering into mycorrhizal relationships, resulting in morphological and functional similarities in mycorrhiza formation. This study, along with those cited above, provides further evidence that some green orchids may associate with two of the same fungal groups as do ericaceous plants—Helotiales and *Serendipita* spp.—raising the possibility that the structurally-analogous ErM and OMF mycorrhizal types may also be analogous at a genetic level. Further genomic work will be required in the search for genetic parallels. Such investigations could compare specific gene sequences between orchids/ericads and Helotiales/*Serendipita*, focusing on genes that code for proteins known to be necessary in maintaining mycorrhizal symbiosis such as those involved in cellular signalling and organisation, membrane transport and plant defence (Dearnaley, Perotto & Selosse 2016).

4.2 Phylogeny of *Bulbophyllum* spp.

Phylogenetic analysis of *Bulbophyllum* spp. rbcL gene sequences indicated a close relationship between *B. exiguum*, *B. bracteatum* and *B. elisae*, which is in agreement with the proposal of Jones (2006) to include these 3 species in the smaller genus *Adelopetalum* based on shared morphological traits. Additionally, *B. shepherdii*, which was moved by Jones to the genus *Oxysepala*, showed a closer phylogenetic relationship to another *Oxysepala* species (*O. gadgarrense*) than to any of the proposed *Adelopetalum* spp. The phylogenetic distance of *B. minutissimum* from both the *Adelopetalum* and *Oxysepala* clades appeared to be approximately equal, and without another *Oncophyllum* rbcL sequence for comparison its taxonomy remains to be clarified.

As noted in Section 4.1.1, groups of closely-related orchids tend to share fungal partners (Waterman et al. 2011). Identification of mycorrhizal fungi may thus be used as an indirect, non-definitive method of validating orchid taxonomic groupings. In this study, the only

Bulbophyllum spp. that harboured *Serendipita, Tulasnella* and Helotiales fungi were those that have been moved by Jones to the genus *Adelopetalum,* i.e. *B. exiguum*, *B. bracteatum* and *B. elisae*. That these fungal associations proved relatively constant over multiple sites supports the view that this group of orchids exhibits some fungal specificity independent of location. In contrast, the roots of the two orchid species proposed to belong to different genera, *B. shepherdii* (*Oxysepala*) and *B. minutissimum* (*Oncophyllum*), were found to contain fungi not shared by the others. *B. shepherdii* harboured the only *Ceratobasidium* sp. identified in this study, as well as the only *Virgaria* sp. (Table 10); *B. minutissimum* harboured the only *Fusarium* and *Phoma* spp. (Table 9). Although the latter 3 fungal genera belong to families known to be pathogenic rather than mycorrhizal, the thick outer layer of epiphytic orchid roots, known as the velamen, is colonised by a wide variety of micro-organisms (Herrera, Suárez & Kottke 2010), and given the characteristic microbial 'fingerprint' of each plant species it is reasonable to assume a degree of uniqueness in the assemblage of these non-mycorrhizal or pathogenic root endophytes (Sánchez-Cañizares et al. 2017).

A further line of evidence to support the notion that *B. shepherdii* is only distantly-related to *B. exiguum*, *B. bracteatum* and *B. elisae* lies in the only two published studies on *Bulbophyllum* mycorrhizal associations. Martos et al. (2012) proposed that *Bulbophyllum* associate with *Tulasnella* (a proposal for which they provided no clear evidence) and *Serendipita* spp., but not with *Ceratobasidium* spp. Furthermore, Těšitelová et al. (2015) included two OMF sequences associated with *Bulbophyllum* spp. in their phylogeny of Sebacinales mycobionts of orchids in the epidendroid *Neottia* genus, both of which show highest (93 and 96%) identity to archived *Serendipita* sequences when compared using BLAST in GenBank. There is therefore no current literature to support a theory that *Bulbophyllum* spp. associate with *Ceratobasidium*. The artificial translocation of the

Ceratobasidium-associated *B. shepherdii* individual sampled here means that mycorrhizal sampling of wild *B. shepherdii* populations is needed to verify these conclusions.

