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The diversity of fungi associated with endemic and endangered orchids of Southern Africa

By

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MAGISTER SCIENTIAE

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Declaration

I, Modjadji Carol Makwela, declare that the dissertation, which I hereby submit for the degree *Magister Scientiae* at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: 

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Preface

Orchids form unique symbiotic relationships and are adapted to growing in extreme environments. The growth and survival of orchids are aided by soil microbes such as bacteria, actinomycetes and fungi, particularly mycorrhizal fungi. This orchid-mycorrhiza interaction is important because mycorrhizal fungi provide nutrients for seed germination, seedling establishment and growth of mature orchids. Without their mycorrhizal partners, orchid propagation in nature is often unsuccessful. Therefore, understanding the taxonomy, distribution and habitat specificity of these fungi is crucial for propagating orchids.

Terrestrial orchids worldwide are going extinct due to the rapid rate of urban development, encroachment of invasive plant species and overcollection. In Southern Africa, populations of most endemic orchids are declining at an alarming rate. Therefore, potential solutions such as orchid translocation to protected habitats and *in vitro* seed germination are urgently required to combat the rapid decline of orchid populations. However, knowledge of the diversity, distribution, and specificity of the mycorrhizal symbionts of orchids in South Africa is sparse. Therefore, the overall aim of the research presented in this dissertation was to report on the diversity of orchid mycorrhizae associated with the South African endemic orchids in the genera *Habenaria* and *Brachycorythis*.

Chapter one: This chapter of the dissertation is a literature review detailing the general background knowledge available on orchid-mycorrhizal associations. The main focus of the review is to highlight the importance of mycorrhizae for the establishment and conservation of orchid populations in nature. Furthermore, this chapter gives an overview of the current knowledge of orchid-mycorrhizal associations.

Chapter two: This chapter investigated the mycorrhizal diversity present in the rhizosphere soil of a critically endangered orchid, *Brachycorythis conica* subsp. *transvaalensis* (Albertina Sisulu Orchid). For this purpose, the fungal diversity of the rhizosphere soil was compared to that in soil from a location where no orchids were previously recorded. Using high throughput sequencing, the fungal diversity present in both soil types was effectively compared. This study showed significant overlap in the fungal diversity in each of the soil samples. Moreover, the orchid rhizosphere soil contained more potential mycorrhizal fungal species.

Chapter three: This chapter focused on comparing the orchid-mycorrhizal relationships of two endemic terrestrial orchids in the genus *Habenaria*. Namely, the *Habenaria barbertoni* which is an endangered orchid and the *Habenaria epipactidea* which is more abundant. Microscopy, fungal isolations, molecular cloning, and high-throughput sequencing were used to identify potential mycorrhizal associates in the roots and tubers of each orchid. Culture-dependent techniques provided limited information on the mycorrhizal symbionts of these orchids as only two potential mycorrhizal fungi were isolated. With culture-independent DNA based techniques, numerous potential mycorrhizal fungi from the Ceratobasidiaceae, Tulasnellaceae, Serendipitaceae, Pezizaceae, Hoehnelomycetaceae and Omphalotaceae were detected. This study showed significant differences in mycorrhizal diversity between the two orchid species.

Conclusion: In the research presented in this dissertation the mycorrhizal fungi associated with orchids in the genus *Habenaria* were successfully recorded and the potential orchid mycorrhizal associates of the *Brachycorythis conica* subsp. *transvaalensis* were identified. Some of the fungi detected could not be identified and thus are potential new mycorrhizal species. Information about the presence and identity of orchid mycorrhizal fungi at different locations can help in choosing sites that harbour fungi that are compatible with orchid species. In future, this data can be used for translocating orchids to conservation sites as well as developing and implementing other suitable conservation measures for orchids in South Africa. This study also contributed to the current knowledge of the types of orchid mycorrhizal fungi that occur in South African soils.

Chapter 1

Significance of mycorrhizal diversity and taxonomy studies for orchid propagation and conservation

1. Abstract

The Orchidaceae is one of the largest angiosperm families comprising over 27 000 known species. Globally orchids are highly sought-after for economic, ornamental, and ethnobotanical purposes. As a result, illegal collection of orchids together with habitat destruction, the encroachment of invasive species and climate change have caused a rapid decline in orchid populations. Orchids produce non-endospermous seeds that rely on mycorrhizal fungi for successful germination. These fungal symbionts also assist in increasing nutrient acquisition, sustaining the growth of mature orchids and enable orchids to colonize a diverse range of habitats. Most orchid mycorrhizae are from the division Basidiomycota including the genera *Ceratobasidium, Thanatephorus, Sebacina,* and *Tulasnella*. Also, non-mycorrhizal fungi associated with orchids can play a role in the survival and fitness of these plants through phytohormone production, microbial antagonism and entomopathogenic activity. This review provides an overview of the history, biology, taxonomy, and importance of these orchid-fungal relationships. Moreover, various studies that have been conducted to understand these associations are described.

Keywords: Mycorrhizae, Orchidaceae, orchid conservation, *Rhizoctonia*-complex

2. Introduction to the Orchidaceae

The Orchidaceae is a highly diverse and widespread plant family encompassing over 27 000 species that are grouped under nearly 900 genera (The Plant List, 2020). Consequently, 10% of all flowering plants worldwide are represented by this family (Roberts & Dixon, 2005). It is the second-largest angiosperm family, following the Asteraceae which comprises 32 000 species. The Orchidaceae is further divided into five subfamilies: Apostasioideae, Epidendroideae, Cypripedioideae, Orchidoideae, and Vanilloideae (Roberts & Dixon, 2005, Chase *et al.*, 2015), the largest of which is the Epidendroideae with over 21 000 species (Valencia-Nieto *et al.*, 2018). Moreover, new orchid species are being discovered and

described annually by researchers and orchid enthusiasts all around the world (Chase *et al.*, 2015).

Orchids have aesthetically appealing flowers that vary greatly in shape, size, colour, texture, and fragrance. Therefore, orchid flowers are in high demand in ornamental, ethnobotanical and horticultural markets (Basu *et al.*, 2016). Orchid trade contributes significantly to the economy of several countries. For example, in the U.S orchid sales amount to approximately $4 billion annually (Zhang *et al.*, 2018). The most commonly cultivated orchids are of the genera *Cymbidium*, *Paphiopedilum, Vanilla* and *Phalaenopsis* (Zhang *et al.*, 2018). These orchids can have multiple uses. For example, the dried seeds of *Vanilla planifolia* are used for flavouring (vanilla essence), manufacturing of perfumes and skincare products as well as in aromatherapy (Dearnaley, 2007, Zhang *et al.*, 2018). Ethnobotanically, orchid roots and tubers are a source of food and traditional medicine (Pant, 2013, Zhang *et al.*, 2018). For instance, in Chinese traditional medicine, *Gastrodia elata* is used to treat headaches, dizziness, tetanus and epilepsy (Thakur *et al.*, 2018, Zhang *et al.*, 2018). In Zambia, the tubers of the orchids *Disa robusta* and *Satyrium buchananii* are used to make a signature dish called *chikanda*, commonly known as African polony (Veldman *et al.*, 2018).

2.1 Global distribution of orchids

Orchids are found on every continent except Antarctica and are adapted to grow in a diverse range of habitats (Roberts & Dixon, 2005, Jacquemyn *et al.*, 2017), which includes tropical and temperate rainforests, deserts, savannas, grasslands, Mediterranean shrublands and the arctic tundra (Jacquemyn *et al.*, 2017). In these environments, orchids are found as either epiphytes, terrestrials, or lithophytes (Roberts & Dixon, 2005, Dearnaley, 2007). Approximately 80% of all orchids grow as epiphytes and their highest diversity is in the tropical regions (Sivasithamparam *et al.*, 2002, Roberts & Dixon, 2005), such as the northern Andes in South America, Madagascar, Sumatra and Borneo. Terrestrial orchids, on the other hand, are more frequently observed where temperatures are moderate, for example in Western Australia which is regarded as the diversity hotspot (Brundrett *et al.*, 2001, Swarts & Dixon, 2009). Moreover, the overall orchid abundance and diversity seems to decrease with an increased distance from the equator (Brundrett *et al.*, 2001).

The current distribution of orchids suggests that their origin dates to 125 million years ago during the fragmentation of Gondwanaland (Roberts & Dixon, 2005). However, due to a lack of substantial fossil records the time in which orchids originated is not yet resolved. The oldest fossil record dates from the Late Cretaceous era 74-85 million years ago (Roberts & Dixon, 2005, Zhang *et al.*, 2018). This is based on the discovery of pollinia from *Meliorchid caribea*. Pollen of *M. caribea* was found on the back of its pollinator bee *Proplebeia dominicana* trapped in amber (Roberts & Dixon, 2005, Ramirez *et al.*, 2007). Following the mass extinctions 65 million years ago, at the Cretaceous-Tertiary boundary, Orchidaceae appear to have undergone dramatic radiation. The survival and high species diversity of orchid species are thought to be a result of their specialized biotic adaptations. (Sivasithamparam *et al.*, 2002, Zhang *et al.*, 2018).

2.2 Adaptive radiation of orchids

Some of the specialized biotic adaptations that may have resulted in the rapid adaptive radiation of orchids include, (1) a crassulacean acid metabolism, a water-conserving photosynthetic pathway evolved from the ancestral C_3 photosynthesis state (Silvera *et al.*, 2009), (2) epiphytism which allows orchids to invade new habitats that are not occupied by other vascular plants. This has significantly increased the abundance of epiphytes compared to terrestrial species (Givnish *et al.*, 2015). Other orchid adaptations are their (3) ability to grow in nutrient-poor substrates, (4) form symbiotic relationships with microbes, (5) specialized associations with pollinators, (6) wind dispersal of seed and production of a large number of seeds from a single pollination event (Sivasithamparam *et al.*, 2002, Roberts & Dixon, 2005, Dearnaley *et al.*, 2012, Zhang *et al.*, 2018).

Orchid seed pods on average produce over a million dust-like seeds which allow for significant genetic variability and high rates of dispersal by wind across large distances (Sivasithamparam *et al.*, 2002, Roberts & Dixon, 2005). Orchid seeds are microspermous and do not have an endosperm, they therefore lack the nutrients required by the developing embryo during germination (Pant, 2013). For successful germination, orchid seeds need to be infected by a mycorrhizal fungus from which they derive all the required nutrients during germination (Zhang *et al.*, 2018). The ability of orchids to use nutrients obtained from their fungal partners is assumed to have contributed to their high species diversity as this permitted orchids to thrive in different types of environmental conditions (Yagame *et al.*, 2017).

3. Orchid propagation and conservation

Despite being the most species-rich family (Taylor & McCormick, 2008), the Orchidaceae has the highest number of plants that are at risk of extinction (Brundrett *et al.*, 2001). The rapid loss of orchid diversity is concerning, especially because the Orchidaceae contains some of the most striking and vulnerable plant species (Basu *et al.*, 2016). Globally, many orchid species are at risk of extinction due to anthropogenic activities (Sivasithamparam *et al.*, 2002). These include habitat alteration or destruction through logging, agriculture, and plantations, habitat fragmentation, urban development and mining (Sivasithamparam *et al.*, 2002, Zhang *et al.*, 2018). In addition, increased collection, trade of wild orchids for horticultural use, amateur/illegal collection of medicinal and consumable orchids decreases orchid populations. Other factors affecting orchid populations include natural incidences such as forest fires, floods, frosts and other drastic climatic conditions (Brundrett *et al.*, 2001). Due to the dramatic decline of orchid populations across a wide range of geographical regions, effective strategies are urgently required for their conservation.

Current orchid conservation initiatives factor in beneficial fungal associates when implementing *in situ* and *ex situ* conservation strategies. The *ex-situ* conservation measures are those that preserve orchid species outside their natural setting including seed banks and orchid tissue culturing (Pant, 2013). For orchid translocation strategies a specific fungus must also be introduced to successfully establish adult orchids in their new habitats (Pereira *et al.*, 2018). Seed baiting techniques are also used in this case to survey the fungal diversity in the new proposed locations (Swarts & Dixon, 2009). Orchid seeds can also be stored for a long period in liquid nitrogen (-196°C). These *ex-situ* strategies are used as backups for critically endangered species that may become extinct in the future (Sivasithamparam *et al.*, 2002). Although this approach is effective, orchid-fungal associates will still be required when planting these seeds in natural environments. For this reason, the simultaneous cryopreservation of orchid seeds and their fungal associates is being implemented. This increases the chances of survival of each orchid in a natural environment in the future (Ercole *et al.*, 2013, Merritt *et al.*, 2014). This dual cryopreservation approach was tested on three orchid species *Dactylorhiza fuchsii, Pterostylis saxicola,* and *Diuris arenaria*. The orchid seeds and the fungal partners were preserved for 6 months without a reduction in germination (Merritt *et al.*, 2014). In addition, the fungal associate did not lose its ability to germinate orchid seeds (Ercole *et al.*, 2013).

Alternatively, *in situ* conservation, which is the preservation of orchids in their natural habitats has been commonly implemented worldwide (Pant, 2013). This approach focuses on habitat protection and management, including conversion of the orchids' natural habitats into protected areas and placing restrictions on the illegal collection of certain orchids. *In situ* and *ex-situ* conservation approaches are currently being used simultaneously to ensure improved conservation success for most orchid species. In both conservation approaches the favourable fungal partners of each orchid species need to be identified and their biology studied for plant growth-promoting properties and their ability to stimulate orchid seed germination.

4. Beneficial plant-associated fungi

Soil inhabiting fungi interact with plant roots in numerous ways. These fungi may have a neutral, beneficial, or detrimental effect on plant survival. Pathogenic fungi are detrimental to plant health by causing plant diseases and reducing overall plant fitness (Doehlemann *et al.*, 2016). Over time plants have developed defences against these fungi and therefore reduce their abundance in the rhizosphere. As a result, beneficial and neutral fungi are found in much higher abundance (Zeilinger *et al.*, 2016). Beneficial plant-fungal interactions provide stability and improved survival of both partners. Therefore, they are often referred to as mutualistic symbiotic relationships (Zeilinger *et al.*, 2016). Commonly found beneficial plant-fungal interactions exist with endophytic and mycorrhizal fungi. Endophytes are abundant and are known to live in plant roots without causing any symptoms (Rodriguez *et al.*, 2009). Some endophytes however have been found to increase the resistance of plants to pathogens and environmental stresses as well as to promote plant growth (Khan & Doty, 2011, Mengistu, 2020). Mycorrhizal fungi are regarded as the most important beneficial symbionts and mainly improve the nutritional status of the plant (Teotia *et al.*, 2017). These beneficial fungal associates are also studied for their potential roles in agriculture, as fertilizer replacements or microbial pesticides (Strobel, 2018).

Over 90% of all plant species form some type of mycorrhizal relationship (Lopez-Raez *et al.*, 2010, Foo *et al.*, 2013). Mycorrhizae or 'root fungus' (Sivasithamparam *et al.*, 2002) are the associations that form between symbiotic soil fungi and roots of vascular plants (Hadley, 1970, Brundrett, 2002). This symbiosis involves the exchange of nutrients between plants and fungi (Lopez-Raez *et al.*, 2010). The fungus obtains a stable source of photosynthetically derived carbohydrates from the plant (Brundrett, 2002), while the host plant receives a variety of benefits from the mycobiont. In addition to improving the uptake of mineral nutrients and

water, mycorrhizae also help to reduce the effect of environmental stress such as drought, heavy metal contaminations and salinity and improve resistance against pests and pathogens (Jacquemyn *et al.*, 2017). Mycorrhizae produce extensive hyphal networks extending from the host plant's roots into the soil and thus increases the overall surface area for nutrient acquisition (Mohammadi *et al.*, 2011). Siderophores and acids are released by mycorrhizae to free "tightly bound" mineral nutrients such as phosphorus, iron and magnesium (Teotia *et al.*, 2017). Also, mycorrhizae can decompose bark and litter to release mineral nutrients from these materials (Rai & De, 2014). The fungal mycelia then transport the acquired nutrients to the root-fungal interface where they are absorbed by the host. These mycobionts can absorb and transport all the essential nutrients required for plant growth (Dearnaley & Cameron, 2017, Teotia *et al.*, 2017). Plants colonized by mycorrhizae can survive under harsh conditions such as drought and nutrient-deficient soils. As a result, plants colonized by mycorrhizae have better chances of survival over plants that do not form these relationships (Mohammadi *et al.*, 2011). Over 6000 fungal species can form mycorrhizal interactions with about 240 000 plant species (Singth, 2007). All these fungal species form different types of mycorrhizal associations with different plant species.

4.1 Types of mycorrhizal associations

Initially, mycorrhizae were classified into three broad groups including the endomycorrhizae (*i.e.* intracellular network), ectomycorrhizae (*i.e.* intercellular network), and ectendomycorrhizae (*i.e.* combination of inter and intracellular hyphal networks) (Mohammadi *et al.*, 2011, Roth-Bejerano *et al.*, 2014). This classification was based mainly on the location of the plant-fungus interface. Later the endomycorrhizal group was divided further into arbuscular mycorrhizae, ericoid mycorrhizae and orchid mycorrhizae (Dighton, 2009, Roth-Bejerano *et al.*, 2014). This group was divided because it contained phylogenetically and functionally diverse fungal lineages (Mohammadi *et al.*, 2011). Also, members of this group vary based on the hyphal structures formed within their plant hosts. Currently, there are seven recognized types of mycorrhizae namely: ericoid, monotropoid, arbutoid, orchid, arbuscular, ectomycorrhizae, and ectendomycorrhizae (Brundrett, 2004, Ramasamy *et al.*, 2011). Each type of mycorrhizal association is unique, and a summary of their differences is shown in Table 1.

The most ancient mycorrhizal associations are the arbuscular mycorrhizae (Glomeromycotan fungi) which arose about 400 million years ago (Mohammadi *et al.*, 2011, Foo *et al.*, 2013).

About 240 species of Glomeromycotan fungi have been described and associated with over 200 000 plant species (Lee *et al.*, 2013). Arbuscular mycorrhizae (AM) are placed in 18 genera of the order Glomerales (Redecker *et al.*,2013).AM are known to have assisted early rootless plants to colonize and disperse on land (Foo *et al.*, 2013). AM are also regarded as the progenitor of plant-fungal symbiosis (Brundrett, 2004) and are associated with bryophytes, pteridophytes, gymnosperms, and angiosperms (Mohammadi *et al.*, 2011). In contrast, ectomycorrhizae (ECM) occur only in gymnosperms (e.g., Pinaceae) and some angiosperms (e.g., Betulaceae). ECM consist of about 2000 fungal taxa from the Basidiomycota, Ascomycota, and Zygomycota (Johnson & Gerhring, 2007). The largest groups of ECM belong to Basidiomycota, including the families Boletaceae and Russulaceae (Brundrett, 2002). ECM and AM are the most commonly observed and well-studied mycorrhizae. This is mainly because the mycorrhizal fungi associate with numerous plant hosts (Johnson & Gerhring, 2007, Roth-Bejerano *et al.*, 2014). The remaining five mycorrhizal groups are restricted to specific plant families and as a result, are less commonly found and not as extensively studied (Brundrett, 2002).

4.2 Factors affecting mycorrhizal interactions

Mycorrhizal associations can be influenced by biotic and abiotic factors such as climate, soil conditions, nutrient availability, host plant responses and root architecture (Majica *et al.*, 2016). Plants control their nutrient uptake by altering their root architecture and morphology (Roth-Bejerano *et al.*, 2014). Increasing their root biomass, root length and the number of root hairs improves the plant's ability to absorb nutrients as well as increase the surface area available for possible mycorrhizal associations (Sathiyadash *et al.*, 2012). Therefore, plants with shorter roots and few root hairs will have less efficient nutrient uptake. This is the case for some orchids which generally have thick succulent roots with large biomass but only a few root hairs (Zhang *et al.*, 2018). The lack of root hairs makes them more dependent on mycorrhizal fungi for nutrient acquisition (Rai & De, 2014).

Soil conditions and levels of atmospheric CO_2 can also affect mycorrhizal associations. Studies suggest that soils with limited nutrients have higher mycorrhizal abundances (Treseder, 2004). The mycorrhizae provide mainly phosphorus and nitrogen to the host plant. Therefore, soils with higher phosphorus and nitrogen content have lower levels of mycorrhizal diversity and colonization (Xu *et al.*, 2017). This is because in these soils plants reallocate their photosynthetically derived carbon to growth rather than to the symbiotic partner, resulting in

decreased mycorrhizal associations. Studies also indicate that elevated levels of CO_2 result in higher levels of mycorrhizal colonization. Higher CO_2 levels increase photosynthesis and thus more photosynthetically derived carbon is available to the mycobionts. As a result, the mycorrhizal fungi will be attracted to these plants (Treseder, 2004). However, most of these factors have only been tested in AM and ECM associations. Whether these factors affect orchid mycorrhizal associations, in the same way, is not yet known.

5. Orchid mycorrhizae

Orchid mycorrhizal fungi are morphologically different from other mycorrhizae and can colonize not only roots but stems, pseudobulbs, protocorms, rhizomes, and tubers of the host plant (Sivasithamparam *et al.*, 2002, Thakur *et al.*, 2018, Zhang *et al.*, 2018). Orchid mycorrhizae are important for all life stages of orchids such as seed germination, growth of protocorms (small, food storing underground stems formed after germination), seedlings, and adult orchids (Zhu *et al.*, 2008). Seed germination is initiated once orchid mycorrhizal fungi penetrate and invade the plant embryo (Suryantini *et al.*, 2015). At this point, the transfer of nutrients is only from the fungus to the plant (Jacquemyn *et al.*, 2017). The fungus in this case does not obtain any photosynthesis-derived carbon sources. This type of nutritional strategy is called 'mycoheterotrophy' (Mosquera-Espinosa *et al.*, 2013, Jacquemyn *et al.*, 2017).

Following penetration, fungal hyphae form coils of undifferentiated hyphae called 'pelotons' (Sivasithamparam *et al.*, 2002, Dearnaley, 2007). These coils develop in the cortical cells of the root, stems, rhizomes, and protocorms (Brundrett *et al.*, 2001, Sivasithamparam *et al.*, 2002, Zhu *et al.*, 2008). The presence of pelotons in the cortical cells shows active mycorrhizal symbiosis between orchid roots and fungi (Zhu *et al.*, 2008) as these are the points of nutrient exchange with the orchid mycorrhizae. The level of cortical cell colonization may also vary between orchid species. For example, analysis of the *Isochilus lineares* orchid revealed that about 75% - 80% of the pelotons were found in the cortical cells of the orchid's roots, whereas in *Epidendrum rigidum* and *Polystachya concreta* only 5% - 10% of the cortical cells were colonized. Furthermore, different fungal species have been shown to associate with distinct parts of the plant *i.e.* the stem collar, root, and rhizomes (Pereira *et al.*, 2005, Suryantini *et al.*, 2015).