4.3 Limitations and potential sources of error

The most fundamental limitation of culture-dependent studies lies in the bias implicit in considering only those fungal species that will readily grow in the laboratory. Such studies will invariably report only species able to live independently of plants, which, in the study of mycorrhizas, is bound to eliminate the most derived, mutalistic biotrophs (Read & Perez-Moreno 2003). Vrålstad (2004) notes the circularity of reasoning inherent in obtaining certain cultivable mycorrhizal fungi from plant roots, and then reporting that the mycorrhizal partners of that plant are easily cultivable. Gene libraries compiled from culture-independent, large-scale sequencing of soils and plant roots continue to reveal a far greater diversity of micro-organisms, including fungi, than has been apparent from culture-based research (Schmidt et al. 2008). Considering that the bulk of the earth's diversity lies in the microbial world, and that >99% of the micro-organisms present in nature are not cultivable using standard methods (Hugenholtz, Goebel & Pace 1998), it is highly likely that many fungi of ecological significance to the orchids studied here have been screened out at the isolation and culturing steps. However, one of the chief benefits of obtaining live cultures for identification is that their living status can be verified. In contrast, culture-independent techniques are prone to error by collecting DNA sequences from inactive, dead or ruptured micro-organisms (Hirsch, Mauchline & Clark 2010). An additional approach would be to sequence microbial RNA or proteins, molecules which are more closely associated with living cell function (Alberts et al. 2015).

Another drawback of working with fungal cultures to isolate OMF is that species known to be orchid mycorrhizal are usually very slow-growing (Zhu et al. 2008). This leads to other,

faster-growing fungal endophytes often enveloping OMF pelotons, at which point sub-culturing of OMF becomes impossible. This was apparent in the seed germination experiments performed in this study, where overgrowth of endophytes obstructed growth of OMF inocula, preventing it from reaching the seeds (see Section 3.3.2). Zhu et al. (2008) have proposed a peloton isolation protocol designed to reduce contamination by purifying pelotons into small agar discs. Although time-consuming, such a process may have increased the number of OMF cultures obtained here.

Yeast or bacterial DNA contamination of the *B. elisae Serendipita* isolate could not be removed despite repeated sub-culturing. Mycorrhizal fungi have demonstrated intimate mutualistic interactions with other root-dwelling micro-organisms (Frey-Klett, Garbaye, & Tarkka 2007), which may explain the difficulty of separating isolate P2 1.9 from the microbial contaminant. Further sub-culturing efforts using microscopy and finer scalpel blades may prove more effective. Another option would be to develop different primer sets designed to exclude the contaminating DNA from amplification.

Seed germination experiments were unable to establish whether any fungal isolates could stimulate orchid seed germination by forming pelotons in orchid cells. This makes definitive characterisation of any isolates as OMF difficult, as no clear mycorrhizal interaction has been observed. The third of Koch's Postulates states that a truly infectious agent, "after being fully isolated from the body and repeatedly grown in pure culture, can induce the disease anew" (Evans 1976). Although pelotons were observed to be present in roots, it is not possible to prove that the cultures that grew from them are peloton-forming fungi unless such fungi are re-introduced to orchid seeds and form pelotons therein. Nevertheless, *Tulasnella, Serendipita* and *Ceratobasidium* are well-known OMF (Dearnaley, Perotto & Selosse 2016), so the isolates identified here may be considered putatively mycorrhizal until further steps are taken to clarify their status. The poorly-developed *B. exiguum* seeds used in the experiments may have

been prematurely harvested, allowing too little time for the seeds to mature, or might have resulted from inbreeding of orchid populations. Inbred plants have been shown to produce 75% less seed than non-inbred individuals, with up to 70% of set seeds exhibiting deformations resulting from arrested development (Mahy & Jacquemart 1999). Given the isolated locations of *B. exiguum* colonies studied here, it is possible that south-east Queensland populations are experiencing low rates of gene flow.

Culturing of root tissue from *B. minutissimum* and *B. shepherdii* yielded few fungal isolates compared to the other *Bulbophyllum* spp. The low number of isolates and lack of OMF obtained from *B. minutissimum* may have been due to this species' small size, with fine roots and ~3mm pseudobulbs providing little tissue from which pelotons could be extracted. Future studies may benefit from dissecting roots and pseudobulbs in order to visually identify pelotons prior to culturing. A similarly low number of isolates from *B. shepherdii* may have stemmed from the plant's removal from its natural location.

Finally, two potential sources of error lie in the bioinformatic analyses of single-gene barcode regions. The ITS gene region was proposed as the universal DNA marker barcode for fungi based on its ease of PCR amplification and broad range across the kingdom (Schoch et al. 2012). However, due to poor species-level resolution stemming from intragenomic ITS variation in some groups, or shared interspecific ITS sequences in others (Kiss 2012), the ITS region is not 100% reliable for species-level identification. Moreover, despite it being tempting to view the phylogenetic tree produced from a single-gene phylogeny as a reflection of organisms' true relatedness, it is in fact a representation of the relatedness of the genes themselves—a much narrower concept. Multi-gene phylogenies can provide a more robust estimation of actual relatedness (Zhang et al. 2011).

The other bioinformatic aspect for which caution should be exercised is bootstrapping. As noted in Section 3.1.5.1, joint confidence in large trees is invariably low (Soltis and Soltis

2003), and although high bootstrap values are usually inferred to represent confidence in actual relationships between loci, this is not quite the case. Felsenstein (1985) notes that "bootstrapping provides us with a confidence interval within which is contained not the true phylogeny, but the phylogeny that would be estimated upon repeated sampling of many characters from the underlying pool of characters". In other words, a bootstrap value indicates only that the analysis returned the same result many times. From this we must be careful of confidently inferring actual evolutionary relationships.

4.4 Future directions and potential applications of findings

For a robust catalogue of orchid mycorrhizal partners, further root sampling would be beneficial for those *Bulbophyllum* spp. that were only represented in this study by plants from a single site: *B. bracteatum, B. elisae, B. minutissimum* and *B. shepherdii.* This would increase sample size and help to confirm associations that have been suggested by identifications gained in this study. Additional seed germination experiments using putatively mycorrhizal isolates as inocula would further verify their mycorrhizal status and clarify whether fungi isolated from adult plants play a role in germination. This information will be important should any of these orchid species become of conservation concern and require *ex situ* propagation. Indeed, with land clearing in SE Queensland showing no signs of deceleration (Field, Burns & Dale 2011; Dept. of Science, Information Technology & Innovation 2017), epiphytic orchid habitat is likely to decrease dramatically over the coming decades.

With increasing global climate instability, agriculture is one of the most vulnerable sectors (Smit & Skinner 2002). For drought-prone Australia the risks are particularly acute. The *Serendipita* isolate obtained in this study deserves further attention in the context of the current surge of interest in crop-improving mycorrhizal fungi, as it belongs to a genus with

well-established agricultural applications (Ghimire & Craven 2011). Pot or glasshouse experiments inoculating major crop species and model plants with this isolate would indicate whether it has any utility in inducing drought, salt or disease tolerance.

Molecular identification of contaminating fungi from seed germination experiments could clarify whether they are orchid endophytes or merely atmospheric contaminants of the experimental procedure. Comparison of endophytic communities isolated from plant fruit and seeds with those isolated from roots may provide insights into the tissue-specificity of orchid endophytes, an area of study linked to the concept of plants as 'holobionts'—interdependent and complex plant-microbial systems (Sánchez-Cañizares et al. 2017).

Additionally, culture-independent identification methods using next-generation sequencing applied to *Bulbophyllum* spp. roots would elucidate whether non-culturable mycorrhizal fungi are present. A wider root-symbiont context for the fungi identified here will be unclear until such an analysis is performed. Structural analysis using scanning electron or transmission electron micrography of orchid root cells inoculated with helotialean fungi would also help to ascertain whether these fungi form intracellular nutrient-exchange structures as reported by Jumpponen (2001). As the uncertain role of root-associated dark septate endophytes is further investigated, their symbiosis in the context of the Orchidaceae will be a critical part of the larger picture.

More broadly, future work on orchid and other mycorrhizas will need to account for the functional and phylogenetic overlap between currently-distinguished mycorrhizal fungi clades. A plethora of independent evolutionary events has given rise to an enormous diversity of mycorrhizal fungi and plants, and the lines between pathogenic, endophytic and mycorrhizal fungi are blurred (Allen et al. 2003). Further understanding of the mycorrhizal dynamics of heavily fungus-dependent orchids like *Bulbophyllum* is likely to reveal far more complex and dynamic interactions than are currently appreciated.