5.1 Types of orchid mycorrhizal infection

There are two known types of orchid mycorrhizal infection, tolypophagy, and ptyophagy (Rasmussen, 2001). Tolypophagy is a type of mycorrhizal colonization whereby the fungi form hyphal coils or pelotons. Initially, the pelotons can be observed as intact coils in the root cortical cells. Then the pelotons are lysed by the orchid and are observed as completely collapsed hyphal masses (Brundrett *et al.*, 2001, Sivasithamparam *et al.*, 2002). This is the most common type of orchid mycorrhizal infection and is often observed in terrestrial orchids. In contrast, ptyophagy is found in only a few orchid species that are myco-heterotrophic (e.g., *Gastrodia*). Ptyophagy is described as the lysis of only the hyphal tips causing the release of fungal cell contents into the plant cell. In both types of orchid mycorrhizal infection, orchids obtain nutrients by absorbing the fungal cell contents released during lysis. The continuous lysis and digestion of the fungal hyphae and pelotons allows for successive rounds of peloton formation, lysis, and reinfection in the same root cells. For this reason, many studies focussing on orchid mycorrhizae rely on the isolation of pelotons and fungal hyphae directly from orchid roots (Dearnaley, 2007, Zhu *et al.*, 2008).

5.2 Mycorrhizal associates of autotrophic, mycoheterotrophic, and mixotrophic orchids

Orchids are either autotrophic, mycoheterotrophic, or mixotrophic and this is determined by the level of dependence on the mycorrhizal partner in their adult stage (Jacquemyn *et al.*, 2017, Yagame *et al.*, 2017). Autotrophs develop green leaves, can photosynthesize, and produce their own carbohydrates (Dearnaley, 2007). These orchids form a mutualistic mycorrhizal symbiosis with saprobic mycorrhizal fungi of the families Ceratobasidiaceae, Tulasnellaceae, Serendipitaceae, and Sebacinaceae to supply them with mineral nutrients (Yagame *et al.*, 2017). Mixotrophs develop partial photosynthesis but obtain both carbohydrates and mineral nutrients from mycorrhizal fungi. Obligate mycoheterotrophs, never develop a photosynthetic capacity and rely solely on their fungal associates for carbohydrates and mineral nutrients (Jacquemyn *et al.*, 2017).

Approximately 235 orchid species are mycoheterotrophic and this form of symbiosis is likely parasitic to the mycorrhizal partner (Girlanda *et al.*, 2011, Jacquemyn *et al.*, 2017). These orchids obtain all their nutritional requirements from mycorrhizal partners which are commonly saprobic fungi including *Marasmius coniatus, Armillaria jezoensis, Erythromyces*

crocicrea, Coprinellus disseminates and the Psathyrellaceae family (Yagame *et al.*, 2017). Mycoheterotrophic orchids are also known to manipulate symbiosis by interacting with mycorrhizae of surrounding trees or plants (Girlanda *et al.*, 2011). In this association, a tripartite relationship is formed comprising of the mycoheterotrophic orchid, a mycorrhizal fungus, and an autotrophic plant. In this relationship, the orchid obtains nutrients from a mycorrhizal fungus which is also associated with an adjacent plant. Some well-studied examples of this tripartite network include a *Ceratobasidium* sp. which forms an orchid mycorrhizal symbiosis with a fully subterranean orchid *Rhizanthella gardneri* and simultaneously forms an ectomycorrhizal symbiosis with the autotrophic shrub *Melaleuca scalene* (Bougoure *et al.*, 2010). Also, a mycoheterotrophic orchid *Corallorhiza trifida* obtains carbohydrates from *Salix repens* through a mycorrhizal associate (*Ceratobasidium cornigerum*) common to both partners (McKendrick *et al.*, 2000).

Orchid-mycorrhizal interactions can also dynamically change. Some orchid species have been shown to change fungal partners depending on environmental pressures (Dearnaley *et al.*, 2012). Fungal switching was observed in *Goodyera pubescens* which was found to switch mycorrhizal partners in the field when faced with drought conditions (McCormick *et al.*, 2006). It is therefore not clear if the environment determines mycorrhizal interactions (Phillips *et al.*, 2011) or if orchid species form specialized interactions only with a predetermined set of fungal taxa (Dearnaley *et al.*, 2012).

5.3 Orchid mycorrhizal specificity

In plant-fungal interactions specificity refers to the number of fungal taxa with which a certain host interacts (Majica *et al.*, 2016). Different orchids in diverse habitats have been observed to have different levels of specificity. Orchids can be mycorrhizal generalists, associating with several taxonomically distinct mycorrhizal taxa (Hadley, 1970), or can be mycorrhizal specialists (Ogura-Tsujita *et al.*, 2009, Jacquemyn *et al.*, 2017). Orchids are generally categorized as having three levels of mycorrhizal specificity: (1) Absolute specificity whereby one orchid associates with only one mycorrhizal fungal species. (2) Taxon level specificity where an orchid interacts with more than one mycorrhizal member of the same taxonomic group *i.e.,* genus or family. (3) Non-specificity where a single orchid species associates with a range of unrelated mycorrhizal taxa (Hadley, 1970, Hirsch *et al.*, 1997, Dearnaley *et al.*, 2012). To add to this complexity, orchids may form associations with different groups of mycorrhizae (Hadley, 1970). Orchid-mycorrhizal specificity can vary even within

the same orchid species found in different regions. It is thus thought that the geographical location of an orchid may affect its mycorrhizal community and specificity (Majica *et al.*, 2016, Jacquemyn *et al.*, 2017). In addition, absolute and taxon level specificity is suspected to play a role in the patchy distribution of orchid species (Phillips *et al.*, 2011).

Many mycorrhizal fungi associating with mature orchid roots may not stimulate successful germination of orchid seeds. However, fungi from a different host and origin may initiate germination. Therefore, the mycorrhizae required to germinate orchid seeds may differ from those species that are required to establish and sustain the growth of orchids in the wild (Hadley, 1970). For example, *Gastrodia elata* relies on *Mycena osmundicola* for germination but relies on *Armillaria mellea* for nutritional supply after germination (Liu *et al.*, 2010). The effectiveness of the mycorrhizal interaction depends on the compatibility between the plant and mycobiont (Van der Heijden & Kuyper, 2001). Once a suitable interaction is formed, successful germination, plant growth, and establishment of the orchid in a natural environment can be achieved (Otero *et al.*, 2011).

6. Taxonomy of orchid mycorrhizae

Orchid mycorrhizae belong to the phyla Basidiomycota and Ascomycota (Dearnaley, 2007). Only a few Ascomycota within the Pezizaceae have been found to form an orchid mycorrhizal symbiosis. The most common genera in this family are species in the genera *Tuber*, *Tricharina,* and *Peziza* (Dearnaley *et al*., 2012, Roth-Bejerano *et al*., 2014). Within the Basidiomycota, orchid mycorrhizae originate from two classes: Atractiellomycetes (Jacquemyn *et al.*, 2017, Zhang *et al.*, 2018) and Agaricomycetes (Sivasithamparam *et al.*, 2002, Dearnaley *et al*., 2012, Jacquemyn *et al*., 2017). The latter is further divided into six orders, Agaricales, Cantharellales, Russulales, Hymenochaetales, Sebacinales, and Thelephorales (Dearnaley *et al*., 2012). The most common orchid mycorrhizae are in the orders Cantharellales, Russulales and Sebacinales. The dominant genera in each order are *Russula* (Russulales), *Sebacina* (Sebacinales) and *Ceratobasidium, Thanatephorus, Tulasnella* of the Cantharellales (Hadley, 1970, Dearnaley *et al*., 2012).

Species in the orders Cantharellales and Sebacinales were the first-ever described orchid mycorrhizal fungi. This discovery was made in 1899 by Noël Bernard who then classified these orchid mycorrhizal fungi as '*Rhizoctonia*'. However recent taxonomic studies reveal that most *Rhizoctonia* species are Basidiomycota from the order Cantharellales (Yang & Li, 2012,

Selosse *et al.*, 2017). The families Ceratobasidiaceae and Tulasnellaceae in this order are now regarded as cosmopolitan orchid mycorrhizal fungi. This is because fungi from each family are known to associate with most orchid species found in different parts of the world (Jacquemyn *et al.*, 2017). As a result, most of the information and descriptions available for orchid mycorrhizae are on fungi in the *Rhizoctonia* complex. Members of this complex are filamentous fungi that do not produce spores (Yang & Li, 2012). In addition to being orchid mycorrhizal symbionts, fungal species in the *Rhizoctonia* complex can be saprophytes or devastating plant pathogens (Hadley, 1970, Otero *et al.*, 2011).

6.1 Distinguishing Rhizoctonia-like fungi

Different fungal taxa are placed in the *Rhizoctonia* complex based on morphological traits (Table 2). These traits include, for example, the number of nuclei per hyphal cell, colony colour, a pattern of concentric circles, and the ability to form sclerotia (Suryantini *et al.*, 2015). Furthermore, *Rhizoctonia*-like fungi can be distinguished by their basidiospores (or monilioid structures) which may vary in length, size, and shape between different species (Mosquera-Espinosa *et al.*, 2013). These fungi also have hyphal branches at right angles, with septa located near the branches (Suryantini *et al.*, 2015). *Rhizoctonia*-like fungi can be divided into three groups based on the number of nuclei per hyphal compartment namely, uninucleate, binucleate, and multinucleate. Most *Rhizoctonia* isolates are either binucleate or multinucleate. However, in rare cases, isolates of *Rhizoctonia* are uninucleate (Zhou *et al.*, 2015). These strains are found in *Ceratobasidium* anamorphs such as *Ceratobasidium bicorne* (Hietala *et al.*, 2001), *Rhizoctonia* isolate JN, and *Rhizoctonia quercus* (Oteo *et al*., 2002).

Based on their ability to anastomose, the *Rhizoctonia* multinucleate group is divided into 14 anastomosis groups (AG). The 14 groups are denoted AG1 to AG13 and the 14th group is denoted AG B1. Anastomosis groups AG 1-4 consist of plant pathogenic fungi, that cause disease symptoms such as damping off, root rot, sheath, and leaf blight in a wide range of hosts. The AG B1 group consists of multinucleate species that are non-pathogenic and are found only in forest soils and plants. Multinucleate *Rhizoctonia* species known to associate with orchids include anastomosis groups AG 1, AG 6, and AG 12 (Oteo *et al.*, 2002). The AG1 group has a subgroup denoted AG1-AG1D which consists of orchid mycorrhizal species *Waitea circinata, Rhizoctonia globulis,* and *Tulasnella* sp. (Suryantini *et al.*, 2015).

Distinctive traits within the *Rhizoctonia* complex are also helpful in separating *Rhizoctonia*-like species that are orchid mycorrhizae from pathogenic taxa (Mosquera-Espinosa *et al.*, 2013). For example, the cultural morphologies differ and orchid mycorrhizae form structures that resemble loose aggregates of hyphae known as poorly developed sclerotia or resting bodies (Sivasithamparam *et al.*, 2002). Such loose sclerotia have been described for *Thanataphorus cucumeris,* the teleomorph state of *Rhizoctonia solani* (Blakeman & Hornby, 1966, Tu & Kimbrough, 1978). However, it is thought that there is an overlap or switching of states between the pathogenic and symbiotic states of *Rhizoctonia* associated with orchids, but the mechanisms are not clearly understood.

6.2 Rhizoctonias: 'Pathogens, endophytes or symbionts'?

Fungi in the *Rhizoctonia* complex have a wide range of habits: plant pathogens, saprotrophs and mycorrhizal symbionts of orchids (Hadley, 1970, Mosquera-Espinosa *et al.*, 2013). These fungi can cause disease in various crops such as rice, tomato and onions and result in major economic losses worldwide (Mosquera-Espinosa *et al.*, 2013). However, when the same fungi are in association with orchids, the fungi can promote growth without causing disease. *Thanatephorus cucumeris*, the basidial stage of *Rhizoctonia* is a devastating plant pathogen of various crops (Hadley, 1970, Mosquera-Espinosa *et al.*, 2013), however, it forms an active symbiosis with orchid species such as *Dactylorhiza purpurella, Coeloglossum viride,* and *Spathoglottis plicata* (Mosquera-Espinosa *et al.*, 2013). A similar scenario has also been described for species in the Agaricales. For example, some orchids associate with plant pathogens such as *Armillaria mellea* (Lok *et al.*, 2009, Liu *et al.*, 2010, Roth-Bejerano *et al.*, 2014). The mechanism(s) underlying this apparent switch from pathogenic form to mycorrhizal has not been studied in depth.

7. Approaches to studying orchid-mycorrhizal relationships

To understand mycorrhizal relationships, a combination of ecological, biochemical, and molecular techniques is used (Dearnaley *et al.*, 2012). Studies using these techniques aim to unravel the numerous factors affecting the formation of mycorrhizal interactions, their importance and ultimately identify associated mycobionts (Novak *et al.*, 2014). Globally orchids are of conservation concern, therefore understanding the complexities involved in the orchid-mycorrhizal association is vital for the conservation of orchid populations (Brundrett *et al.*, 2001). Besides, orchids are difficult to propagate without their mycorrhizal symbionts (Zhu

et al., 2008). Thus, propagating orchids in the presence of their identified mycorrhizal partners contribute to solving this problem (Brundrett, 2002).

Orchid mycorrhizal studies often use a combination of both culture-dependent and culture-independent approaches (Ma *et al.*, 2016). Earlier research predominantly used culture-dependent methods focusing on the morphological characteristics of these fungi (Athipunyakon *et al.*, 2004). This approach includes microscopic analysis of orchid mycorrhizal structures within root cells (Sathiyadash *et al.*, 2012) as well as isolation of fungi from colonized orchid roots, rhizomes, and tubers which are then further characterized based on their morphological features. Currently, with improving technologies, molecular-based approaches are used in the study of orchid mycorrhizae. Most contemporary studies prefer to use both approaches simultaneously for three main reasons: (1) orchid mycorrhizal isolates can be used for further studies such as their characterization and screening for new biologically active metabolites (Sun & Guo, 2012); (2) obtaining fungal isolates also allows for germination and growth trials to be conducted (Hosomi *et al.*, 2012); (3) a comprehensive list of the overall mycorrhizal diversity including unculturable species associated with each orchid species can be identified using the culture-independent approach (Huang *et al.*, 2014, Zhao *et al.*, 2014).

7.1 Culture-dependent techniques: Microscopy

Microscopic techniques were first used in studies by Wahrlich (1886) and Janse (1897) to observe orchid mycorrhizal associations of over 50 orchid species as referenced in (Sally *et al.*, 2008). In both these studies, pelotons were observed in the cortical cells of orchid protocorms (Zhu *et al.*, 2008). Since then, microscopy has been used in studies to confirm positive mycorrhizal interactions (Table 3). However, the association with stems, thalli, tubers, and corms are less frequently analysed microscopically.

Common observations using different microscopy techniques revealed that orchid mycorrhizal infection of orchid seeds occurs through suspensor cells of the embryo or the root hairs of protocorms (Peterson *et al.*, 1996, Baskin & Baskin, 2014). Furthermore, hyphal penetration and peloton formation occur in the cortical cells (Peterson *et al.*, 1996). The layer of cortical cells colonized by either intact or lysed pelotons varies between different orchid species (Table 3). Interestingly, microscopic analysis revealed the first record of orchid-associated arbuscular mycorrhizae in *Disperis neilgherrensis*. Appressoria-like structures and arbuscules were observed in this orchid's root cells, thus confirming the association.

Furthermore, it was shown that cells infected by pelotons had no arbuscules, and *vice versa* (Muthukumar *et al.*, 2013). Once the presence of mycorrhizal infection is confirmed in the orchid tissues the next step is to obtain the orchid mycorrhizae in culture.

7.2 Culture-dependent techniques: Fungal Isolations

Isolation and screening of potential orchid mycorrhizal fungi from orchid tissue are important for subsequent orchid studies such as seed germination and growth trials (Brundrett *et al.*, 2001). Having a collection of orchid mycorrhizal isolates will also make it easier to implement *ex situ* conservation (Sivasithamparam *et al.*, 2002). To obtain these isolates several techniques can be used. Most commonly, orchid roots and tubers are surface sterilized and plated on different types of growth media, such as potato dextrose media (PDA), cornmeal agar (CMA), water agar (WA), and malt extract media (MEA), just to name a few (Currah *et al.*, 1987). Another technique that is currently used is transferring individual pelotons observed microscopically onto selective media (Zhu *et al.*, 2008). Seed baiting techniques are also used to obtain orchid mycorrhizal isolates directly from soils close to growing adult orchids. Orchid seeds are carefully placed in mesh and embedded in the soil (Rasmussen & Whigham, 1993). Once protocorms or seedlings develop, fungal isolations are conducted.

Following several sub-culturing steps, pure cultures are described based on morphological traits such as colony texture, colour, and growth rate (Currah *et al.*, 1987), (Table 2). To better classify these isolates, microscopic features such as the number of nuclei, hyphal septa, and presence or absence of monilioid cells are also used (Athipunyakon *et al.*, 2004). The colony colour of *Rhizoctonia*-like fungi ranges from white to dark brown and is often used to classify orchid mycorrhizae to genus level (Suryantini *et al.*, 2015).

DNA can be extracted from the fungal isolates and PCR amplicons sequenced for preliminary identification by sequence comparison with known taxa. The sequences can also be used in a phylogenetic analysis to confirm either their identity based on sequence comparisons or confirming the presence of a unique species. The most commonly amplified gene region is the internal transcribed spacer (ITS) region, the universal DNA barcode for fungi (Schoch *et al.*, 2012). Other gene regions that are often used for better identification of these orchid mycorrhizae include the 28S ribosomal DNA (rDNA), β-tubulin, glyceraldehyde-3-phosphate dehydrogenase, actin, and the mitochondrial large subunit (Muthukumar *et al.*, 2013, Ma *et al.*, 2016, Raja *et al.*, 2017).

Culture dependent approaches are frequently used for studying orchid mycorrhizae. However, this approach has some limitations. Fungal isolations rely on surface sterilization which can influence which of the orchid mycorrhizae are isolated (Zhu *et al.*, 2008) and in some cases can result in surface fungal contaminants to be identified as orchid-fungal associates (Ma *et al.*, 2016). Other limitations to fungal isolations include: (1) fungal and bacterial contaminations, (2) isolating non-mycorrhizal fungi, and (3) not isolating unculturable mycobionts (Zhu *et al.*, 2008). Zhu *et al.,* (2008) published a method that may resolve some of these problems. In this technique, orchid mycorrhizae are obtained directly from individual pelotons in each orchid root. The pelotons are then treated with antibiotics such as streptomycin sulphate and potassium penicillin to prevent bacterial contamination. By using this approach only the fungi that form these pelotons are cultured (Zhu *et al.*, 2008).

Fungal identifications based on morphology alone are thought unreliable. A significant limitation of using morphology is that the taxonomy of many fungi cannot be determined accurately. This is because the taxonomy of fungi relies strongly on the characteristics of the reproductive structures and these are often not produced in culture (Sun & Guo, 2012). Some studies also showed morphological plasticity in fungi; thus, two or more species may look similar but are distinct species based on other characteristics (Muller *et al.*, 2007, Taylor & McCormick, 2008, Gonzalez *et al.*, 2016). The grouping of orchid mycorrhizae based solely on morphology resulted in the formation of the *Rhizoctonia*- complex where closely related species could not be differentiated (Carling *et al.*, 1999). Studies that incorporate DNA-based methods, however, revealed species in the complex can be differentiated more accurately based on DNA sequence differences (Taylor & McCormick, 2008, Amaradasa *et al.*, 2013, Gonzalez *et al.*, 2016).

7.3 Culture-independent - DNA based techniques

Direct sequencing of DNA extracted from orchid tissues has revealed a large diversity of orchid mycorrhizal associates (Setaro *et al.*, 2011, Ma *et al.*, 2016). For this approach, ITS primers were developed specifically for the amplification of orchid mycorrhizal fungi. These primers also help to reduce the chances of amplifying DNA from the plant material (Taylor & McCormick, 2008). The use of this approach circumvents the need for isolations, thus reducing the possibility of false-positive results. This approach has led to the identification of unculturable mycorrhizae and understanding of their interactions with their hosts (Sun & Guo, 2012).

Molecular cloning and next-generation sequencing are the more commonly used culture-independent techniques to study orchid mycorrhizal communities (Table 4). Molecular cloning is a combination of molecular techniques that involving the isolation of DNA from a sample and inserting a fragment of the DNA into a modified cloning vector that is then propagated in a suitable bacterial host. The clones can be used to make multiple identical copies of the DNA fragment or express a resulting protein (Bertero *et al.*, 2017). Over the past decade, there has been a general shift from the molecular cloning approach and conventional Sanger sequencing in favour of next-generation sequencing approaches (Zhao *et al.*, 2014, Forin *et al.*, 2018). Next-generation sequencing allows for fungal communities to be studied much more efficiently and rapidly. For example, orchid mycorrhizal associates of different orchid species, or orchids in different habitats and between orchid roots and their immediate soils can be compared (Huang *et al.*, 2014, Oja *et al.*, 2015, Pecoraro *et al.*, 2018). The cloning approach, on the other hand, is time-consuming, especially if multiple gene regions are used in the study. Also, this approach only allows for the sequencing of a limited number of clones which are representatives of only the dominant fungal taxa in the microbial community (Douterelo *et al.*, 2014). Despite these limitations, the cloning approach can be used to effectively identify orchid mycorrhizal taxa (Ma *et al.*, 2016). Most of the studies (Table 3) showed that the Cantharales – *Ceratobasidium* sp. and *Tulasnella* sp. are frequently reported as the most dominant orchid mycorrhizal taxa associated with various orchids growing in different parts of the world (Hadley, 1970, Jacquemyn *et al.*, 2017).

8. Chemical studies of orchid mycorrhizae

Techniques such as mass spectrometry and isotope analysis are often used in fungal ecology and are also being explored in orchid- mycorrhizal associations (Griffith, 2004, Liebel & Gebauer, 2011, Lekberg *et al.*, 2012). While DNA based studies provide information on the identity and variations of mycorrhizal diversity at different orchid life stages, between sites and among orchid species (Huynh *et al.*, 2004, Baskin & Baskin, 2014, Jacquemyn *et al.*, 2016, Lee *et al.*, 2017), chemical studies can reveal the nutritional interactions between the symbiotic partners. For example, orchid mycorrhizal studies involving isotope analysis and spectrometry provide information about the mechanism and direction of nutrient transfer and the type and amount of nutrients transferred (Ljungquist & Stenstrom, 1983, Bidartondo *et al.*, 2004, Dearnaley *et al.*, 2012, Ercole *et al.*, 2014). Therefore, these studies also provide important knowledge about the nutrient acquisition in orchid mycorrhizal associations.