5. Conclusions

This study provided the first catalogue of fungi associated with the roots of Australian *Bulbophyllum* orchids, identifying putatively mycorrhizal species in well-established orchid mycorrhizal clades as well as several helotialean DSE with potentially mutualistic roles. Additionally, evidence from shared mycorrhizal associations and plant DNA analysis supported the taxonomic re-classifications of 4 of 5 *Bulbophyllum* sp. proposed by Jones (2006). Seed germination experiments, which were unable to yield suitable data due to rapid overgrowth of endophytic fungal species, revealed the difficulties in working with very small seeds and seed pods.

Evidence was obtained for orchid-fungus species specificity for a *Tulasnella* mycobiont that is new to science. Two additional new fungal species, one *Serendipita* and one Helotiales, were also identified, highlighting the diversity of mycorrhizal and endophytic fungi that have not yet been described. The common OMF specificity of *B. exiguum, B. bracteatum* and *B. elisae* for *Tulasnella* correlates with current understanding of epiphytic orchid mycorrhizal ecology, supporting the notion that epiphytes tend to evolve narrow specificity due to stressful abiotic conditions (Martos et al. 2012). Evidence of dark septate endophytes from the Helotiales suggests that OMF may be more diverse than is currently appreciated, and that clear-cut functional categorisation of mycorrhiza types may not always be appropriate.

Taxonomic revisions proposed by Jones (2006) were largely supported by comparison of fungi harboured by *Bulbophyllum* orchids and phylogenetic analysis of orchid rbcL genes. Thus, *B. exiguum, B. bracteatum* and *B. elisae*, with a shared *Tulasnella* mycorrhizal partner and closely-aligned gene sequences, appear to have been appropriately re-assigned to the genus *Adelopetalum. B. shepherdii*, which was the sole *Bulbophyllum* species in this study found to associate with a *Ceratobasidium* fungus and to phylogenetically cluster with an Australian *Oxysepala* orchid, appears to have been accurately re-assigned to the genus

Oxysepala. Insufficient comparative data for *B. minutissimum* was obtained. Its mycorrhizal status and phylogenetic placement remain unclear.

Seed germination experiment protocols for working with very small seeds and seed pods will need to be developed to prevent overgrowth of endophytic fungi. Future symbiotic seed germination studies will help to verify the mycorrhizal status of the fungi isolated in this study, addressing the question of whether developmental shifts occur in the mycorrhizal associations of these orchids.

The outcomes of the hypotheses outlined in Section 1.5 are as follows:

1. *Five south-eastern Queensland* Bulbophyllum *orchids associate with the same group of OMF as the* Bulbophyllum *on Réunion Island studied by Martos & Selosse (2008 unpub.):* Serendipita *(family Sebacinaceae, Clade B).* **Disproved**. Of the 5 orchid spp. only 2 (*B. exiguum* and *B. elisae*) were found to harbour fungi belonging to the Serendipitaceae.
2. B. exiguum *exhibits OMF specificity across multiple sites in south-east Queensland.* **Disproved**. Although *B. exiguum* shared fungal partners with other orchids in the study, at each site *B. exiguum* plants harboured different fungi.
3. *OMF cultures isolated from adult plants are able to stimulate germination and developmental shifts in* B. exiguum. Insufficient data.
4. *Five SE Queensland* Bulbophyllum *orchids belonging to new genera proposed by Jones (2006)*—Adelopetalum, Oxysepala *and* Oncophyllum— *have shared, genus-specific OMF partners that differ from those of other Bulbophyllum spp.* **Proven** in the case of *Adelopetalum* (*B. exiguum, B. bracteatum* and *B. elisae*) and *Oxysepala* (*B. shepherdii*). Insufficient data for *B. minutissimum.*

5. *Chloroplast DNA sequences of 5 SE Queensland* Bulbophyllum *orchids belonging to new genera proposed by Jones (2006)*—Adelopetalum, Oxysepala *and* Oncophyllum*—do not cluster together in phylogenetic analysis, but belong to different clades.* **Proven**. Orchid rbcL genes formed two distinct clades, supporting hypothesis 4.

This investigation has demonstrated that three south-east Queensland *Bulbophyllum* orchids appear to exhibit narrow mycorrhizal specificity for a fungus in the *Tulasnella* genus. Orchids were also shown to harbour dark septate endophytes, hinting at mycorrhizal associations outside the commonly-accepted OMF clades. Furthermore, this study has provided evidence that both mycorrhizal and DNA data are in agreement with proposed taxonomic re-classifications of *Bulbophyllum* based on plant morphology.