8.1 Stable and Radioactive isotope analysis

The natural abundances of stable carbon and nitrogen isotopes between orchids and their mycobionts have been analysed. This approach has been used mainly to determine the degree of trophism (autotrophy, mixotrophy, and mycoheterotrophy) and the flow of nutrients between the symbionts (Girlanda *et al.*, 2011, Dearnaley *et al.*, 2012, Kuga *et al.*, 2014). Radioactively labelled isotopes and isotope imaging studies are normally used to trace carbon that comes from the mycobiont (Girlanda *et al.*, 2011, Kuga *et al.*, 2014).

8.1.1 Trophism

The stable and radioactive isotope approach was used to determine the level of mycorrhizal specificity of photosynthetic orchids (Girlanda *et al.*, 2011). This was done by comparing the levels of fungus-derived carbon and nitrogen levels in the mycorrhizal fungi and orchid leaves (Girlanda *et al.*, 2011). Autotrophic orchids generally have lower levels of natural isotopes compared to their mycorrhizal partners (Dearnaley *et al.*, 2012). This is expected for orchids that are less reliant on their mycobionts. Mycoheterotrophic orchids on the other hand are largely dependent on their mycorrhizal partners and therefore have either similar or higher amounts of ^{15}N and ^{13}C isotopes (Dearnaley *et al.*, 2012). Mixotrophs naturally have photosynthetic levels that are intermediate between autotrophs and mycoheterotrophs. Therefore, mixotrophs have natural isotope abundances that are intermediate between autotrophs and mycoheterotrophs (Dearnaley *et al.*, 2012).

8.1.2 Flow of nutrients

Using the stable and radioactive isotope analysis it was shown that C and N can flow bi-directionally between the orchid and fungus. The parasitic nature of mycoheterotrophic orchids has also been shown using this approach (Ogura-Tsujita *et al.*, 2009, Liebel & Gebauer, 2011). In a study done by McKendrick *et al*. 2000, it was shown that ^{14}C labelled photosynthates moved from one tree species to the mycoheterotrophic orchid *Corallorhiza trifida* via ectomycorrhizal fungi (Dearnaley *et al.*, 2012, Ma *et al.*, 2016). It was also shown that Mediterranean meadow orchids had a much higher ^{15}N compared to neighbouring non-orchid species (Girlanda *et al.*, 2011). In a study by Hynson *et al*. (2009), the bidirectional transfer of carbon between *Goodyera repens* and *Ceratobasidium cornigerum* has also been demonstrated using this method (Hynson *et al.*, 2009, Girlanda *et al.*, 2011). In the same study, it was demonstrated that the net transfer of carbon from *G. repens* to *C. cornigerum* was five times

more than the transfer from fungus to plant (Hynson *et al.*, 2009). This further supports the type of tropism of this orchid in showing its lack of dependence on fungus-derived nutrients (Dearnaley *et al.*, 2012).

8.2 Gas chromatography – detection of phytohormones

Gas chromatography-mass spectrometry analysis (GC/MS) is frequently used to identify growth-promoting hormones produced by mycorrhizal species (Barroso *et al.*, 1986). Phytohormones produced by mycorrhizae play important roles in seed germination (Hirsch *et al.*, 1997, Yeung, 2017), plant growth, and sustaining mycorrhizal interactions (Ljungquist & Stenstrom, 1983, Xu *et al.*, 2018). These phytohormones or their derivatives have been identified as indole acetic acids (IAA), indole-3-ethanol (IEt), jasmonic acids, salicylic acid, brassinosteroids, oligosaccharides, gibberellin (GA), ethylene, and abscisic acid (Barroso *et al.*, 1986, Hirsch *et al.*, 1997, Tsavkelova *et al.*, 2006, Foo *et al.*, 2013). It is anticipated that many derivatives will be identified as more attention is afforded to research on phytohormones produced by different mycorrhizal species.

Phytohormones produced by arbuscular mycorrhizae extracted directly from mycelia or liquid cultures have been extensively studied (Zhang *et al.*, 1999, Foo *et al.*, 2013). It was found that hormones such as auxin, cytokinin, and ethylene increase in concentration during mycorrhizal associations with these fungi (Hirsch *et al.*, 1997). For example, inoculation of *Picea abies* (spruce) seedlings with mycorrhizal fungi *Cenococcum* sp., *Hebeloma* sp. and *Laccaria* sp. showed that increased ethylene production helped with the formation of mycorrhiza (Tsavkelova *et al.*, 2006). Ectomycorrhizal fungi also produce auxin, cytokinin, GA, and B-vitamins (Hirsch *et al.*, 1997). Due to limited evidence, it is assumed that the transfer of these plant hormones from the mycobiont to the host is important for the development of protocorms (Barroso *et al.*, 1986). Moreover, unlike ectomycorrhizae and arbuscular mycorrhizae, only a few studies show that orchid mycobionts produce phytohormones.

Orchid mycobionts are known to secrete the hormones gibberellins (GA3), IAA, and ABA (Liu *et al.*, 2010, Yeung, 2017). These hormones have been shown to play a role in improving seed germination and growth of orchids in, for example, *Gastrodia elata*, *Dendrobuim hancokii*, *Ophrys lutea,* and *Liparis nervosa* (Ljungquist & Stenstrom, 1983, Liu *et al.*, 2010). Non-pathogenic *Fusarium* sp. that are commonly associated with terrestrial

orchids also produce the plant hormones GA, ethylene, and auxins (Tsavkelova *et al.*, 2008). *Fusarium* spp. such as the rice pathogen *Fusarium fujikuroi* and *F. proliferatum* belonging to the *Giberella fujikuroi* complex secrete GA (Tsavkelova *et al.*, 2008). Orchid-associated *F. proliferatum* is non-pathogenic to the host and can induce seed germination of terrestrial orchids *Cypripedium reginae* and *Dendrobium desiflorum* (Tsavkelova *et al.*, 2008). The extent to which these phytohormones affect orchids is not yet fully understood. However, these phytohormones may be important for mycorrhizal establishment similar to what has been described in arbuscular mycorrhizae.

9. Role of non-mycorrhizal fungi in their association with orchids

A broad range of non-mycorrhizal endophytes is frequently isolated from orchid roots representing over 110 genera (Ma *et al.*, 2016). Some of these fungi form a mutualistic symbiosis with orchids and assist in producing compounds that promote plant growth, alleviate the effects of environmental stressors, and aid in the formation of stable mycorrhizal associations. In return, plant hosts provide diverse niches for endophytes (Xia *et al.*, 2019). Common endophytes associated with orchids are categorized as class two endophytes (Rodriguez *et al.*, 2009). These consist mainly of fungi residing in the Ascomycota (Pezizomycotina) and a few Basidiomycota (specifically in the Agaricomycotina and Pucciniomycotina) (Rodriguez *et al.*, 2009). These class 2 endophytes are mutualistic, and able to colonize plant roots whereby they confer some benefits to the hosts while also obtaining nutrition in exchange (Rodriguez *et al.*, 2009, Ma *et al.*, 2016). Commonly isolated genera include various species of *Guignardia, Conocybe, Cryptococcus, Trichosporiella, Beauveria, Trichosporiella, Gliocladium, Cephalosporium, Papulaspora, Fusarium, Phacodium, Cladosporium, Alternaria, Thyridaria,* and *Chaetomium* (Huang *et al*., 2014). These fungi do not form characteristic orchid mycorrhizal associations and are not of the *Rhizoctonia* complex. However, these fungi directly or indirectly improve orchid growth (Pant *et al.*, 2017).

Endophytes have been tested in several growth assays and germination trials on various orchid species to confirm their impact (Hiruma *et al.*, 2016, Pant *et al.*, 2017). These studies revealed that germination and growth of orchids are improved by this association. For example, the root endophyte *Colletotrichum tofieldiae* has been shown to transfer phosphorus to its host plant which aids plant growth under phosphate-deficient conditions (Hiruma *et al.*, 2016). Besides improved nutrient uptake, these endophytes may assist in producing plant growth hormones, are nematophagous fungi that protect orchids against nematodes and *Phoma,*

Alternaria and *Aspergillus* spp. are metal-resistant and play roles in endophyte-assisted phytoremediation (Huang *et al.*, 2014, Ma *et al.*, 2016). These endophytes can enhance phytoremediation by helping with the degradation of toxins *in planta* and reducing the toxic contaminants from the soil (Khan & Doty, 2011).

Dark septate endophytes (DSE) are commonly isolated from plant roots and are known to provide nutrients such as phosphorus to their hosts (Dighton, 2009, Hiruma *et al.*, 2016). These endophytes are conidial Ascomycete fungi that colonize plant roots without causing any disease (Jumpponen, 2001). Common DSE are of the fungal genera *Chloridium, Leptodontidium, Phialocephala* and *Phialophora.* These fungi are usually found to associate with plants growing in cool and nutritionally poor environments. They penetrate the root hairs and form dark melanized runner hyphae between the cortical cells and characteristic multibranched structures called microsclerotia which are the points at which nutrients are exchanged (Dighton, 2009). These fungi are considered to be mycorrhizal and possibly ectendomycorrhizae. Similar to ectendomycorrhizae DSE form incomplete mantles and colonize root cells both intra- and intercellularly (Jumpponen, 2001). However, further studies are required to confirm the host range and mycorrhizal capacity of these fungi. A diverse assemblage of these fungi has also been isolated from orchids (Jumpponen & Trappe, 1998, Ma *et al.*, 2016). For example, the dark septate endophyte *Leptodontidium* infecting the *Dendrobium nobile* orchid was shown to increase the overall fitness of the plant by increasing shoot height, number of new buds, number of roots and stem diameters compared to uncolonized plants (Pant *et al.*, 2017). More studies are required to further understand these orchid-endophyte relationships.

10. Conclusion

Mutualistic interactions such as mycorrhizae are important for the survival of both plants and fungi in nature. Fungi forming these associations mostly depend on the plant to provide photosynthetically derived carbon. All orchid mycorrhizal interactions follow this same pattern, except during the mycoheterotrophic growth state or germination stage. In nature, orchids cannot germinate and survive without mycorrhizal fungi. Thus, studying these mycorrhizal associates gives insights into the adaptability, distribution, and dispersal of orchids. In addition, recent studies and techniques revealed the complexities of the symbiosis. For example, single orchid species may form multiple mycorrhizal interactions, orchids may associate with fungi that can cause disease on other plant species, mycorrhizae required for

germination may be different from those involved in growth and mycoheterotrophic orchids form complex tripartite interactions. Taxonomic studies to determine the diversity of orchid mycorrhizae associated with different orchids worldwide are needed before the mechanisms involved in forming these associations can be fully understood. Moreover, the number of endangered orchids is rapidly increasing requiring conservation measures.

Since mycorrhizae are an important part of the orchid's life cycle, they are studied for ways in which they can be used in conservation approaches. In South Africa, this is limited by the lack of knowledge of mycorrhizal fungi associated with endemic orchid species. Also, in this region, it is not clear how the distribution of orchid mycorrhizae may affect the occurrence of orchids. Therefore, the overall aim of this dissertation is to address these knowledge gaps by identifying the mycorrhizal associates of the endemic orchids *Habenaria barbertoni*, *Habenaria epipactidea* and *Brachycorythis conica* subsp. *transvaalensis*. Thus, this study will provide information that could be used in orchid conservation measures and will be a useful reference for future orchid mycorrhizal studies in South Africa.

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12. List of tables

Table 1: Summary of the characteristic differences between mycorrhizal types.

	Arbuscular mycorrhizae	Arbutoid mycorrhizae	Monotropoid	Ericoid mycorrhizae	Orchid mycorrhizae	Ectendomycorrhizae	Ectomycorrhizae	References
Plant taxa	Bryophyta Pteridophyta Gymnosperms Angiosperms	Ericaceae Pyrolaceae	Ericaceae (Monotropoideae)	Ericaceae Epacridaceae Empetraceae	Orchidaceae	Gymnosperms Angiosperms	Gymnosperms Angiosperms	Johnson & Gerhring, 2007
Age of association	>400 My	~80 My	~80 My	~80 My	~100 My	Unknown	>100 My	Strullu-Derrien *et al.*, 2018
Fungal lineage	Glomeromycota Zygomycota	Basidiomycota	Basidiomycota	Ascomycota	Basidiomycota Ascomycota	Basidiomycota Ascomycota	Basidiomycota Zygomycota Ascomycota (E-strain fungi of Pezizales)	Dighton, 2009

Table 1 continued: Summary of the characteristic differences between mycorrhizal types

	Arbuscular mycorrhizae	**Arbutoid mycorrhizae**	**Monotropoid**	**Ericoid mycorrhizae**	**Orchid mycorrhizae**	**Ectendomycorrhizae**	**Ectomycorrhizae**	**References**
Specificity	Low	Medium-high	Medium-high	Medium-high	Variable	Unknown	Variable	(Brundrett, 2002)
Septation	Aseptate	Septate	Septate	Septate	Septate	Septate	Septate	(Strullu-Derrien *et al.*, 2018)
Colonization type	Intracellular	Intracellular	Intracellular	Intracellular	Intracellular	Intra & Intercellular	Intercellular	(Barman *et al.*, 2016)
Type of vesicle	Arbuscules (cortical cells) Appressorium (root surface)	Thin hyphal sheath (root surface) Hartig net (paraepidermal) Hyphal coils invade cortical cells	Fungal sheaths Hartig nets Hyphal peg in cortical cell wall	Hyphal coils Absent fungal sheath	Pelotons (Cortical cells) Absent fungal sheath	Thin fungal sheath (root surface)	Hartig net (outside cortical cells) Hyphal sheath (root surface)	(Brundrett, 2002, Brundrett, 2004)

Table 2: Summary of characteristics used to identify *Rhizoctonia* species isolated from orchids.

Mycorrhizal species	Location	Host orchid	Colony/hyphae description	# Nuclei	Sclerotia/monilioid cells	Reference
Rhizoctonia repens	Alberta	*Platanthera obtusata*	White to cream Yellow patches of aerial mycelia	–	Minute sclerotia Spherical monilioid cells	Currah *et al.* (1987)
Rhizoctonia anaticula	Alberta	*Platanthera obtusata*	Aerial mycelia thin and cream Hyphae septate	–	Chains of monilioid cells with beak-like projections	Currah *et al.* (1987)
Rhizoctonia solani/ Thanatephorus cucumeris	South Wales	*Pterostylis acuminata*	Light tan turned dark brown & concentric rings	Multinucleate	Randomly scattered sclerotia	Carling *et al.* (1999)
	Australia	*Dactylorhiza pupurpella*	Hyphae dolipore septate	–	–	Williamson & Hadley (1970)
Rhizoctonia zeae/ Waitea circinate	Thailand	*Paphiopedilum niveum*	Pink to brown drying pinkish buff Hyphae convoluted branching & Septate	Multinucleate	Basidia/Basidiospore	Athipunyakon *et al.* (2004)
Rhizoctonia globularis/ Endoperplexa enodulosa	Thailand	*Goodyera procera*	White to pale pink Hyphae septate branching at upright angles	–	Monilioid cells globose in branched chains of 3-8 cells	Athipunyakon *et al.* (2004)

Table 2 continued: Summary of characteristics used to identify *Rhizoctonia* species isolated from orchids.

Mycorrhizal species	Location	Host orchid	Colony/hyphae description	# Nuclei	Sclerotia/monilioid cells	Reference
Rhizoctonia sp. AG-G	Indonesia	*Sacoila australis*	Dark brown	Binucleate	Sclerotia	Pardo *et al.* (2015)
Rhizoctonia sp. AG-G	Indonesia	*Sacoila australis*	Light brown	Multinucleate	Sclerotia	Pardo *et al.* (2015)
Rhizoctonia sp. AG-A	Indonesia	*Cyclopogon elatus*	Golden yellow	Multinucleate	Sclerotia	Pardo *et al.* (2015)
Rhizoctonia sp. AG-H	Indonesia	*Habenaria hexaptera*	White	Multinucleate	Sclerotia	Pardo *et al.* (2015)
Ceratobasidium obscurum	Alberta	*Acianthus reniformis*	Pale yellow becoming orange Hyphae dolipore septa no clamp connections	Binucleate	–	Currah *et al.* (1987)
Ceratorhiza pernacatena	Thailand	*Calanthe rubens*	White to light buff, cottony with concentric zones/ Hyaline septate	Binucleate	Globose monilioid cells with narrow tubular connections and septa between adjacent cells Loose sclerotia	Athipunyakon *et al.* (2004)

Table 2 continued: Summary of characteristics used to identify *Rhizoctonia* species isolated from orchids.

Mycorrhizal species	Location	Host orchid	Colony/hyphae description	# Nuclei	Sclerotia/monilioid cells	Reference
Ceratorhiza cerealis/ Ceratobasidium cerealis	Thailand	*Goodyera procera,*	White to light brown Hyphae Septate	Binucleate	Barrel-shaped monilioid cells Brown sclerotia	Athipunyakon *et al.* (2004)
Ceratobasidium sp. AG-O	Indonesia	*Dendrobium lancifolium*	White turns yellow with concentric zones/ Hyphae Septate	_	Globose monilioid cells	Agustini *et al.* (2016)
Epulorhiza sp.	Indonesia	*Sacoila australis*	Light brown	Binucleate	Sclerotia	Agustini *et al.* (2016)
Ceratorhiza goodyerae – repentis/ Ceratobasidium cornigerum	Thailand	*Goodyera procera*	White cream when young Orange-dark brown at maturity with concentric zones Hyphae Septate	Binucleate	Sclerotia Elliptical monilioid cells	Athipunyakon *et al.* (2004)
Ceratorhiza ramicola/ Ceratobasium ramicola	Thailand	*Paphiopedilum* sp.	White hyphae turned light brown/ Hyphae septate and anastomosed	Binucleate	Monilioid cells Sclerotia absent	Athipunyakon *et al.* (2004)

Table 2 continued: Summary of characteristics used to identify *Rhizoctonia* species isolated from orchids.

Mycorrhizal species	Location	Host orchid	Colony/hyphae description	# Nuclei	Sclerotia/monilioid cells	Reference
Epulorhiza sp.	Indonesia	*Aa achalensis*	White	Binucleate	Sclerotia absent	Agustini *et al.* (2016)
Epulorhiza sp.	Indonesia	*Pelexia bonariensis*	Dark brown	Binucleate	Sclerotia	Agustini *et al.* (2016)
Epulorhiza repens/ Tulasnella calospora	Thailand	*Calanthe rosea,*	White to cream submerged with concentric zonation/Hyphae septate with constricted branch points	Binucleate	Monilioid cells spherical in short branches	Athipunyakon *et al.* (2004)
	Brazil	*Oeceoclades maculata*	White to cream/ Hyphae with no clamp connections		Globulose monilioid cells Sclerotia absent	Pereira *et al.* (2005)
Sistrotema sp.	Thailand	*Paphiopedilum godefroyae*	Cream to yellow/ Aerial septate hyphae with constricted branch points	_	Monilioid cells hyaline to yellow with clamp connections	Agustini *et al.* (2016)
Trichosporiella multisporum	Thailand	*Paphiopedilum niveum*	Yellow to white/ Hyphae septate lateral conidia	--	Conidia smooth-walled globose to ellipsoidal	Agustini *et al.* (2016)

Table 3: Summary of observations made using different microscopy types to identify active orchid mycorrhizal colonization.

	Orchid Species	Habit	Mycorrhizae	Lifestyle	Plant organ	Geographic region	Observation	Reference
Light Microscopy	*Spathoglottis plicata*	Terrestrial	*Tulasnella calospora*	Mycorrhizal	Roots	Western Ghats	**Cortex**: Specific sections with monilioid cells **Pelotons**: Intact and lysed **Entry**: Fungal entry through the root hairs	Sathiyadash *et al.* (2012)
	Gastrodia similis	Terrestrial	*Resinicium* sp.	Saprophyte	Roots	Caribbean islands	**Cortex**: Middle layer **Pelotons**: Collapsed and Intact **Hyphae**: Dolipore septa	Selosse *et al.* (2010)
	Stelis concinna	Epiphyte	*Tulasnella* sp.	Mycorrhizal	Roots	South of Ecuador	**Cortex**: All layers **Pelotons**: Collapsed and Intact **Hyphae**: Clamp connections	Suarez *et al.* (2006)
	Dactylorhiza purpurella	Terrestrial	*Rhizoctonia* sp.	Pathogen	Roots	Scotland Aberdeen	**Hyphae**: Young (circular/elliptical) &older (angular/empty)	Hadley *et al.* (1971)
	Dactylorhiza majalis	Terrestrial	*Epulorhiza* sp.	Mycorrhizal	Roots	North Zealand, Denmark	**Pelotons**: Collapsed and Intact **Entry**: Fungal infection through suspensor cells	Rasmussen (1990)

Table 3 continued: Summary of observations made using different microscopy types to identify active orchid mycorrhizal colonization.

	Orchid Species	Habit	Mycorrhizae	Lifestyle	Plant organ	Geographic region	Observation	Reference
Electron Microscopy	*Dendrobium officinale*	Epiphyte	*Mycena* sp.	Saprophyte	Roots	Yunnan, China	**Cortex**: Inner layer **Pelotons**: Collapsed and intact **Hyphae**: Dolipore septa **Entry**: penetrate epidermal cells & pass-through passage cells	Zhang *et al.* (2012)
	Wullschlaegelia aphylla	Terrestrial	*Mycena* sp.	Saprophyte	Roots	Caribbean islands	**Cortex**: Inner layer **Hyphae**: Dolipore septa	Selosse *et al.* (2010)
	Stelis concinna	Epiphyte	*Tulasnella* sp. *Sebacina* sp. *Ceratobasidium* sp.	Mycorrhizal	Roots	South of Ecuador	**Hyphae**: Imperforate dolipore septa and no hyphal clamps	Suarez *et al.* (2006)

Table 3 continued: Summary of observations made using different microscopy types to identify active orchid mycorrhizal colonization.

	Orchid Species	Habit	Mycorrhizae	Lifestyle	Plant organ	Geographic region	Observation	Reference
Electron Microscopy	*Crepidium acuminatum*	Terrestrial	*Tulasnella* sp.	Mycorrhizal	Roots	Uttarakhand India	**Cortex**: All layers **Pelotons**: Collapsed and intact **Entry**: Fungal penetration of epidermal cells via root hairs	Thakur *et al.* (2018)
	Platanthera hyperborea	Terrestrial	*Ceratorhiza* sp.	Mycorrhizal	Protocorms	Alberta, Canada	**Peloton**s: Collapsed and intact **Hyphae**: Dolipore septa **Entry**: Penetrate suspensor cells	(Peterson *et al.*, 1996)
	Neottia nidus-avis	Terrestrial	*Rhizoctonia* sp.	Mycorrhizal	Roots	Germany	**Cortex**: Outer layer **Pelotons**: Collapsed and Intact	(Dorr & Kollmann, 1969)

Table 3 continued: Summary of observations made using different microscopy types to identify active orchid mycorrhizal colonization.

	Orchid Species	Habit	Mycorrhizae	Lifestyle	Plant organ	Geographic region	Observation	Reference
Fluorescence Microscopy	*Spathoglottis plicata*	Terrestrial	*Eupholorhiza repens*	Mycorrhizal	Roots	Sri Lanka	**Cortex:** Inner and outer layer **Pelotons:** Collapsed and Intact **Hyphae:** Individual hyphae observed **Entry:** Penetrate epidermal cells & pass-through passage cells	Senthilkumar & Krishnamurthy (2000)
	Disperis neilgherrensis	Terrestrial	Unknown species	Mycorrhizal	Roots Rhizome	southern India	**Cortex:** All layers **Pelotons:** Intact **Hyphae:** Septate	(Muthukumar *et al.*, 2013)
	Spirnnthes sirzensis	Terrestrial	*Ceratobasidiurn cornigerum*	Mycorrhizal	Protocorm	Canada	**Pelotons:** Collapsed and Intact **Entry:** Penetrate suspensor cells and enter parenchyma cells.	Uetake & Peterson (1997)

Table 4: Summary of results from molecular techniques used to capture orchid mycorrhizal diversity.

Technique	Orchid Species	Habit	Location		Mycorrhizal diversity	Dominant taxa	Reference
Illumina GAIIx paired-end sequencing	*Phalaenopsis* KC1111	Autotroph	Taiwan	Roots	◊ ■ ▼	Ceratobasidiaceae	Huang *et al.* (2014)
454 amplicon Pyrosequencing	*Platanthera bifolia* *Platanthera chlorantha*	Mixotrophs	Belgium	Roots	◊ ◆ ○ ● □	Ceratobasidiaceae	Esposito *et al.* (2016)
Illumina HiSeq2000	*Cymbidium hybridum*	Mixotroph	China	Roots	◊ ◆ ▼	Ceratobasidiaceae	Zhao *et al.* (2014)
454 pyrosequencing	*Cypripedium calceolus*	Autotroph	Estonia	Roots	◊ ◆ ○ □	Tulasnellaceae Sebacinales	Oja *et al.* (2015)
				Soil	◊ ◆ ○ □	Sebacinales	
454 pyrosequencing	*Orchis militaris*	Autotroph	western Estonia	Roots	◊ ◆ ○ ▼	Tulasnellaceae	Oja *et al.* (2017)
	Platanthera chlorantha			Roots	◊ ◆ ○ ▼	Ceratobasidiaceae	
		Autotroph		Transect soil	◊ ◆ ○ ▼	Sebacinales	

◊ Ceratobasidiaceae ◆Tulasnellaceae ○ Sebacinaceae ● Pezizaceae □ Ectomycorrhizae ■ Endomycorrhizae ▼Other

Table 4 continued: Summary of results from molecular techniques used to capture orchid mycorrhizal diversity.

Technique	Orchid Species	Habit	Location		Mycorrhizal diversity	Dominant taxa	Reference
ITS - RFLP	*Hexalectris spicata*	Mycoheterotroph	Arizona Mexico Virginia	Roots	◊ ◆ ○ □	Ceratobasidiaceae	Taylor *et al.* (2003)
Cloning	*Phalaenopsis* KC1111	Autotroph	Taiwan	Roots	◊ ■ ▼	Ceratobasidiaceae	Huang *et al.* (2014)
Cloning	*Chamaegastrodia sikokiana*	Mycoheterotroph	Japan	Rhizomes	◊ □	Ceratobasidiaceae	Yagame *et al.* (2007)
Cloning	*Gymnadenia conopsea*	Autotroph	Germany	Roots	◊ ◆ ○ □ ● ▼	Ectomycorrhizae	Stark *et al.* (2009)
Cloning	*Coryciinae*	Autotrophs	South Africa	Roots	◊ ◆ ○ ●	Ceratobasidiaceae	Waterman *et al.* (2011)
Cloning	*Gymnadenia conopsea*	Autotroph	South America	Roots & Protocorms	◊ ◆ ● ▼	Tulasnellaceae	Tesitelova *et al.* (2013)
Cloning	*serapias vomeracea*	Autotrophs	Italy	Roots	◊ ◆ ○	Tulasnellaceae	Girlanda *et al.* (2011)
	Anacamptis laxifl ora				◊ ◆ ○	Tulasnellaceae	

◊ Ceratobasidiaceae ◆ Tulasnellaceae ○ Sebacinaceae ● Pezizaceae □ Ectomycorrhizae ■ Endomycorrhizae ▼ Other

Table 4 continued: Summary of results from molecular techniques used to capture orchid mycorrhizal diversity.

Technique	Orchid Species	Habit	Location		Mycorrhizal diversity	Dominant taxa	Reference
Illumina paired-end (PE) sequencing	*Gastrodia flavilabella*	Mycoheterotroph	Taiwan	Tuber Soil	◊ ◆ ● □ ▼	Mycena species	Oja *et al.* (2017)
Illumina MiSeq paired-end sequencing	*Anacamptis morioa* *Ophrys sphegodes*	Autotrophs	Italy	Roots Soil	◊ ◆ ○ ● ▼ ◊ ◆ ○ ● ▼	Ceratobasidiaceae Tulasnellaceae	Voyron *et al.* (2017)
454 pyrosequencing	*Anacamptis, Dactylorhiza, Neotinea, Ophrys, Orchis,*		Italy	Roots	◊ ◆ ○ □ ▼	Tulasnellaceae	Jacquemyn *et al.* (2015)

◊ Ceratobasidiaceae ♦Tulasnellaceae ○ Sebacinaceae ● Pezizaceae □ Ectomycorrhizae ■ Endomycorrhizae ▼Other

Chapter 2

Fungal diversity associated with the rhizosphere of a critically endangered South African terrestrial orchid, *Brachycorythis conica* subsp. *transvaalensis*

1. Abstract

The Albertina Sisulu orchid, *Brachycorythis conica* subsp. *transvaalensis* is a critically endangered terrestrial orchid with a single population remaining in the Gauteng Province of South Africa. For the conservation of this endemic orchid, several strategies are being implemented such as protection of habitat, identifying pollinators and *in vitro* propagation. For symbiotic germination, it is essential to identify the mycorrhizal associates of this orchid using non-destructive sampling. In this study, high-throughput sequencing was used to catalogue and compare the fungal diversity in soils sampled from the orchid's rhizosphere and non-orchid rhizosphere soils collected from the same coordinates. Bioinformatics and statistical analyses of the data showed that despite the substantial overlap in the community composition of fungi associated with the two soil types, several unique species were identified from orchid rhizosphere soils. These included several potential orchid mycorrhizal species from the orders Agaricales, Cantharellales and Sebacinales. This study provides the first insight into the soil fungal diversity associated with the rhizosphere of this critically endangered orchid. In the future, data from this study can be used for optimising conservation measures and isolation of suitable mycorrhizal species required for *in vitro* symbiotic germination of this orchid.

Keywords: Agaricales, Albertina Sisulu orchid, Cantharellales, Sebacinales, soil microbiome

2. Introduction

South Africa has over 13 000 endemic plant species including more than 400 orchid species of which 40% are currently of conservational concern (Bytebier & Johnson, 2015, SANBI, 2020). Habitat destruction, the encroachment of invasive species, and over-collection for illegal trade, traditional medicine, and food are the leading causes of orchid population decline in this region (Jacquemyn *et al.*, 2012, Fay, 2018, Gale *et al.*, 2018, Sanchez-Bayo & Wyckhuys, 2019). Loss of orchid biodiversity will negatively affect the country's economy,

eco-tourism, indigenous cultural practices, and biological diversity (Chinsamy *et al.*, 2011, Basu *et al.*, 2016, Hinsley *et al.*, 2018). Maintaining orchid populations in their natural habitats is therefore of great importance for the survival of these plants.

To maintain orchid populations in nature, the presence of specific insect pollinators and mycorrhizal fungi is essential (Dearnaley *et al.*, 2012, Jacquemyn *et al.*, 2012). Pollinators adapted to specific floral morphologies are required by many orchids for fertilization of flowers and outcrossing (Johnson & Liltved, 2008, Suetsugu & Tanaka, 2014, Valencia-Nieto *et al.*, 2018). Successful fertilization of orchids results in millions of wind-dispersible seeds that lack endosperms (Roberts & Dixon, 2005). Consequently, these seeds are dependent on mycorrhizal fungi to supply the embryo with all the nutrients required to germinate (Rasmussen & Rasmussen, 2009, Basu *et al.*, 2016). In later life stages, mycorrhizal fungi continue to supplement the orchids' nutrients in exchange for plant-derived carbohydrates (Rasmussen & Rasmussen, 2009). This high level of dependence of orchids on their fungal symbionts makes them sensitive to disturbances in their habitat, as this can result in the loss of compatible pollinators and mycorrhizal associates (Bellgard & Williams, 2011, Jacquemyn *et al.*, 2016). The patchy distribution of orchid mycorrhizae was found to directly restrict the distribution of some orchids (McCormick, 2018, Pecoraro *et al.*, 2018) and orchids associated with a narrow range of mycorrhizal taxa may be more vulnerable to environmental changes (Oteo *et al.*, 2002, Valadares *et al.*, 2012).

Habitat destruction due to rapid urban development has led to the decline of many orchid populations in South Africa, including the critically endangered orchid, *Brachycorythis conica* subsp. *transvaalensis* (SANBI, 2020). This perennial plant grows up to 400 mm in height in grasslands and is renowned for its beautiful, sweet-scented white flowers with pale to dark pink spots (Figure 1). Additionally, the root system of this orchid includes tubers but lacks a well-defined lateral root system (Figure 1). In African traditional medicine, the Ndebele people use infusions of its tuber to ward off evil spirits (Raimondo *et al.*, 2009, Chinsamy *et al.*, 2011, Bytebier & Johnson, 2015). It was first officially described from a collection in 1918 in Pretoria, Gauteng, and later also sighted in several locations in the Limpopo and Mpumalanga provinces. In 2007, a survey of this orchid found only one remaining population with approximately 100 surviving plants in the Gauteng, Krugersdorp region (Raimondo *et al.*, 2013, Peter *et al.*, 2019). In 2019, a similar survey revealed that a single viable population of 68 plants remains in this same location. This is the last known population of *B. conica* subsp. *transvaalensis* and is now the most critically endangered orchid in South Africa (Chinsamy *et*

al., 2011, Raimondo *et al.*, 2013). Unfortunately, the area where the last colony of *B. conica* subsp. *transvaalensis* is found was proposed by the Mogale City Municipality for a high-intensity housing project which meant this orchid population would be lost. However, swift community-based initiatives aimed at conserving the orchid temporarily halted this development (Hankey & Cooper, 2018).

Orchid conservationists at the Wild Orchids of Southern Africa (WOSA) in collaboration with SANBI and Proteadal Conservation Association (PCA) launched the 'Save Mogale City's critically endangered Orchid initiative' (Hankey & Cooper, 2018, Peter *et al.*, 2019), to preserve the orchid's natural habitat and raise awareness of its importance. To achieve this, the orchid was given a common name that commemorates the anti-apartheid activist Mama Albertina Sisulu who was born in 1918, the same year when *B. conica* subsp. *transvaalensis* was first discovered (Downing & Hastings-Tolsma, 2016, Peter *et al.*, 2019). In 1955, the orchid was named and described by the Kew botanist V.H. Summerheyes, which was the same year Albertina Sisulu together with the African National Congress Women's League launched the freedom charter (Hankey, 2016). Therefore, it seemed fitting to name *B. conica* subsp. *transvaalensis* after Albertina Sisulu. However, despite increased awareness and initiatives in 2011, the decision to prevent urban expansion in the area was overturned by the provincial Minister of Agriculture and Rural Development (Peter *et al.*, 2019). Further engagements continue in the hope to change this decision. Meanwhile, several other conservation measures for the Albertina Sisulu orchid including restriction of access to its habitat, removing alien invasive species, fundraising for specific projects, germination of seeds in the laboratory, and identifying pollinators and mycorrhizal symbionts are being implemented (Hankey & Cooper, 2018, Peter *et al.*, 2019).

Since orchid mycorrhizae are indispensable for orchid growth, they are an important focus of orchid conservation approaches. Confirming the presence of mycorrhizal fungi at transplanting sites increases the success of translocating orchids to conservation sites (Gale *et al.*, 2018, McCormick, 2018, Reiter *et al.*, 2018). Laboratory grown orchids inoculated with suitable orchid mycorrhizal associates or orchid seeds germinated in the presence of appropriate mycorrhizal symbionts have higher survival rates when transplanted into the soil (Sivasithamparam *et al.*, 2002, McCormick, 2018). Such measures allowed the successful propagation of endangered orchids including *Dactylorhiza hatagirea, Caladenia cruciformis,* and *Spiranthes Brevilabris* (Stewart *et al.*, 2003, Aggarwal & Zettler, 2010, Reiter *et al.*, 2016). This symbiotic germination technique, although utilized across the globe, has not been reported

in Africa because the mycorrhizal associates of most African orchids, including *B. conica* subsp. *transvaalensis,* are not known.

Orchid mycorrhizal fungi mainly belong to the genus *Rhizoctonia* which comprises a group of phylogenetically and ecologically diverse fungi. Since most of these fungi are unculturable, next-generation sequencing (NGS) is currently employed to capture orchid mycorrhizal diversity and abundance in root and soil samples (Huang *et al.*, 2014, Zhao *et al.*, 2014, Oja *et al.*, 2015, Mujica *et al.*, 2016). Moreover, the mycorrhizal diversity in various habitats surrounding *B. conica* subsp. *transvaalensis* is unknown. Using a high-throughput sequencing platform, the present study aimed to catalogue and compare the fungal diversity associated with the rhizosphere soil of *B. conica* subsp. *transvaalensis* and non-orchid rhizosphere soil collected from the same area. It was hypothesised that the fungal community composition and richness will be influenced by the soil types, and the rhizosphere soil of the orchid will include a diversity of mycorrhizal species. Analyses of diversity data in this study confirmed that the fungal diversity substantially varied between the two soil types yet there were overlapping species, and that the rhizosphere soil contained previously undescribed taxa from orchid mycorrhizal fungal orders of the Agaricales, Cantharellales, and Sebacinales.

3. Methods and Materials

3.1 Collection of soil samples

Due to the current conservation status of *B. conica* subsp. *transvaalensis,* collection of live plant samples is not feasible. Therefore, rhizosphere soil samples from the orchids were used in the present study.

Rhizosphere soil samples from three *B. conica* subsp. *transvaalensis* plants were collected near the Walter Sisulu National Botanical Garden, Krugersdorp (26°04'31.4"S 27°49'02.3"E) in April 2018. A 12 cm^2 soil core was extracted 20 cm away from the plants at a depth of 10 cm after removing the topsoil. Three samples of non-rhizosphere soil were randomly collected from a site 50 m to the north of the *B. conica* subsp. *transvaalensis* population where orchids had never been observed previously.

3.2 Soil sample preparation and extraction of environmental DNA

All the soil samples were dried at room temperature (21-23 °C) for three weeks. Approximately 50 g of each soil sample was pulverized using a Retsch grinding jar attached to

a Qiagen TissueLyser II for 2 min at 20 frequency/sec. After each pulverization step, the grinding jars were surface sterilized using 4 % (v/v) sodium hypochlorite solution and 4N hydrochloric acid. Thereafter, the jars were rinsed a few times with sterile distilled water and dried using a blow dryer.

Environmental DNA was extracted from 0.5 g of each soil sample using the Mo-Bio PowerSoil® DNA Isolation Kit (Carlsbad, CA) following the manufacturer's protocols. All DNA samples were stored at -20 °C until the preparation of the fungal amplicon library.

3.3 Preparation of amplicon library

Each soil DNA sample was amplified in triplicate using two sets of primers targeting the complete Internal Transcribed Spacer (ITS1-5.8S gene-ITS2) region. Primers ITS1F and ITS4 (White, 1990, Gardes & Bruns, 1993) were used for amplifying the total fungal diversity. To exclusively capture Tulasnellaceae, each DNA sample was separately amplified using primers ITS1 and ITS4-Tul (Bruns *et al*., 1991, Taylor & McCormick, 2008). PCR conditions for both the amplifications were 96 °C for 1 min, followed by 35 cycles of 96 ˚C for 30 sec, 60 ˚C for 40 sec, 72˚C for 2 min and final elongation for 72 ˚C for 10 min. Positive amplifications were verified using gel electrophoresis.

3.4 Pooling of amplicons and amplicon sequencing

For each soil sample, three separate PCR replicates for each primer pair were pooled into a single sample. Thereafter, 25 µL of each pooled PCR product was cleaned using Agencourt AMPure XP PCR purification beads (Beckman Coulter Genomics, USA). Amplicon library preparation and Illumina MiSeq sequencing were outsourced to Inqaba Biotechnical Industries (Pty) Ltd, SA. The raw Illumina data were deposited in the NCBI Sequence Read Archive (https://submit.ncbi.nlm.nih.gov/ subs/sra/) under the accession number PRJNA693177.

3.5 Analyses of high-throughput sequencing data

The Illumina MiSeq sequencing data were demultiplexed by Inqaba Biotechnical Industries (Pty) Ltd. Further analyses of the data were performed using Quantitative Insights into Microbial Ecology 2 (QIIME2) v2020.8 (Bolyen *et al.*, 2019). The plugin 'q2-dada2' (Callaham *et al.*, 2016) was used for filtering, trimming, denoising, and deletion of singletons and chimeras. During filtering, sequences shorter than 200 bp with more than 6 bp

homopolymers and a Phred quality score below 30 were discarded from the analysis. The 'q2-vsearch' plugin (Rognes *et al.*, 2016) was used for the *de novo* assembly of the reads at a 98 % sequence similarity. Taxonomy was assigned to Operational Taxonomic Units (OTUs) using the plugin 'qiime feature-classifier' (Bokulich *et al.*, 2018). The UNITE fungal ITS database v8.2 (Abarenkov *et al.*, 2020) was used as the reference for assigning taxon names to the OTUs.

3.6 Statistical analyses of microbiome data

The statistical analyses of the microbiome data were performed using the pipeline available through Calypso v8.84 (Zakrzewski *et al.*, 2017). Principal coordinate analysis (PCoA) using Bray and Curtis distance was used for plotting the community composition of fungi recorded from two soil types. Statistical assessment of differences in fungal community composition between the two different soil types was done using permutational multivariate analysis of variance (PERMANOVA) available through the 'vegan' package of R version 4.0 (R Core Team, 2018). Krona plots were generated with Krona tools V2.7.1(Ondov *et al.*, 2011)

4. Results

4.1 Fungal diversity associated with soil samples

A total of 182 797 raw reads were obtained from high-throughput sequencing of environmental DNA extracted from rhizosphere and non-rhizosphere soil samples. All these sequences were from amplicons obtained from both the (ITS1F and ITS4) and (ITS1 and ITS4-Tul) primer sets. After quality filtering, 162 222 (88.75 %) reads were used for downstream analyses. A substantial portion of these reads was recovered from the rhizobiome of three orchids (92 004). A total of 100 fungal OTUs were identified after *de novo* assembly of the filtered reads recovered from both soil types. The majority of these OTUs were represented by Ascomycota (69 %) and Basidiomycota (25 %). The remaining OTUs were from Muccuromycota (4 %), and Mortierellomycota (2 %) (Figure 2A and 3).

Based on soil types, 74 fungal OTUs were detected from rhizosphere soil of *B. conica* subsp. *transvaalensis*, whereas non-rhizosphere soil contained 72 OTUs (Figure 4). Among these, 48 OTUs were mutually shared between the two soil types (Figure 2B). Orchid rhizosphere and non-rhizosphere soils included 28 and 26 exclusive fungal OTUs, respectively (Figure 2B).

Fungal species richness was significantly influenced by the soil types (p=<0.5). In the PCoA plot the data points clustered by soil types without any overlap (Figure 5). PERMANOVA also suggested that the soil type was a significant factor influencing fungal diversity (r^2=0.32).

4.2 Community composition of fungi associated with the rhizobiome of B. conica *subsp.* transvaalensis

The rhizobiome of the orchid included a higher percentage of 'unclassified fungi' compared to non-rhizosphere soils (Figure 3). The proportion of Basidiomycota was higher in the rhizosphere than in the non- rhizosphere soil (Figure 2C and D). The Basidiomycota included some unclassified fungi from known orchid mycorrhizal taxa in the order Sebacinales (unclassified) and the families Entolomataceae and Psathyrellaceae and Tulasnellaceae (Figure 2C, D and Figure. 4).

The orchid rhizosphere soil contained several exclusive fungi from the Ascomycota (Figure 3 and 4). These included known orchid mycorrhizal fungi from the Pleosporales. Compared to non-rhizosphere soils, the diversity of fungi from the Pleosporales was substantially higher in the orchid's rhizosphere (Figure 3 and 4).

5. Discussion

In the present study, high-throughput sequencing was used for cataloguing and comparing the fungal diversity associated with the rhizosphere of *B. conica* subsp. *transvaalensis* and soil samples collected 50 m north from the rhizosphere soil collection where no orchids have ever been observed. Analyses of the sequence data showed that the fungal diversity substantially overlapped in the two soil types, yet there were also striking differences in that more than 20 fungal taxa were unique to each soil type. Furthermore, the rhizosphere soil included a diversity of undescribed taxa from the Agaricales, Cantharellales and Sebacinales, fungal orders which are known to contain orchid mycorrhizae.

Plants significantly influence the microbial diversity associated with their rhizosphere which include organisms that may be beneficial and pathogenic to the plants (Berendsen *et al.*, 2012, Baldrian, 2017). In the present study, the fungal diversity associated with the orchid's rhizosphere was significantly different from non-rhizosphere soil, but there was also substantial overlap in their mycobiota. This rhizosphere mycobiota included an assortment of fungi

taxonomically related to previously described orchid mycorrhizae (Dearnaley *et al.*, 2012, Jacquemyn *et al.*, 2017). The overlapping fungal species are possibly members of the core fungal microbiome associated with the grassland ecosystem from where both soil types were collected.

Earlier research showed that the majority of orchid mycorrhiza reside in the Basidiomycota (Jacquemyn *et al.*, 2017). This was also reflected in the present study in which most of the OTUs identified as known mycorrhizal fungi belonged to this phylum. Furthermore, unique, and previously undescribed taxa from the orders Agaricales, Cantharellales, and Sebacinales, all residing in the Basidiomycota, were detected from the orchid's rhizosphere. OTUs belonging to the genera *Clitopilus* (Agaricales) and *Coprinellus* (Agaricales), as well as one OTU identified as belonging to the Tulasnellaceae (Cantharellales), were identified, and belonged to the Basidiomycota. The rhizosphere soil also had a high percentage of unidentified Sebacinales. Therefore, it is possible that fungi from these taxa symbiotically associate with *B. conica* subsp. *transvaalensis* as mycorrhizae.