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7. Appendices

Appendix A Complete site data for mycorrhizal root sampling of 5 *Bulbophyllum* orchid spp. over 7 sites.

Orchid species	Sp./location code	Location	Date
B. exiguum	BE DA 14.2	D'Aguilar NP	14.02.2017.
B. exiguum	BE MR 10.3	Main Range (QMF)	10.03.2017.
B. bracteatum	BB MR 10.3	Main Range (QMF)	11.03.2017.
B. minutissimum	BMYA 4.4	Yalangur	04.04.2017.
B. elisae	BLGW 2.5	Girraween NP	02.05.2017.
B. exiguum	BEST 2.5	Mt. Tully	02.05.2017.
B. exiguum	BESP 23.5	Springbrook	23.05.2017.
B. shepherdii	BSST 20.6	Stanthorpe	20.06.2017.

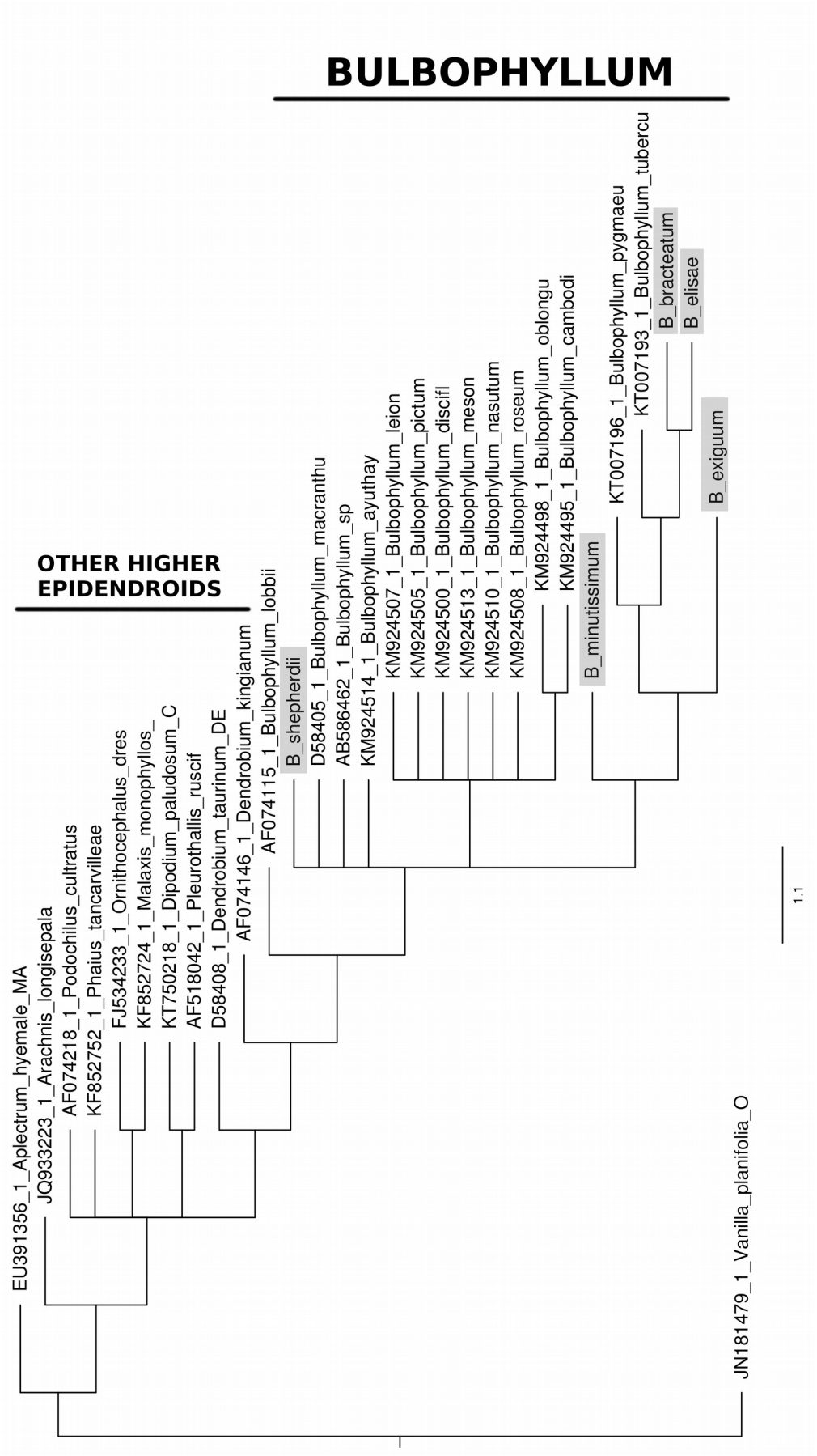
Sp./location code	Distance from ground (m)	Dominant tree species
BEDA 14.2	1.5	*Archontophoenix cunninghamiana, L. confertus, Eucalyptus microcorys, E. saligna*
BEMR 10.3	2	*A. cunninghamii, Syzygium smithii, Streblus brunonianus*
BBMR 10.3	20	*A. cunninghamii, Eucalyptus punctata, Eucalyptus propinqua, L. confertus, Eucalyptus viminalis, Plectranthus, Themeda, Leucopogon*
BMYA 4.4	15	*Alphitonia excelsa, Geijera parviflora, Asparagus setaceus, Notelaea longifolia*
BLGW 2.5	2	*Eucalyptus youmanii, Casuarina, Banksia spinulosa*
BEST 2.5	2	*Jacksonia scoparia, Eucalyptus andrewsii, Angophora floribunda, Acacia implexa, Commersonia bartramia*
BESP 23.5	4	*Eucalyptus microcorys*
BSST 20.6	1	*Ficus* sp.