Pleosporales is the only order of the Ascomycota known to have a symbiotic association with various species of orchids (Jacquemyn *et al.*, 2017, Schweiger, 2019). In the current study, Pleosporales was one of the most common fungal orders recovered from all six soil samples. Among these, at least eight taxa were exclusively identified from the orchid's rhizosphere including unidentified species of *Coniothyrium*, *Pyrenochaeta*, *Dictyosporiaceae*, *Keissleriella* and *Phaeosphaeriaceae*, as well as *Dictyosporium heptasporum* and *Pseudocoleophoma bauhiniae*. The majority of these taxa are either plant pathogens or saprophytes (Zhang *et al.*, 2009). But, fungal species in the genera *Coniothyrium* and *Pyrenochaeta* have also been identified as endophytes from orchids (Tan *et al.*, 2012, Novotna *et al.*, 2018). Therefore, the Pleosporales exclusively detected from the rhizosphere soil might live in symbiosis with *B. conica* subsp. *transvaalensis*. It is known that pathogenic fungi can sometimes also form beneficial associations with their plant hosts. Most notably, *Rhizoctonia sensu stricto* which includes several plant pathogenic fungi also contains several orchid mycorrhizal species (Andersen & Rusmussen, 1996).

This study identified candidate orchid mycorrhizal taxa from the rhizosphere of *B. conica* subsp. *transvaalensis*. However, their symbiotic association with this orchid will remain unconfirmed until live plant sampling to perform fungal isolations coupled with high-throughput sequencing of the tubers becomes feasible. Additionally, the rhizosphere of this

orchid contained a large diversity of unidentified fungi, including some which might form orchid mycorrhizal associations. Further identification using long-read sequencing together with an updated fungal reference database will allow us to identify these cryptic fungi.

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7. Figures

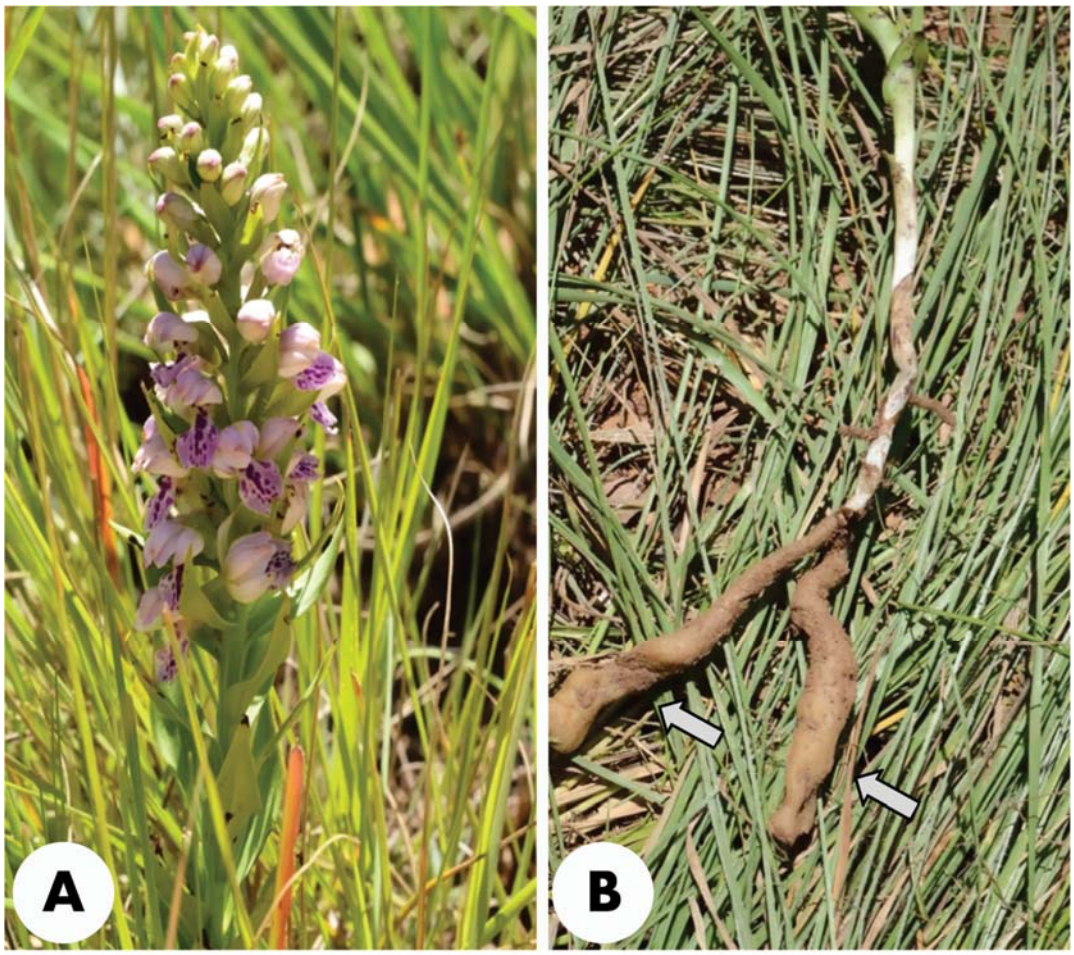

Figure 1. *Brachycorythis conica* subsp. *transvaalensis*. (A) Above-ground plant with inflorescence, and (B) subterranean tuberous structure (indicated by arrows) lacking a lateral root system. Photo credit: Gerrit van Ede.

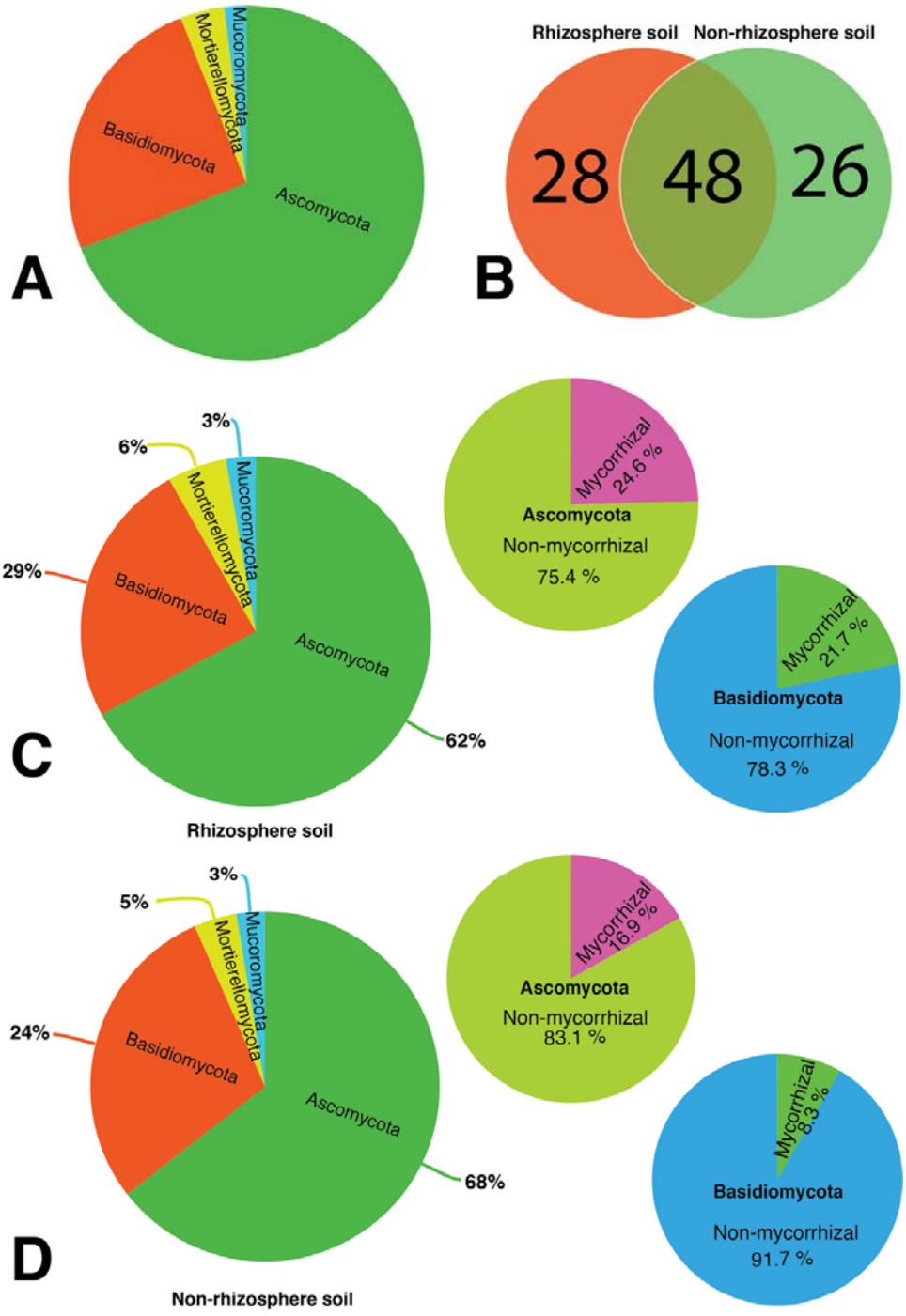

Figure 2. Pie charts showing the prevalence of fungal phyla identified. (A) Phyla detected from both soil types; (B) number fungal OTUs that were found exclusively in the different soil types and number of OTUs shared between two soil types; (C) phyla detected from rhizosphere soil of *Brachycorythis conica* subsp. *transvaalensis* with percentage of mycorrhizal and non-mycorrhizal taxa; and (D) phyla detected from non- rhizosphere soil with percentages of mycorrhizal and non-mycorrhizal taxa. In (C) and (D) the percentage of mycorrhizal and non-mycorrhizal fungal taxa are shown.

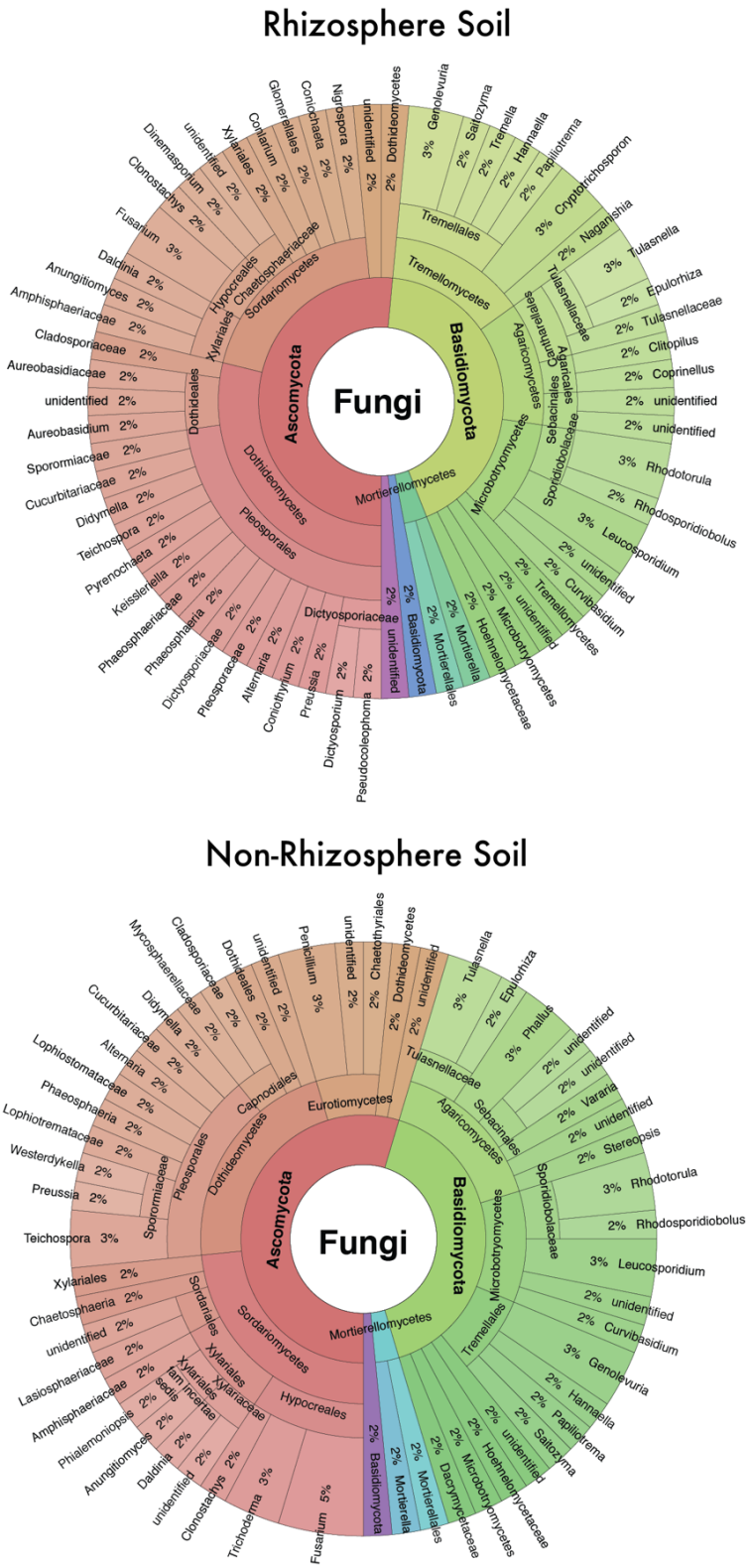

Figure 3. Krona plots showing the diversity of fungal genera (where available) detected from high-throughput sequencing of soil samples collected from the rhizosphere of *Brachycorythis conica* subsp. *transvaalensis* and from the soil where no orchids have been recorded previously.

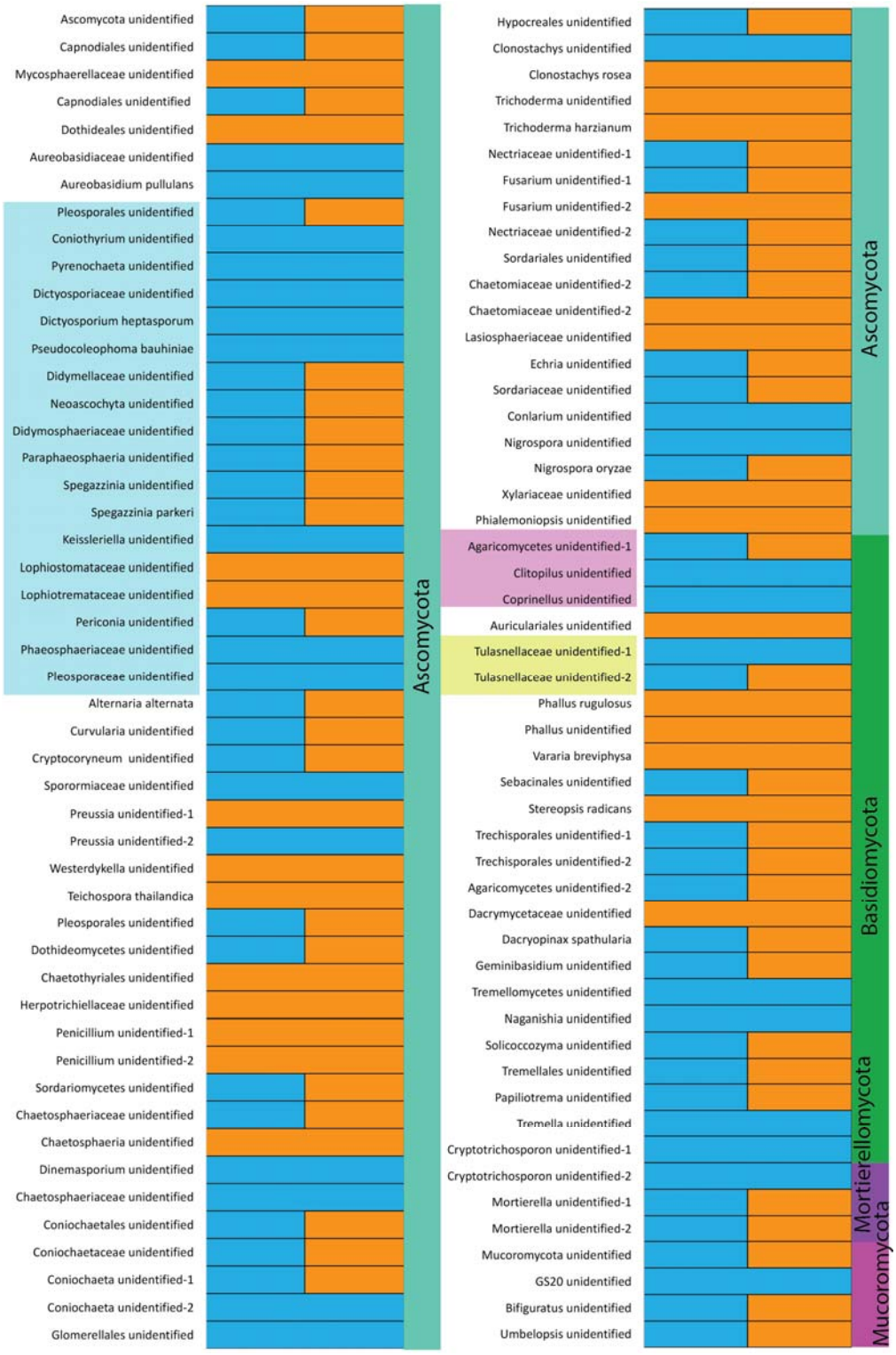

Figure 4. Distribution of fungal OTUs (up to species level where available) detected from two types of soil. OTUs exclusively detected from *B. conica* subsp. *transvaalensis* rhizosphere soil are shown with blue bars, OTUs in non- rhizosphere soil with orange bars and OTUs present in both soil types are shown with blue and orange bars. Orchid mycorrhizal fungal orders are highlighted in blue = Pleosporales, pink = Agaricales, and yellow = Cantharellales.

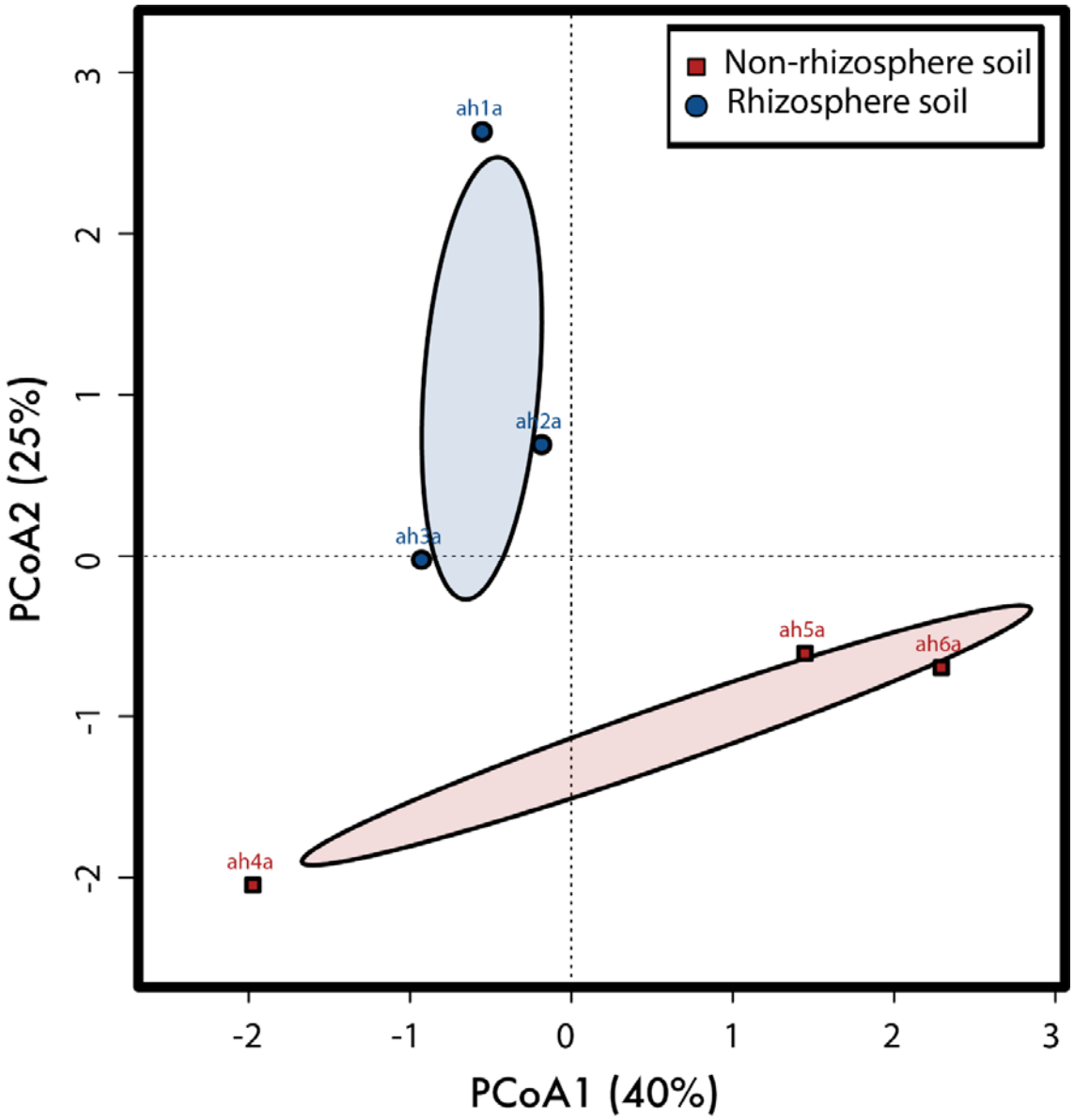

Figure 5. Principle coordinate analysis (PCoA) predicted that the fungal species richness varied across orchid rhizosphere and non-rhizosphere soils.

Chapter 3

Mycorrhizal diversity associated with two South African endemic and endangered orchid species from the genus *Habenaria*

1. Abstract

The symbiosis between orchid mycorrhizae and orchids is recognized as the most important factor affecting the germination, growth, and distribution of these plants. Mycorrhizae provide all the nutrients required for the survival of the orchid and in their absence orchid propagation and germination are often unsuccessful. Due to the high level of dependence of orchids on their symbiotic partners, it is suggested that mycorrhizal specificity may be directly linked to the rarity of orchids. It has been proposed that endangered orchids may be associated with specific mycorrhizal species while non-endangered orchids form symbiosis with multiple mycorrhizal species. To determine whether this is true for South African terrestrial orchids in the genus *Habenaria*, we compared the mycorrhizal associates of an endangered orchid *Habenaria barbertoni* and a commonly found species, *Habenaria epipactidea*. To achieve this aim, microscopic analysis, fungal isolations, and culture-independent techniques such as molecular cloning and high-throughput sequencing were used. Overall, six known orchid mycorrhizal families were detected from the plants namely Ceratobasidiaceae, Tulasnellaceae, Serendipitaceae, Pezizaceae, Hoehnelomycetaceae and Omphalotaceae. Our results also showed that these orchids have distinct mycorrhizal associates and that the orchid mycorrhizal species richness in *Habenaria* may be influenced by environmental factors and not mycorrhizal specificity. This is the first study to record the orchid mycorrhizal diversity associated with *Habenaria* spp. in South Africa and provides information that can be used in the implementation of mycorrhizae based *in-situ* conservation techniques.

Keywords: Ascomycota, Agaricomycetes, pelotons, *Rhizoctonia*, symbiosis, terrestrial orchids

2. Introduction

Plants are associated with an assortment of microorganisms on their subterranean and aerial parts (Montesinos, 2003, Bonfante & Genre, 2010). These microbes may have a neutral, detrimental, or beneficial effect on plant health (Jacoby *et al.*, 2017). Beneficial microbes such as mycorrhizal fungi and nitrogen-fixing bacteria form mutualistic associations with various plant species (Bonfante & Genre, 2010, Jacoby *et al.*, 2017).

Mycorrhizae are a diverse group of soil-inhabiting fungi that colonise the roots of all Gymnosperms and the majority of all known Angiosperms (Harrison, 1997, Brundrett, 2002, Dighton, 2009). This plant-mycorrhiza association is ancient, dating back to at least 407 million years during the Silurian period of the Paleozoic era (Barman *et al.*, 2016). Mycorrhizae assist plants with nutrient uptake, improving their defence and overcoming stresses (Dighton, 2009, Lee *et al.*, 2013). In exchange, the fungus obtains photosynthetically derived carbohydrates and shelter to proliferate (Dighton, 2009). Most land plant species including orchids depend on mycorrhizal associations to complete their life cycles and sustain their growth (Lee *et al.*, 2015, Jacoby *et al.*, 2017)

Orchidaceae includes over 28 000 species growing in a variety of habitats globally (The Plant List, 2020). For over 50 million years all orchids have been in association with mycorrhizae that aid in their seed germination and growth (Roberts & Dixon, 2005, Van der Heijden *et al.*, 2014). Orchid seeds are minute, lacking any nutrient reserves and cannot germinate in the absence of their mycorrhizal partners (Arditti, 1967, Ghimire *et al.*, 2009). Therefore, during germination, orchid seeds obtain water and nutrients such as carbon, nitrogen, and phosphorus from their mycorrhizal partners. In return, the fungal symbionts obtain ammonium that is produced by the embryo (Dearnaley & Cameron, 2017, Fochi *et al.*, 2017, Yeh *et al.*, 2019). The nutrient exchange between the orchid and fungus is maintained in the mature stage of the plant whereby carbohydrates are transferred to the orchid mycorrhizal fungi (Sally *et al.*, 2008, Yeh *et al.*, 2019).

An estimated 25 000 fungal Operational Taxonomic Units (OTUs) have been identified as mycorrhizal fungi associating with orchids in earlier studies (Van der Heijden *et al.*, 2014, Bolduc *et al.*, 2015). These comprise mainly Basidiomycota in the orders Agaricales, Cantharellales, Hymenochaetales, Russulales, Sebacinales and Thelephorales (Athipunyakon *et al.*, 2004, Chang & Chou, 2006, Rasmussen & Rasmussen, 2009, Dearnaley *et al.*, 2012).

The only Ascomycota order known to associate with orchids is the Pezizales (Dearnaley *et al.*, 2012, Jacquemyn *et al.*, 2017). Orchids can associate with more than one of these mycorrhizal fungi at a time (Hadley, 1970, Zhu *et al.*, 2008, Girlanda *et al.*, 2011, Huang *et al.*, 2018). Some mature orchid species are mycorrhizal generalists that associate with several mycorrhizal fungi that are not evolutionary related (Ogura-Tsujita *et al.*, 2009, Majica *et al.*, 2016). Other species are specialists that interact with fungi in the same taxonomic group, i.e., family, genus, or species (Sherrerson *et al.*, 2005, Bailarote *et al.*, 2012, Tesitelova *et al.*, 2015). Additionally, the mycorrhizal species that assist with seed germination can differ from those that associate with the mature orchid (McCormick *et al.*, 2012, Meng *et al.*, 2019). Mycorrhizae are an inseparable part of the orchid's life cycle and thus are a key component that that should be considered in conversation efforts worldwide (Ercole *et al.*, 2013, Fay, 2018, Gale *et al.*, 2018, Reiter *et al.*, 2018).

Southern Africa hosts a high level of endemic and unique orchid species (Bytebier & Johnson, 2015, SANBI, 2020). Amongst the approximately 500 described species in the region, 471 are endemic (Chinsamy *et al.*, 2011, Bytebier & Johnson, 2015). South African orchids are highly sought after for their unique floral morphologies and various medicinal properties (Johnson & Anderson, 2010, Chinsamy *et al.*, 2011, Pant, 2013). However, illegal orchid trade together with habitat destruction due to urbanization and agriculture have led to a rapid decline in orchid populations (Basu *et al.*, 2016, IUCN, 2020). According to the South African Biodiversity Institute (SANBI), over 200 orchid species in South Africa are critically endangered or of conservational concern (Chinsamy *et al.*, 2011, Bytebier & Johnson, 2015, SANBI, 2020).

The conservation of orchids in South Africa and elsewhere in the world relies on efforts such as seed banks, tissue culture and maintaining native ecosystems (Brundrett *et al.*, 2001, Ercole *et al.*, 2013, Merritt *et al.*, 2014). Orchids in their natural habitats, however, require specialized biotic interactions with pollinators and mycorrhizal fungi (Swarts & Dixon, 2009, Aggarwal & Zettler, 2010, Johnson & Anderson, 2010). In Southern Africa, the challenge to orchid conservation is that while most orchid pollinators are known, the mycorrhizal associates of most endemic orchids remain unidentified (Grobler, 2005, Johnson & Liltved, 2008).

The aim of this study was to bridge the important knowledge gap of unknown mycorrhizal symbionts of most South African endemic orchids. This was done by cataloguing and comparing the mycorrhizal diversity associated with South African orchids of conservation

concern. For the implementation of future orchid conservation programmes in this region, it is important to identify the orchid mycorrhizal diversity and also to isolate the mycorrhizal fungi from orchid roots. Orchid species chosen for this study are endemic terrestrial orchids of the genus *Habenaria*. This genus consists of 839 species distributed worldwide across tropical and subtropical regions including eastern Asia, Brazil, central and southern Africa (Suetsugu & Tanaka, 2014, The Plant List, 2020). About 30 of these species occur in shrub and grassland habitats of South Africa (Bytebier & Johnson, 2015).

Two species, *Habenaria barbertoni* and *H. epipactidea,* were selected for this study mainly because of their different conservation status. Both are small terrestrial orchids growing up to 400 mm and 500 mm, respectively (Figure 1). They are characterised by inverted flowers that are mostly green with yellow or white petals and lips, which flower between January and March every year (Bytebier & Johnson, 2015). Both orchids differ in their root structure, *H. barbertoni* have shorter and thicker roots as compared to the much longer and thinner roots of *H. epipactidea. Habenaria epipactidea* is commonly found throughout the country, except in the Western and Northern Cape, while *H. barbertoni* is of conservation concern with only a few populations catalogued in eastern South Africa, in Mpumalanga and Gauteng provinces (Bytebier & Johnson, 2015, SANBI, 2020).

3. Materials and Methods

In this study, different methods were used to identify and catalogue mycorrhizae associated with *H. barbertoni* and *H. epipactidea* (Figure 2). A short overview is given here to guide the reader while details are presented in the sections below. The presence of orchid mycorrhizal fungi in orchid roots and tubers was observed using light microscopy. Then culturable fungi were isolated from the roots and tubers with the aim of obtaining orchid mycorrhizal isolates. Following identification of the fungal isolates characteristic morphological and microscopic features of the orchid mycorrhizal isolates were recorded. A molecular cloning approach was then used to identify any mycorrhizal fungi in DNA extracted from the orchid root and tuber samples. Phylogenetic analysis was used to confirm the identity of the sequences representing orchid mycorrhizae obtained from the fungal isolations and cloning methods. To capture a higher diversity of fungi associated with the orchids the high throughput Illumina sequencing approach was used.

3.1 Sample collection and preparation

Habenaria barbertoni samples were collected in March 2017 and May 2018. *Habenaria epipactidea* was collected only in May 2018 because no plants were found in the earlier season. All orchids used in the present study were collected from a plot near Pretoria (25°54'43.6"S 28°25'06.2"E). During each collection trip, at least three live plants were obtained for each species growing at a distance of 400 m.

After collection, roots and tubers from each plant were cleaned with tap water followed by surface sterilization with 1% (w/v) silver nitrate solution. Surface sterilized roots and tubers were repeatedly washed with sterile deionized water. The plant samples were then dried with paper towels and either used directly for isolations and microscopy or stored at -20 ºC for DNA extractions.

3.2 Fungal isolations and identifications

Fungi were isolated by placing segments (5mm x 5mm) of surface-sterilized roots and tubers on Petri dishes containing either malt extract agar (MEA; 20 g malt extract, Merck, South Africa; 15 g Difco agar and 1 L of deionized water) or half-strength potato dextrose agar (PDA; 19.5 g PDA powder, Merck, South Africa; 7 g Difco agar; 1 L of deionized water) growth medium supplemented with 0.1% (w/v) streptomycin sulphate (Merck, Germany). After three days of incubation at room temperature, all the different types of growing fungi were transferred to new half-strength PDA plates. Following two subsequent sub-culturing steps, pure cultures were obtained and maintained on a half-strength PDA growth medium at 4˚C for the duration of this study.

Genomic DNA was extracted from isolates grown on half-strength potato dextrose medium for five days using the PrepMan Ultra Sample Preparation Reagent kit (Applied Biosystems, Inc., Foster City, CA) following the manufacturer's instructions. For each cultured isolate, the complete Internal Transcribed Spacer region (that included the ITS1 region, 5.8S gene and ITS2 region) was amplified with the primer pair ITS1F and ITS4 (White, 1990, Gardes & Bruns, 1993). Each PCR reactions included 5µl of 5× MyTaq reaction buffer (Bioline, Inc, USA), 5 U/µL MyTaq DNA Polymerase (Bioline, Inc, USA), 0.5 µl (10 mM) each of forward and reverse primers, and the final volume was made up to 25µl with PCR grade water. The PCR protocol was 96 °C for 1 min, 35 cycles of 96 ˚C for 30 sec, 55 ˚C for 40 sec, 72˚C for 2 min and final elongation of 72 ˚C for 10 min. PCR products were visualized using

agarose gel electrophoresis. Amplicons were sequenced at the DNA Sequencing Facility of the University of Pretoria and assembled using the CLC Main Workbench v8.0.1 (Qiagen, Hilden, Germany). Preliminary identification of the isolates was done by comparing the sequences using a BLASTn (Altschul *et al.*, 1990) similarity search algorithm against know sequences available at the NCBI GenBank database. Only sequences with above 90% similarity were used for identification of isolates in this study.

The partial ribosomal large (LSU) and small (SSU) subunit gene regions were amplified for isolates that were putatively identified as orchid mycorrhizal species based on the BLASTn results. Primer pairs used for amplifying LSU were LR0R/LR7 (Vilgalys lab, 1992) and LR3R/LR9 (Vilgalys lab, 1992, Lutzoni lab, 2001) whereas NS1/NS4 and NS3/NS8 were used for amplifying SSU (White, 1990). PCR reaction mix, amplification protocol, and assembling of amplicons were the same as above. The annealing temperatures for these primer sets were between 54 ºC to 56 ºC.

3.3 Microscopic analysis

Transverse and longitudinal sections of surface-sterilized roots and tubers from *H. barbertoni* and *H. epipactidea* were inspected for characteristic orchid mycorrhizal structures such as pelotons. The sections were stained with 0.1% (w/v) lactophenol cotton blue solution and microscopy was done using a Nikon Eclipse Ni compound microscope. All images were captured at 100x magnification using the Nikon RIS elements camera.

The morphological characters for the isolates identified as orchid mycorrhizae were also investigated using microscopy. Characters such as septum type, branching angle, constrictions of hyphae and presence of monilioid cells were assessed and photographed. Published descriptions of fungi and monographs were used for morphological identification of each isolate (Warcup & Talbot, 1967, Athipunyakon *et al.*, 2004, Pereira *et al.*, 2005, Basalyan *et al.*, 2014, Hussain *et al.*, 2018).

3.4 Molecular cloning and insert sequencing

Total genomic DNA was extracted from root and tuber samples using the MOBIO PowerPlant®Pro DNA isolation kit (MOBIO Laboratories, Inc, Carlsbad, USA) following the manufacturer's instructions. The complete ITS region from fungal DNA present in the DNA extraction samples was amplified using the primers ITS1F and ITS4 (Bruns *et al.*, 1991, Gardes

& Bruns, 1993). In separate reactions, the ITS1 forward primer was paired with the Tulasnellaceae-specific reverse primer ITS4-Tul. The LSU and SSU gene regions were also amplified from the samples, using primers in section 3.2. The PCR protocol was 96 °C for 1 min, 35 cycles of 96 ˚C for 30 sec, 60 ˚C (ITS) / 55 ºC for 40 sec, 72˚C for 2 min, and final elongation at 72 ˚C for 10 min. Positive amplification was confirmed using agarose gel electrophoresis. Thereafter, amplicons were purified and concentrated using the Zymo Research DNA Clean and Concentrator kit (Irvine, USA) following the manufacturer's instructions.

Purified and pooled PCR products were cloned using the pGEM T-Easy vector system (Promega, Mannheim, Germany) following the manufacturer's instructions. After the ligation reactions, TOP 10 *E. coli* competent cells were transformed with the recombinant plasmids. Transformed bacteria were then transferred onto individual selective media plates containing 100ug/ml ampicillin, 40 µl Xgal (20 mg/ml) and 40 µl of IPTG (100mM). From each plate, five or six recombinant white colonies were picked and mixed with 6 µl PCR grade water. The mixture was then used directly in colony PCR amplifications using the vector-specific primers SP6 and T7. Both the PCR amplifications and sequencing were conducted as described in section 3.2.

CLC Bio (Qiagen, Hilden, Germany) software was used to assemble and edit the sequences obtained from the sequencing facility. BLASTn searches against DNA sequences in GenBank was done to identify fungi from which the sequences originated. Based on this preliminary search, sequences were separated into either Basidiomycota or Ascomycota, after which they were further identified to different taxonomic levels.

3.5 Phylogenetic analysis

Phylogenetic analyses were done to confirm the identity of all the orchid mycorrhizal sequences detected. The analyses used DNA sequences from the ITS, LSU and SSU gene regions obtained from the pure cultures and cloning experiments as well as well-characterised sequences of closely related taxa from the respective fungal families retrieved from GenBank. For each fungal family representative datasets were created for the individual gene regions and alignments were done using the online version of MAFFT (http://mafft.cbrc.jp/alignment/server/). Alignments were manually adjusted where necessary using Mesquite 3.61 (Maddison & Maddison, 2018). A separate concatenated dataset that

included the ITS-LSU gene regions belonging to the *Coprinellus* isolate was generated using MEGA 7 and MEGA X (Kumar *et al.*, 2016). Phylogenetic analyses were performed using RAxML 8.2 (Stamatakis, 2014). The general time-reversible model along with a gamma distribution (GTR GAMMA) was selected using jModelTest 2.1 (Guindon & Gascuel, 2003, Posada, 2008). Ten replicated likelihood searches were performed followed by 1000 bootstrap replicates. Trees were viewed and rooted in FigTree 1.4. Clades with < 70 % bootstrap support were considered unsupported.

3.6 High throughput Illumina Sequencing

For amplicon library preparation the complete fungal internal transcribed spacer (ITS1-5.8S-ITS2) region was amplified from each genomic DNA extracted from the roots of *H. barbertoni* and *H. epipactidea* using two sets of primers. Primers ITS1F and ITS4 *(Bruns et al*., 1991, Gardes & Bruns, 1993) were used for capturing most of the fungal diversity. For exclusively amplifying Tulasnellaceae, each DNA sample was separately amplified using primers ITS1 and ITS4-Tul (Bruns *et al*., 1991, Taylor & McCormick, 2008). For each primer pair, PCR reactions were done in triplicate. PCR conditions for both the amplifications were 96 °C for 1 min, followed by 35 cycles of 96 ˚C for 30 sec, 60 ˚C for 40 sec, 72˚C for 2 min and final elongation for 72 ˚C for 10 min. Positive amplifications were verified using gel electrophoresis.

3.6.1 Pooling of amplicons and amplicon sequencing

For each root sample, three separate PCR replicates for each primer pair were pooled into a single sample. Thereafter, 25 µL of each pooled PCR product was cleaned using Agencourt AMPure XP PCR purification beads (Beckman Coulter Genomics, USA). Amplicon library preparation and Illumina MiSeq sequencing were conducted by Inqaba Biotechnical Industries (Pty) Ltd, SA.

3.6.2 Analyses of high-throughput sequencing data

The bioinformatics pipeline Quantitative Insights into Microbial Ecology 2 (QIIME 2) v2020.8 (Bolyen *et al.*, 2019) was used to analyse all the sequencing data. Demultiplexing of sequencing data was done by Inqaba Biotechnical Industries (Pty) Ltd. The ‘q2-dada2’ plugin (Callaham *et al.*, 2016) was used to filter, trim, denoise, remove singletons and chimeras. Filtering parameters were set to a Phred quality score of 30 and cut off sequence length of 200

bp with more than 6 bp homopolymers. All sequences that did not meet these criteria were discarded from the analysis. For *de novo* assembly of the reads at a 98% sequence similarity the 'q2-vsearch' plugin (Rognes *et al.*, 2016) was used. The 'qiime feature-classifier' plugin (Bokulich *et al.*, 2018) was then used to assign taxonomy to the Operational Taxonomic Units (OTUs) using the UNITE fungal ITS database v8.2 as a reference. For visualisation of the microbiome data 'phyloseq' (McMurdie & Holmes, 2013) and 'ggplot' (Wickham, 2016) packages available through R packages (R Core Team, 2018) were used to rarefy and generate figures that allowed for the comparisons of species richness between the two orchid species. A PCoA plot was also generated using R.

4. Results

4.1 Identification of culturable isolates

In total, 112 fungal isolates were recovered from the roots and tubers of *H. barbertoni* and *H. epipactidea* (Table 1). Ninety-five of the isolates belonged to 28 fungal genera representing 30 species based on DNA sequence comparisons with known sequences on GenBank. The remaining 17 isolates could only be identified as uncultured fungal species previously identified from DNA metabarcoding studies. Most isolates were from the Ascomycota (Sordariomycetes (74%), Dothideomycetes (18%) and Eurotiomycetes (6%), while 2% were identified as Agaricomycetes (Basidiomycota).

A total of 42 fungal isolates were obtained from *H. epipactidea* (Table 1). These included 31 Sordariomycetes, 8 Dothideomycetes and 3 Eurotiomycetes. No isolates with high similarity to described orchid mycorrhizal fungi were obtained from this orchid species.

Fifty-four isolates were obtained from *H. barbertoni*. These isolates belonged to Sordariomycetes (41 isolates), Dothideomycetes (10 isolates), Eurotiomycete (1 isolate) and two mycorrhizal isolates belonging to the Agaricomycetes. The mycorrhizal isolates were assigned to the Ceratobasidiaceae and Psathyrellaceae using BLASTn search analysis for three gene regions (nLSU, nSSU, and ITS). Based on ITS sequence data, the isolates had 91% sequence similarity to an undescribed *Ceratobasidium* sp. (JQ247397) and 99% sequence similarity to *Coprinellus micaceus* (KY48384), respectively. In the case of the nLSU gene region, isolates were found to have a 97% sequence similarity to *Rhizoctonia solani* (MH873231) and a 99% sequence similarity to *Coprinellus* sp. (MF140461), while results from

the nSSU sequences were 99% similar to *Rhizoctonia* sp. (HQ427477) and 99% similar to *Psathyrella gracilis* (DQ851582), respectively.

Six fungal species, *Mycoleptodiscus geniculatus*, *Aspergillus spelaeus*, *Purpureolilium lilacinum, Fusarium oxysporum*, *Chaetomium homopilatum* and *Clonostachys rosea,* were isolated from both *Habenaria* species (Table 1). Seventeen fungal genera represented by 30 isolates were unique to *H. barbertoni* and 14 genera (18 isolates) were only found in *H. epipactidea* roots.

4.2 Morphological and microscopic descriptions of mycorrhizal isolates

The colony morphology of the isolate of *Ceratobasidium* sp. (Figure 3A-B) on PDA was observed as dark brown hyphae having light brown aerial mycelium. Hyphae were often submerged in the growth medium (Figure 3B). Often hyphae aggregated to form dark brown sclerotia on the surface of the medium. Light microscopy analysis showed that this isolate had characteristics common to Basidiomycota and were similar to *Rhizoctonia*-like fungi (Figure 3C-H). These include hyphal branching patterns such as hyphal knots (Figure 3C), thick-walled hyphae with constrictions at 90-degree angles, and dolipore septa (Figure 3D-F), thick-walled chlamydospores occurring between hyphae (Figure 3G) and encrustations of crystals were observed in the hyphae (Figure 3H). These features are consistent with those of Basidiomycota fungi (Potter K *et al.*, 2006) within the *Rhizoctonia* complex, and those found to associate with orchid species (Athipunyakon *et al.*, 2004).

The vegetative hyphae of the isolate identified as a *Coprinellus* sp. were white with concentric circles observed at the bottom of the PDA medium (Figure 4A). The isolate had well developed aerial mycelium with a cottony texture with a white and light-yellow colour (Figure 4B). Light microscopy revealed morphological characteristics typical of Basidiomycota, including septate hyphae with constrictions at 90-degree angles (Figure 4C). Hyphal clamp connections and anastomosis between hyphae of the same strain were also observed in this isolate (Figure 4D-E).

4.3 Mycorrhizal colonization of orchid root and tubers

Light microscopy was used to observe mycorrhizal colonization within the roots and tubers of both *H. barbertoni* and *H. epipactidea* (Figure 5). Mycorrhizal colonization differed in the two orchid species. In *H. barbertoni* (Figure 5 A-D), no pelotons were observed in the

roots or tubers; instead, only monilioid structures and characteristic fungal hyphae with dolipore septa and basidiospores were observed. In contrast, in *H. epipactidea* mainly pelotons were observed in both the roots and tubers (Figure 5). Furthermore, the pelotons were collapsed with several hyphae extending from each loose coil (Figure 5G) indicating that the orchid mycorrhizal association in those cells was at the peloton lysis stage. Fungal hyphae with established (Figure 5E) and newly forming (Figure 5H) clamp connections were also observed (Figure 5E and 5H) in the *H. epipactidea* roots.