Sp./location code	# of roots sampled	# of colonies	# of plants in colony
BEDA 14.2	6	1	>100
BEMR 10.3	5	2	>500
BBMR 10.3	5	2	>10
BMYA 4.4	5	>10	>1000
BLGW 2.5	5	3	>500
BEST 2.5	5	2	>1000
BESP 23.5	5	1	>500
BSST 20.6	3	1	1

Sp./location code	MASL (m)	Host	Slope aspect
BEDA 14.2	477	*Rhodamnia* sp.	S
BEMR 10.3	753	Rock	NE
BBMR 10.3	799	Rock	NE
BMYA 4.4	588	Rock	S
BLGW 2.5	1079	Rock	SE
BEST 2.5	1035	Rock	SE
BESP 23.5	766	*Acacia melanoxylon*	ESE
BSST 20.6	969	Rock	E

Sp./location code	Lat	Long	Side of tree/rock
BEDA 14.2	-27.401625	152.799643	S
BEMR 10.3	-28.339464	152.369823	NE
BBMR 10.3	-28.340127	152.371041	NNE
BMYA 4.4	-27.2521	151.5214	S
BLGW 2.5	-28.521	151.5957	SE
BEST 2.5	-28.432	151.5751	SE
BESP 23.5	-28.1327	153.1623	S
BSST 20.6	-28.3814	151.5549	E

Appendix B Low-resolution Maximum Parsimony phylogenetic analysis of rbcL genes from 5 *Bulbophyllum* orchid spp. based on a 700 bp ClustalW alignment. Generated using the web server version of TNT (Goloboff, Farris & Nixon 2008) at www.phylogeny.fr. Scale bar represents average number of nucleotide substitutions per site.



Appendix C Data matrix for presenting germination (GRI) and developmental rate (DRI) indices of seed germination experiments (Sections 2.3 & 3.3), which were impeded by overgrowth of contaminants. Figures were to represent means of three replicates. p-values were to be derived from Fisher's exact tests of all treatments at each developmental stage at 0.05% probability.

Developmental Stages →	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Treatments ↓	**GRI** (%)	**DRI** (% per 5 days)	**DRI** (% per 5 days)	**DRI** (% per 5 days)	**DRI** (% per 5 days)
Serendipita sp. (P5 1.1)	-	-	-	-	-
Tulasnella sp. (P1 1.2)	-	-	-	-	-
Helotiales sp. (P5 1.7)	-	-	-	-	-
Phoma sp. (P3 1.12)	-	-	-	-	-
Uninoculated control	-	-	-	-	-
Fisher's exact test **p-value**	-	-	-	-	-