4.4 Fungal community structure: molecular cloning

The cloned inserts of 24 LSU amplicons, 3 SSU amplicons and 231 ITS amplicons were sequenced. Sequences from 140 amplicons belonged to the Ascomycota and 118 amplicons to the Basidiomycota. *Habenaria epipactidea* was associated with putative Basidiomycota orchid mycorrhizae of the Tulasnellaceae represented by 16 cloned inserts. From *H. barbertoni,* 67 cloned amplicons were identified as possible orchid mycorrhizal fungi belonging to the families Ceratobasidiaceae (34), Serendipitaceae (10), Hoehnelomycetaceae (7), Tulasnellaceae (6), and Helicogloeaceae (4) in the phylum Basidiomycota. Also, from this phylum, one clone was identified as a fungus from the Omphalotaceae family which is known to contain ectomycorrhizal fungi. From the Ascomycota, only five sequences of the Pezizaceae family were detected. Overall, using the molecular cloning approach 84 cloned putative mycorrhizal amplicons were identified which represented approximately 32% of all the sequences obtained.

Collectively 124 fungal cloned inserts were identified as non-mycorrhizal fungi represented by the subphyla Pezizomycotina, Pucciniomycotina, Agaricomycotina, and Ustilagomycotina from both orchids. The remaining sequences could not be identified to any taxonomic rank lower than the phylum level using the Genbank NCBI database and were therefore assigned as either Basidiomycota sp. (28 isolates), Ascomycota sp. (22 isolates), or unclassified soil fungus (3 isolates).

Considering the overall fungal diversity, variation was observed between the orchid species. Only five fungal species represented by 104 cloned amplicons were common to both *H. barbertoni* and *H. epipactidea*. These included fungi from the genera *Cladosporium, Paeciliomyces, Alternaria, Davidiella* and *Epiccocum,* as well as 22 sequences of orchid mycorrhizal Tulasnellaceae (Table 2). Moreover, 25 different fungal taxa (132 sequences) were

unique to *H. barbertoni* and 25 sequences in 13 taxa were only found in *H. epipactidea* samples.

4.5 Phylogenetic analysis

All the sequences representing orchid mycorrhizal taxa obtained from the molecular cloning and fungal isolation techniques were used in phylogenetic analysis. Using GenBank taxonomy these sequences were further grouped into their respective families. For phylogenetic analysis, the dataset for each taxon were analysed separately because of the taxonomic diversity of the fungi based on their ITS sequence BLASTn results. All phylogenetic trees were converted into cladograms to better visualize the phylogenetic relationships.

4.5.1 Psathyrellaceae

The family Psathyrellaceae was represented by one isolate identified as *Coprinellus* sp. Three gene regions (nLSU, nSSU, and ITS) were sequenced for this isolate but only the nLSU and ITS sequences were used to construct a multigene phylogeny (Figure 6). The nSSU gene region was not included in the analysis due to insufficient representative sequences of this gene region for *Coprinellus* species in the NCBI database. The multigene phylogenetic analysis showed that the *Coprinellus* strain isolated in this study was closely related to a *Coprinellus micaceus* with 100% statistical bootstrap support (Figure 6).

4.5.2 Ceratobasidiaceae

In total, the Ceratobasidiaceae was represented by 35 cloned sequences and one isolate belonging to *Ceratobasidium* sp. (Table 2). All sequences were obtained from *H. barbertoni* samples through the amplification of three gene regions (nLSU, nSSU, and ITS). Individual phylogenetic trees were generated for each gene region including both the isolate sequence and cloned sequences.

Phylogenetic analysis of the ITS sequence data (Figure 7) grouped the 11 cloned sequences denoted as Orchid mycorrhiza MMC.CLN and MCF.CLN into one unique monophyletic clade with the sequence from the isolated fungus (Orchid mycorrhizae MMOR 005 isolate). The group was well supported with a 96% bootstrap value. The group was most closely related to a sequence (AJ549123) belonging to a mycorrhizal fungus isolated from *Dactylorhiza incarnata* with a bootstrap support value of 77%.

The LSU phylogenetic tree (Figure 8) reflected the results from the ITS phylogenetic analysis. Sequences from the *Ceratobasidium* isolate and cloned sequences grouped into a single monophyletic clade with high 100% bootstrap support. The cloned LSU sequence (Orchid mycorrhiza LSOR109.CLN) was the exception as it was placed sister to the other cloned LSU sequence (74 bootstrap support). This clade containing all sequences obtained in this study was distantly related to a *Ceratorhiza sp.* KX611342 with 72% bootstrap support.

The Ceratobasidium SSU phylogenetic tree (Figure 9) grouped the sequences obtained from this study in a monophyletic group with 97% bootstrap support. This clade was paraphyletic to a clade containing uncultured species of *Ceratobasidium* (HM453874) and *Thanatephorus* (HM446472).

4.5.3 Tulasnellaceae

A total of 22 ITS sequences were obtained from both *Habenaria* orchids using the molecular cloning technique. These sequences grouped in the Tulasnellaceae family. Results from the phylogenetic analysis (Figure 10) showed that the sequences obtained in this study clustered into four separate clades: Group 1 (orange), Group 2 (pink), Group 3 (yellow) and Group 4 (green). Sequences in Group 1 clustered with 89% bootstrap support and consisted of nine cloned sequences and were obtained from *H. epipactidea.* Group 2 had seven cloned sequences also obtained from the *H. epipactidea* and grouped with a 76% bootstrap support. Isolates in Group 3 (three cloned sequences) and Group 4 (three cloned sequences) clustered with 71 % and 77% bootstrap support in their respective groups. Group 3 and Group 4 formed sister groups, distant to Groups 1 and 2. Groups 1 and 2 were closely related to *Tulasnella dilquescens* (GenBank accession: AY37329), while Groups 3 and 4 were closely related to *Tulasnella calospora* (KP053823), but the relationships were not supported by bootstrap analysis.

4.5.4 Serendipitaceae

The phylogenetic analysis of cloned sequences identified as belonging to the Serendipitaceae grouped the sequences in two highly supported clades (Figure 11). All sequences were from *H. barbertoni.* The two groups formed sister clades, and together they formed a monophyletic clade with sequences representing *Sebacina vermifera* (EU626000, FN663133, FN663146) with 76% bootstrap support.

4.5.5 Hoehnelomycetaceae

Seven cloned sequences were obtained from *H. barbertoni* and identified as orchid mycorrhizae belonging to the order Atractiellales. Phylogenetic analysis (Figure 12) showed that all cloned sequences grouped in a monophyletic clade with 99% bootstrap statistical support. This monophyletic group included sequences belonging to *Atractiellales* sp. (GenBank accession no.: KF428369, KF428718, KF428447) and formed a sister clade to *Proceropycnis pinicola* (GenBank accession no.: DQ198780).

4.5.6 Omphalotaceae

Based on a BLASTn search, the best match for one of the cloned sequences (Orchid mycorrhiza MMC3 clone) obtained from *H. barbertoni* samples was an uncultured Trichlomataceae (FJ475747). However, *Gymnopus* sp. (Family: Omphalotaceae) had a 99% similarity to the query sequence. Alignments were made for the cloned sequence with reference ITS sequences that included species from *Gymnopus* and the Uncultured Trichlomataceae FJ475747 sequence. The maximum-likelihood phylogenetic tree generated based on ITS sequence data showed that the uncultured Trichlomataceae (FJ475747) sequence was nested within the *Gymnopus* species clade with bootstrap support of 65%. (Figure 13). The identity of the cloned sequence could not be established as it formed a polytomy with various *Gymnopus* spp.

4.5.7 Pezizaceae

Five cloned sequences from *H. barbertoni* were preliminarily identified as taxa belonging to the Pezizaceae. These were the only Ascomycota orchid mycorrhizae identified in this study. Phylogenetic analysis (Figure 14) grouped the sequences in a single clade that included a sequence from an uncultured Pezizaceae (FJ88742) with 98% bootstrap support. Together these sequences form a monophyletic clade that included a sequence from *Terfezia* sp. (DQ061109) and an uncultured Pezizaceae (FJ788742) with bootstrap support of 100%.

4.6 High-throughput Illumina sequencing

Using the high-throughput sequencing platform (Illumina HiSeq 2500) a total of 227042 raw sequences were obtained from all samples. Following data curation, 110419 sequences remained and were clustered into 160 OTUs (97% sequence similarity). In total, 59804 of the

reads were from *H. barbertoni* and 50615 from *H. epipactidea.* Taxonomic assignment grouped all sequences into eight fungal classes (Figure 15A), the most abundant of which was the Dothideomycetes (67 OTUs) followed by the Sordariomycetes (34 OTUs), Agaricomycetes (19 OTUs), Microbotryomycetes (7 OTUs), Tremellomycetes (6 OTUs), Atractiellomycetes (1 OTU), Mortierellomycetes (4 OTUs), and Mucormycotina class *incertae sedis* (1 OTU).

Orchid mycorrhizal fungi identified in *Habenaria* samples were represented by the Tulasnellaceae (17 OTUs), Serendipitaceae (2 OTUs), and Hoehnelomycetaceae (1 OTU) (Figure 15B). Orchid mycorrhizae of the Tulasnellaceae (*Tulasnella calospora*) and Serendipitaceae (*Serendipita* sp.) were found in both *Habenaria* species (Figure 15). Those unique to each orchid species were *Atractiella rhizophila* (Hoehnelomycetaceae) for *H. barbertoni* and *Epulorhiza* sp. (Tulasnellaceae) for the *H. epipactidea*. The 21 OTUs remaining were either Ascomycota or Basidiomycota but were unidentified at lower taxonomic levels. The other six classes were represented by 27 fungal families which have a broad range of non-mycorrhizal ecological roles (Figure 15B).

A principal coordinate analysis was used to determine overlap in fungal taxa in *H. barbertoni* and *H. epipactidea.* PC1 (principal component 1) explained 32.9% of the variation in the data and PC2 explained 28.9%. The analysis showed that there was some overlap between the two orchid species, indicating more shared than unique occurring species (Figure 16). The only differences can be linked to 15 OTUs which had the largest differences in sequence abundance between the two orchid species. The OTUs that were more abundant in *H. barbertoni* than in *H. epipactidea* based on the frequency of their sequence reads belonged to *Phoma* sp. (OTU12), *Mortierella* sp. (OTU17), *Pleosporales* sp. (OTU27), *Tulasnella* sp. (OTU37), *Mortierella* sp. (OTU41), *Colletotrichum* sp. (OTU47), *Mortierella* sp. (OTU52), *Gibberella* sp. (OTU58), *Papiliotrema* sp. (OTU64), *Fusarium* sp. (OTU69), *Epicoccum* sp. (OTU135), *Didymella* sp. (OTU137), *Humicola* sp. (OTU209), and *unidentified* (OTU460). The only OTUs more abundant in *H. epipactidea* represented *Didymella* sp. (OTU15) and *Epicoccum* sp. (OTU 135).

Variations in fungal diversity were also observed within each orchid sample (Table 3). Fungal genera found in all six samples of *Habenaria* orchids included *Alternaria, Ascochyta, Aureobasidium, Colletotrichum, Mortierella, Phoma, and Tulasnella*. Fungal genera found in all three samples of *H. barbertoni* were only *Atractiella* and *Humicola*. In contrast, none of the fungal genera identified was found only in the three *H. epipactidea* samples. The remaining

fungal genera (Table 1) were only found in one or two of the samples and not all three samples collected from *H. barbertoni* and *H. epipactidea*.

4.7 Overlap in results obtained from different methods

Three different methods were used to identify the orchid mycorrhizal diversity associated with each *Habenaria* species, namely fungal isolations, molecular cloning, and Illumina sequencing. An overlap of only seven fungal genera was detected among these methods. These included *Alternaria* sp., *Epicoccum* sp., *Humicola* sp., *Coprinellus* sp., *Setophoma* sp., *Fusarium* sp., *Colletotrichum* sp.

5. Discussion

Orchid mycorrhizal fungi can affect the geographic distribution and establishment of orchids in nature (McCormick & Jacquemyn, 2013, Jacquemyn *et al.*, 2017). Therefore, data from studies on the diversity of orchid mycorrhizae can aid in developing and implementing effective conservation strategies (Liu *et al.*, 2010, Merritt *et al.*, 2014). The orchid mycorrhizal diversity in South Africa has rarely been studied, thus limited information is available that can be used in conservation measures for orchids occurring in this region. Moreover, the effect of mycorrhizal associates on the occurrence of orchids in various habitats in South Africa is unknown. To address this, we investigated the orchid-mycorrhizal relationships of two South African orchids *H. barbertoni* and *H. epipactidea*. A combination of culture-dependent and independent methods was used to catalogue the mycorrhizal diversity associated with each orchid species and to investigate the variation in fungal diversity between the two orchid species. The results from this study showed that a substantial portion of the mycorrhizal taxa associated with *H. barbertoni* and *H. epipactidea* overlapped. Yet, each orchid species was found associated with a set of exclusive fungal taxa. Furthermore, the diversity of mycorrhizal fungi associated with *H. barbertoni* substantially varied between two collection times. This study therefore highlights the complexity in mycorrhizal diversity associated with two endemic *Habenaria* species from South Africa.

5.1 Mycorrhizal colonization

Based on microscopic observations all orchid roots and tubers examined were colonized by mycorrhizal fungi. However, the extent of mycorrhizal colonization differed between the two orchid species. In *H. barbertoni* no pelotons were detected in the roots or tubers; instead,

only unbranched monilioid structures, fungal hyphae with dolipore septa, located between the cortical cell walls, and basidiospores were observed. This possibly indicates an early stage of infection before peloton formation in new cortical cells (Huynh *et al*., 2004). In *H. epipactidea* pelotons were mainly observed in the roots and tubers indicating a later stage of colonization. Furthermore, the pelotons were observed as collapsed with several hyphae extending from each loose coil indicating that the orchid mycorrhizal association was at the peloton lysis stage (Huynh *et al.*, 2004, Suryantini *et al.*, 2015).

Habenaria species from India also showed different colonization patterns. Similar to *H. barbertoni*, *Habenaria ovalifolia* and *Habenaria multicaudata* were exclusively colonized by Basidiomycetous fungi with hyphae with dolipore septa but without pelotons (Anjali *et al.*, 2016). In contrast, *Habenaria marginata* and *Habenaria roxburghii* were mainly colonized by peloton forming fungi similar to *H. epipactidea* from this study (Sathiyadash *et al.*, 2012, Anjali *et al.*, 2016). Different Basidiomycota root colonization forms were also observed in orchid species from the genera *Dendrobium, Cymbidium* and *Gastrodia* (Zhu *et al.*, 2008, Martos *et al.*, 2009, Li *et al.*, 2020, Zhang *et al.*, 2020). These studies together with our results show that the patterns of mycorrhizal colonization can vary between orchid species from the same genus (Sathiyadash *et al.*, 2012). This study also provides a point of reference for future studies into the mycorrhizal colonization patterns of *Habenaria* orchids in South Africa.

5.2 Orchid mycorrhizal associates of Habenaria *orchids*

The orchid mycorrhizal associates identified in *Habenaria* orchids from South Africa were from the Ceratobasidiaceae, Serendipitaceae, Tulasnellaceae, Pezizaceae, Psathyrellaceae, Hoehnelomycetaceae, and Omphalotaceae. Orchid mycorrhizae belonging to the Tulasnellaceae and Serendipitaceae were identified in both *H. barbertoni* and *H. epipactidea*. However, each orchid is associated with different species from either orchid mycorrhizal family. The remaining five families were only found in *H. barbertoni*. From our results, the three most abundant orchid mycorrhizal taxa were the Ceratobasidiaceae, Serendipitaceae, and Tulasnellaceae. These are also known to be the most widespread and prevalent orchid mycorrhizal associates of photosynthetic terrestrial orchids (Dearnaley, 2007, Girlanda *et al.*, 2011, Selosse *et al.*, 2011).

Most terrestrial orchids native to Europe, Asia, America and Australia (including *Cypripedium calceolus*, *Neottia ovata*, *Cyclopogon elatus*, *Orchis militaris*, *Pterostylis nutans*

and *Pheladenia deformis*) are predominantly associated with the genera *Ceratobasidium*, *Tulasnella* or *Serendipita* (Irwin *et al.*, 2007, Dearnaley *et al.*, 2012, Davis *et al.*, 2015, Oja *et al.*, 2015, Pardo *et al.*, 2015, Anjali *et al.*, 2016, Jacquemyn *et al.*, 2017). Moreover, the main associates of *Habenaria* orchids growing in other parts of the world including *H. radiata* from Japan (Cowden & Shefferson, 2012), *H. hexaptera* from Argentina (Pardo *et al*., 2015) and *H. repens, H. macroceratitis and H. quinquiseta* from North America (Stewart & Zettler, 2002, Keel *et al.*, 2011, McCormick, 2018) are members of the Tulasnellaceae, Ceratobasidiaceae, and Serendipitaceae. The consistent occurrence of these orchid mycorrhizal taxa could suggest that they play key roles in germination and protocorm development (Keel *et al.*, 2011, Calevo *et al.*, 2020). Other mycorrhizal associates from the Pezizaceae, Psathyrellaceae, Hoehnelomycetaceae and Omphalotaceae were also identified from *H. barbertoni* but not from *H. epipactidea*. These fungi were previously thought to be saprophytic but have only recently been recognized as orchid mycorrhizae and their role in seed germination and protocorm development is still under investigation (Kottke *et al.*, 2010, Waterman *et al.*, 2011, Suarez & Kottke, 2016).

The mycorrhizal diversity of *Habenaria* orchids was studied using both molecular cloning and Illumina sequencing techniques. Results from the Illumina sequencing indicated that *Habenaria epipactidea* formed associations with Tulasnellaceae and Serendipitaceae. In contrast to the next-generation sequencing approach, sequence data from cloned ITS amplicon inserts did not identify any members of the Serendipitaceae from *Habenaria epipactidea*. The detection of additional mycorrhizal taxa using an Illumina sequencing approach highlights the advantages of using next-generation sequencing in biological diversity studies compared to molecular cloning (Mostafa *et al.*, 2015, Castro *et al.*, 2018, Forin *et al.*, 2018).

5.3 Orchid mycorrhizal associations shift over time.

The orchid mycorrhizal diversity associated with *H. barbertoni* emerged from two sampling time points in 2017 and 2018. In the 2017 samples, using molecular cloning of the ITS region, seven orchid mycorrhizal taxa (Ceratobasidiaceae, Serendipitaceae, Tulasnellaceae, Pezizaceae, Psathyrellaceae, Hoehnelomycetaceae, and Omphalotaceae) were identified. In 2018, only the Tulasnellaceae, Hoehnelomycetaceae, and Serendipitaceae were detected through the Illumina sequencing approach. Many more taxa were thus identified from *H. barbertoni* in 2017 compared to 2018, despite the low resolution of the cloning approach.

This difference in species abundance might be explained by differences in climate in the two sampling seasons or by the later sample collection date in 2018.

A change in mycorrhizal diversity over time has been observed in other orchids such as *Neottia ovata* (Oja *et al.*, 2015, Jacquemyn *et al.*, 2016), *Anacamptis morio* (Ercole *et al.*, 2014), *Pseudorchis albida* (Kohout *et al.*, 2012), *Gymnadenia conopsea* (Gao *et al.*, 2020) *Cyrtochilum retusum* and *Epidendrum macrum* (Cevallos *et al.*, 2018). Changes in orchid mycorrhizal colonizing orchid roots of *Neottia ovata* were observed after 4 weeks (Oja *et al.*, 2015), while Kohout *et al.* (2012) observed changes in the mycorrhizal associates of *Pseudorchis albida* between summer and autumn of the same year. Also, the succession of orchid mycorrhizae through the different life stages of orchid growth has been observed (Bidartondo *et al.*, 2004). For example, for germination, *Gastrodia elata* requires *Mycena osmendicula* but requires *Armillaria* sp. for further growth (Chen *et al.*, 2019). Similarly, the *Dendrobium nobile* orchid requires different mycorrhizae for protocorm development (*Epulorhiza* spp.) and adult growth (Sebacinales and Cantharellales) (Chen *et al.*, 2012). These studies support the temporal change observed in orchid mycorrhizal associates of *Habenaria* spp.

Climate variations might also influence mycorrhizal colonization of orchid roots. While temperatures in 2017 and 2018 were similar, the overall mean rainfall observed in 2017 was higher than that observed in 2018 (http://www.weathersa.co.za/home/historicalrain). Moreover, several months after the first sample collection (2017) the rainfall was below average, therefore indicating drought conditions before orchid collections in April 2018. In March 2018, the first big rains were experienced in the region resulting in sudden waterlogging (Mkhwanazi *et al.*, 2018). Thus, the environmental conditions before the second collection of orchid roots in our study could have resulted in the replacement of non-adapted mycorrhizae with more tolerant species

The effect of abiotic factors on the mycorrhizal associates of the two orchid species is to a large extent supported by several previous studies. These studies showed that mycorrhizal relationships can fluctuate with changes in climate such as temperature, drought, and flooding (Osono *et al.*, 2003, Bellgard & Williams, 2011, Pecoraro *et al.*, 2018). Of particular relevance is the discovery that high amounts of rainfall can cause a decline in the abundance and diversity of drought-tolerant ectomycorrhizal species in the soil while increasing the occurrence of saprophytes and pathogens (Osono *et al.*, 2003, Pickles *et al.*, 2012). Furthermore, arbuscular

mycorrhizae are also negatively affected by increased water because they are sensitive to lower oxygen levels (Millar & Bennett, 2016, Jamiolkowska *et al.*, 2018). Therefore, orchid mycorrhizae of *Habenaria* spp. investigated in the current study may also be affected by these abiotic factors, but more studies are required to confirm this notion.

5.4 Habitat-driven variations in mycorrhizae diversity

Orchid mycorrhizal diversity and abundance of specific taxa can vary between habitats (Esposito *et al.*, 2016, Mujica *et al.*, 2016). Although the *Habenaria* orchids were found growing 190 m from each other, their immediate habitats varied. *Habenaria epipactidea* samples were collected in an open area surrounded mainly by indigenous grasses, while *H. barbertoni* orchids sampled were growing in a shady area surrounded by different trees and shrubs. In grassland-like habitats similar to where *H. epipactidea* was growing, orchid mycorrhizal fungi frequently occur at higher abundances (Voyron *et al.*, 2017, Izuddin *et al.*, 2019). This is possibly because the dispersal of fungal spores from these areas is unhindered by other plants and trees (Jacquemyn *et al.*, 2016, Oja *et al.*, 2017). The *H. barbertoni* habitat, on the other hand, is favourable for the growth of saprophytic orchid mycorrhizal fungi due to the presence of decaying plant matter and plant exudates required for independent survival of these fungi (Izuddin *et al.*, 2019). Moreover, the proximity of *H. barbertoni* to neighbouring plants and trees may have allowed for the formation of tripartite associations with ectomycorrhizae (Bidartondo *et al.*, 2004, Yeh *et al.*, 2019). This may explain the association of *H. barbertoni* with a more diverse range of saprophytic orchid mycorrhizae. Thus, the mycorrhizal diversity observed in the *Habenaria* orchids sampled in this study could have resulted from the co-occurring flora in their immediate habitats.

Variations in habitat have been shown to affect the mycorrhizal diversity of several orchid species. For example, in a study by Jacquemyn *et al.* (2014) *Orchis purpurea* and *Orchis mascula* occurring in 25x 25m plots were found to harbour distinct orchid mycorrhizal associates. Similar observations were made for other South African orchids in the subtribe Coryciinae (Waterman *et al.*, 2011), as well as for *Dactylorhiza* spp. from various parts of Europe, Asia, and Africa (Jacquemyn *et al.*, 2016). While orchids growing in close proximity had generally different mycorrhizal associates, the dominant mycorrhizal taxa were consistent (Bayman *et al.*, 2016, Cevallos *et al.*, 2018). These studies together with our results, therefore, support the hypothesis that mycorrhizal diversity and abundance may be influence by the

orchid's habitat (Oja *et al.*, 2017, Izuddin *et al.*, 2019). Habitat therefore likely influenced the diversity of the orchid mycorrhizae in the two *Habenaria* species included in this study.

5.5 Non-mycorrhizal fungal associates

Non-mycorrhizal associates identified in this study were dominated by fungi from the Ascomycota which were more abundant in the roots than orchid mycorrhizal fungi. In accordance with similar studies, genera such as *Cladosporium*, *Paeciliomyces*, *Phoma*, *Alternaria*, *Davidiella*, *Epiccocum*, *Mycoleptodiscus*, *Fusarium*, *Chaetomium*, *Clonostachys,* and *Purpureocillium* were identified in both *Habenaria* orchids (Ma *et al.*, 2016, Sarsaiya *et al.*, 2019). *Habenaria barbertoni* was associated uniquely with isolates from *Xyalaria* and *Trichoderma* sp. while the *Phaeosphaeria* and *Leucosporidium* genera were uniquely found in *H. epipactidea* roots.

The fungi identified as non-mycorrhizal in this study may play important roles in the orchids' development. For example, they might be involved in promoting seed germination and growth, by producing hormones such as indoles and gibberellins or might protect the orchid by producing antimicrobial compounds as was shown in previous studies (Kawaide, 2006, Ma *et al.*, 2016, Wang *et al.*, 2016). The co-inoculation of arbuscular mycorrhizae and associated endophytes (*Xylaria* sp., *Phialophora* sp., and *Phoma* sp.) showed significantly higher seedling growth compared to a mycorrhizal inoculation alone (Wezowicz *et al.*, 2017, Wazny *et al.*, 2018). This might be the same for *Habenaria* spp. Although the exact roles of orchid-associated endophytes are unclear, there is potential that these fungi assist in orchid propagation. Exploring the roles of fungi associated with South African orchids could therefore result in the identification of native fungi which could be used to enhance the growth of orchids as well as of other crop plants.

6. Conclusions

This is the first study to successfully document and compare the fungal community composition of two co-existing orchids of the genus *Habenaria*. It is clear from this study that the fungal associates of these orchids significantly differ. Fungi within the *Rhizoctonia-complex* (Serendipitaceae and Tulasnellaceae) were shown to be the only orchid mycorrhizae associated with both orchid species. Also, a considerable number of orchid mycorrhizae were unique to *H. barbertoni* in this study, including members of the Ceratobasidiaceae, Pezizaceae, Psathyrellaceae, Hoehnelomycetaceae, and Omphalotaceae. The biology and role of these

identified mycorrhizal associates were not assessed in this study. Therefore, future studies on their roles in seed germination and orchid growth are still required.

Successful orchid translocations have been conducted on 66 orchid species worldwide (Reiter *et al.*, 2016). The success of these translocations relied strongly on first screening new locations for compatible mycorrhizal associates and the presence of suitable pollinators for the displaced species (Reiter *et al.*, 2016). To conduct similar translocations for South African orchids, knowledge of their orchid mycorrhizal partners is pivotal. This study provides a list of orchid mycorrhizae associated with two endemic orchid species, *H. barbertoni* and *H. epipactidea,* collected from the same site. Our results could provide the first step in introducing mycorrhizae-based conservation approaches for South African orchids. This study also highlights the importance of considering environmental factors such as climate change and habitat when working with orchid mycorrhizal fungi in conservation strategies. Moreover, when selecting new sites for orchid translocation, data from this study may help to show which habitats are best suited for each orchid species by first comparing the endemic fungal population at the new sites with our results.

7. References

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8. Tables and figures

Table 1: List of fungal strains isolated from both *H. barbertoni* and *H. epipactidea* identified using ITS sequence data. Included in the table: Orchid species, habitat, number of isolates obtained and BLASTn search results (Closest related taxa, classification. % Similarity and GenBank accession number)

Orchid species	Collection date	Habitat	# Isolates	Closest related taxa	Classification	% Similarity	GenBank accession no
H. barbertoni	2017	Shrubland	1	*Chaetomium cochliodes*	Sordariomycetes/Chaetomiaceae	99	MH550490
H. barbertoni	2017	Shrubland	4	*Mycoleptodiscus geniculatus*	Sordariomycetes /Mycoleptodiscus	98	MH862555
H. barbertoni	2017	Shrubland	2	*Setophoma terrestris*	Dothideomycetes/Pleosporineae	99	EF154351
H. barbertoni	2017	Shrubland	1	*Ceratobasidium* sp.	Agaricomycetes/Ceratobasidiaceae	91	JQ247397
H. barbertoni	2017	Shrubland	1	*Purpureocillium lilacinum*	Sordariomycetes/Ophiocordycipitaceae	100	HQ607867
H. barbertoni	2017	Shrubland	4	*Periconia macrospinosa*	Dothideomycetes/Pleosporineae	97	HG936260
H. barbertoni	2017	Shrubland	1	*Periconia macrospinosa*	Dothideomycetes/Pleosporineae	96	KY031641
H. barbertoni	2017	Shrubland	2	*Alternaria longissima*	Dothideomycetes/Pleosporaceae	100	HG328076
H. barbertoni	2017	Shrubland	1	*Coprinellus micaceus*	Agaricomycetes/ Psathyrellaceae	100	GU187887
H. barbertoni	2018	Shrubland	1	*Trichoderma gamsii*	Sordariomycetes/Hypocreomycetidae	100	MK185387

Table 1 continued: List of fungal strains isolated from both *H. barbertoni* and *H. epipactidea* identified using ITS sequence data. Included in the table: Orchid species, habitat, number of isolates obtained and BLASTn search results (Closest related taxa, classification. % Similarity and GenBank accession number)

Orchid species	Collection date	Habitat	# Isolates	Closest related taxa	Classification	% Similarity	GenBank accession no
H. barbertoni	2017	Shrubland	2	*Purpureocillium lilacinum*	Sordariomycetes/Ophiocordycipitaceae	99	KY951911
H. barbertoni	2017	Shrubland	1	*Chaetomium indicum*	Sordariomycetes/Chaetomiaceae	100	MH864199
H. barbertoni	2017	Shrubland	1	*Phomopsis columnaris*	Sordariomycetes/ Diaporthaceae	99	KU712245
H. barbertoni	2017	Shrubland	1	*Phomopsis columnaris*	Sordariomycetes/Diaporthaceae	99	KC145883
H. barbertoni	2017	Shrubland	1	*Acremoniopsis suttonii*	Sordariomycetes/ Nectriaceae	99	NR_145059
H. barbertoni	2017	Shrubland	1	*Alternaria longissima*	Dothideomycetes /Pleosporaceae	99	GQ924020
H. barbertoni	2017	Shrubland	3	*Neurospora dictyophora*	Sordariomycetes /Sordariaceae	99	MH862539
H. barbertoni	2018	Shrubland	1	*Fusarium oxysporum*	Sordariomycetes/Nectriaceae	100	MH879859
H. barbertoni	2018	Shrubland	3	*uncultured Clonostachys*	Sordariomycetes /Bionectriaceae	99	KF493907
H. barbertoni	2018	Shrubland	6	*Mycoleptodiscus geniculatus*	Sordariomycetes /Mycoleptodiscus	98	MH862555
H. barbertoni	2018	Shrubland	1	*Chaetomium homopilatum*	Sordariomycetes/Chaetomiaceae	99	JQ666671
H. barbertoni	2018	Shrubland	1	*Humicola* sp.	Sordariomycetes/Chaetomiaceae	99	FJ612990
H. barbertoni	2018	Shrubland	1	*Fusarium redolens*	Sordariomycetes/Nectriaceae	99	KU350711
H. barbertoni	2018	Shrubland	4	*Paecilomyces* sp.	Eurotiomycetes /Clavicipitaceae	98	FJ765032

Table 1 continued: List of fungal strains isolated from both *H. barbertoni* and *H. epipactidea* identified using ITS sequence data. Included in the table: Orchid species, habitat, number of isolates obtained and BLASTn search results (Closest related taxa, classification. % similarity and GenBank accession number)

Orchid species	Collection date	Habitat	# Isolates	Closest related taxa	Classification	% Similarity	GenBank accession no
H. barbertoni	2018	Shrubland	1	*Trichoderma gamsii*	Sordariomycetes/Hypocreomycetidae	100	MK185387
H. barbertoni	2018	Shrubland	2	*Paecilomyces* sp.	Eurotiomycetes /Clavicipitaceae	98	GU108582
H. barbertoni	2018	Shrubland	1	*Fusarium* sp.	Sordariomycetes/Nectriaceae	100	HG936875
H. barbertoni	2018	Shrubland	1	*Daldinia loculata*	Sordariomycetes /Xylariales	99	JQ758777
H. barbertoni	2018	Shrubland	1	*Fusarium oxysporum*	Sordariomycetes/Nectriaceae	99	MG571600
H. barbertoni	2018	Shrubland	1	*Fusarium oxysporum*	Sordariomycetes/Nectriaceae	99	MK641475
H. barbertoni	2018	Shrubland	1	*Clonostachys rosea*	Sordariomycetes/Bionectriaceae	99	MH067954
H. barbertoni	2018	Shrubland	1	*Fusarium oxysporum*	Sordariomycetes/Nectriaceae	100	MK396896
H. barbertoni	2018	Shrubland	1	*Fusarium oxysporum*	Sordariomycetes/Nectriaceae	99	MK713411
H. barbertoni	2018	Shrubland	1	*Aspergillus spelaeus*	Eurotiomycetes/Aspergillaceae	98	HG915907
H. barbertoni	2018	Shrubland	1	*Hypocreales* sp.	Sordariomycetes/Hypocreomycetidae	99	GQ923987
H. barbertoni	2018	Shrubland	1	*Trichoderma gamsii*	Sordariomycetes/Hypocreomycetidae	100	KX343107
H. epipactidea	2018	Grassland	2	*Chaetomium homopilatum*	Sordariomycetes/Chaetomiaceae	99	JQ666671
H. epipactidea	2018	Grassland	2	*Fusarium oxysporum*	Sordariomycetes/Nectriaceae	100	MH575293
H. epipactidea	2018	Grassland	1	*Fusarium proliferatum*	Sordariomycetes/Nectriaceae	99	MH055399

Table 1 continued: List of fungal strains isolated from both *H. barbertoni* and *H. epipactidea* identified using ITS sequence data. Included in the table: Orchid species, habitat, number of isolates obtained and BLASTn search results (Closest related taxa, classification. % similarity and GenBank accession number)

Orchid species	Collection date	Habitat	# Isolates	Closest related taxa	Classification	% Similarity	GenBank accession no
H. epipactidea	2018	Grassland	1	*Chaetomium flavigenum*	Sordariomycetes/Chaetomiaceae	97	MH858989
H. epipactidea	2018	Grassland	1	*Periconia igniaria*	Dothideomycetes/Periconiaceae	98	DQ420988
H. epipactidea	2018	Grassland	1	*Beauveria bassiana*	Sordariomycetes/Cordycipitaceae	99	AJ560686
H. epipactidea	2018	Grassland	1	*Fusarium oxysporum*	Sordariomycetes/Nectriaceae	99	KU671046
H. epipactidea	2018	Grassland	1	*Purpureocillium lilacinum*	Sordariomycetes/Ophiocordycipitaceae	99	HQ607867
H. epipactidea	2018	Grassland	1	*Fusarium oxysporum*	Sordariomycetes/Nectriaceae	100	MH512964
H. epipactidea	2018	Garden	1	*Aspergillus fumigatus*	Eurotiomycetes/Aspergillaceae	96%	KY523044
H. epipactidea	2018	Garden	1	*Pochonia bulbillosa*	Sodariomycetes/Clavicipitaceae	99%	AB709835
H. epipactidea	2018	Garden	1	*Phoma* sp.	Dothideomycetes/ Didymellaceae	99%	KP278172
H. epipactidea	2018	Garden	1	*Colletotrichum dematium*	Sordariomycetes/Glomerellaceae	99%	JQ677042
H. epipactidea	2018	Garden	1	*Cordyceps taishanensis*	Sordariomycetes/Cordycipitaceae	100%	FJ008928
H. epipactidea	2018	Garden	1	*Epicoccum nigrum*	Dothideomycetes/ Didymellaceae	100%	MH645206
H. epipactidea	2018	Garden	1	*Epicoccum sorghinum*	Dothideomycetes/ Didymellaceae	99%	KX758542
H. epipactidea	2018	Garden	1	*Pleosporales* sp.	Dothideomycetes	94%	GQ923978
H. epipactidea	2018	Garden	1	*Talaromyces cellulolyticus*	Eurotiomycetes /Trichocomaceae	96%	JN624892

Table 1 continued: List of fungal isolates obtained from both *H. barbertoni* and *H. epipactidea* identified using ITS sequence data. Included in the table: Orchid species, date of collection, habitat, number of isolates obtained and BLASTn search results (Closest related taxa, classification. % similarity and GenBank accession number)

Orchid species	Collection date	Habitat	# Isolates	Closest related taxa	Classification	% Similarity	GenBank accession no
H. epipactidea	2018	Garden	2	*Fusarium oxysporum*	Sordariomycetes/Nectriaceae	99%	MH454072
H. epipactidea	2018	Garden	1	*Aspergillus spelaeus*	Eurotiomycetes/ Aspergillaceae	99%	MH911347
H. epipactidea	2018	Garden	2	*Colletotrichum dematium*	Sodariomycetes/Glomerellaceae	99%	JQ684863
H. epipactidea	2018	Garden	1	*Fusarium oxysporum*	Sordariomycetes/Nectriaceae	99%	MH879861
H. epipactidea	2018	Garden	3	*Fusarium oxysporum*	Sordariomycetes/Nectriaceae	100%	MH879859
H. epipactidea	2018	Garden	1	*Aureobasidium pullulans*	Dothideomycetes /Saccotheciaceae	100%	AM160630
H. epipactidea	2018	Garden	1	*Fusarium oxysporum*	Sordariomycetes/Nectriaceae	99%	LC428050
H. epipactidea	2018	Garden	2	*Fusarium oxysporum*	Sordariomycetes/Nectriaceae	100%	MG571602
H. epipactidea	2018	Garden	1	*Epicoccum sorghinum*	Dothideomycetes /Didymellaceae	99%	MH824378
H. epipactidea	2018	Garden	1	*Pochonia bulbillosa*	Sordariomycetes/Clavicipitaceae	99%	AB378552
H. epipactidea	2018	Garden	1	*Fusarium oxysporum*	Sordariomycetes/Nectriaceae	100%	MK396896
H. epipactidea	2018	Garden	2	*Fusarium oxysporum*	Sordariomycetes/Nectriaceae	100%	LT841236
H. epipactidea	2018	Garden	2	*Clonostachys rosea*	Sordariomycetes/Bionectriaceae	99%	MH067954
H. epipactidea	2018	Garden	1	*Mycoleptodiscus geniculatus*	Sordariomycetes /Mycoleptodiscus	98%	MH862555

Table 2: List of sequences obtained by cloning PCR-amplified fungal DNA from both *H. barbertoni* and *H. epipactidea*. ITS, LSU and SSU gene regions were amplified, cloned into a sequencing vector, and sequenced. Included in the table: Orchid species, habitat, number of cloned inserts obtained and BLASTn search results (Closest related taxa, classification, similarity and GenBank accession number)

Orchid species	Collection Date	Habitat	Gene region	# Clones	Closest related taxa	Classification	% Similarity	Accession no
H. barbertoni	2017	Shrubland	ITS	10	*Ceratobasidium* sp.	Cantharellales/Ceratobasidiaceae	92	JQ247397
H. barbertoni	2017	Shrubland	ITS	1	*Ceratobasidium* sp.	Cantharellales/Ceratobasidiaceae	93	AF472285
H. barbertoni	2017	Shrubland	ITS	3	Uncultured Tulasnellaceae	Cantharellales/ Tulasnellaceae	99	JX545219
H. barbertoni	2017	Shrubland	ITS	3	Uncultured Tulasnellaceae	Cantharellales/ Tulasnellaceae	99	KP053823
H. barbertoni	2017	Shrubland	ITS	10	*Sebacina vermifera*	Sebacinales/Serendipitaceae	97	EU626000
H. barbertoni	2017	Shrubland	ITS	5	Uncultured Pezizaceae	Pezizales/Pezizaceae	99	FJ788742
H. barbertoni	2017	Shrubland	ITS	1	Tricholomataceae	Agaricales/Tricholomataceae	95	FJ475747
H. barbertoni	2017	Shrubland	ITS	7	*Atractiella rhizophila*	Atractiellales/ Hoehnelomycetaceae	99	KX499292
H. barbertoni	2017	Shrubland	LSU	2	*Thanatephorus cucumeris*	Cantharellales/Ceratobasidiaceae	96	AF354111
H. barbertoni	2017	Shrubland	LSU	9	*Uthatobasidium fusisporum*	Cantharellales/Ceratobasidiaceae	93	AF518664
H. barbertoni	2017	Shrubland	LSU	6	*Rhizoctonia solani*	Cantharellales/Ceratobasidiaceae	93	MH874912
H. barbertoni	2017	Shrubland	LSU	1	*Rhizoctonia solani*	Cantharellales/Ceratobasidiaceae	90	MH874413
H. barbertoni	2017	Shrubland	LSU	2	*Ceratobasidium* sp.	Cantharellales/Ceratobasidiaceae	98	KM280400
H. barbertoni	2017	Shrubland	LSU	4	*Infundibura adhaerens*	Atractelliales/Helicogloeaceae	91	KF061296

Table 2 continued: List of sequences obtained by cloning PCR-amplified fungal DNA from both *H. barbertoni* and *H. epipactidea*. ITS, LSU and SSU gene regions were amplified, cloned into a sequencing vector, and sequenced. Included in the table: Orchid species, habitat, number of cloned inserts obtained and BLASTn search results (Closest related taxa, classification, similarity and GenBank accession number)

Orchid species	Collection Date	Habitat	Gene region	# Clones	Closest related taxa	Classification	% Similarity	Accession no
H. barbertoni	2017	Shrubland	SSU	1	*Rhizoctonia solani*	Cantharellales/Ceratobasidiaceae	99	KJ652019
H. barbertoni	2017	Shrubland	SSU	1	*Rhizoctonia* sp.	Cantharellales/Ceratobasidiaceae	99	FJ515314
H. barbertoni	2017	Shrubland	SSU	1	*Thanatephorus cucumeris*	Cantharellales/Ceratobasidiaceae	99	DQ917659
H. barbertoni	2017	Shrubland	ITS	6	*Pleosporales* sp.	Pleosporales	97	KY965431
H. barbertoni	2017	Shrubland	ITS	1	*Pleosporales* sp.	Pleosporales	98	KF800272
H. barbertoni	2017	Shrubland	ITS	1	Uncultured fungus	-	98	KT224885
H. barbertoni	2017	Shrubland	ITS	25	*Fusarium oxysporum*	Hypocreales/ Nectriaceae	99	MK828120
H. barbertoni	2017	Shrubland	ITS	1	*Uncultured Hygrocybe*	Agaricales/Hygrophoraceae	88	JQ347153
H. barbertoni	2017	Shrubland	ITS	1	*Fusarium oxysporum*	Hypocreales/ Nectriaceae	99	KX387580
H. barbertoni	2017	Shrubland	ITS	1	*Phaeosphaeria* sp.	Pleosporales/ Phaeosphaeriaceae	95	EU490166
H. barbertoni	2017	Shrubland	ITS	1	Uncultured fungus	-	98	KT224885
H. barbertoni	2017	Shrubland	ITS	25	*Fusarium oxysporum*	Hypocreales/ Nectriaceae	99	MK828120
H. barbertoni	2017	Shrubland	ITS	1	Uncultured *Hygrocybe*	Agaricales/Hygrophoraceae	88	JQ347153
H. barbertoni	2017	Shrubland	ITS	1	*Fusarium oxysporum*	Hypocreales/ Nectriaceae	99	KX387580
H. barbertoni	2017	Shrubland	ITS	1	*Pleosporales* sp.	Pleosporales	95	MH425303

Table 2 continued: List of sequences obtained by cloning PCR-amplified fungal DNA from both *H. barbertoni* and *H. epipactidea*. ITS, LSU and SSU gene regions were amplified, cloned into a sequencing vector, and sequenced. Included in the table: Orchid species, habitat, number of cloned inserts obtained and BLASTn search results (Closest related taxa, classification, similarity and GenBank accession number)

Orchid species	Collection Date	Habitat	Gene Region	# Clones	Closest related taxa	Classification	% Similarity	Accession no
H. barbertoni	2017	Shrubland	ITS	1	*Vonarxula javanica*	Microbotryomycetes /Chrysozymaceae	90	KY105849
H. barbertoni	2017	Shrubland	ITS	1	Basidiomycota clone	Basidiomycota	96	GU328584
H. barbertoni	2017	Shrubland	ITS	6	Nigrospora oryzae	Trichosphaeriales/Trichosphaeriaceae	99	KF709558
H. barbertoni	2017	Shrubland	ITS	1	*Malassezia restricta*	Malasseziales/Malasseziaceae	99	CP030254
H. barbertoni	2017	Shrubland	ITS	1	*Peniophora* sp.	Chaetothyriales	98	HQ607853
H. barbertoni	2017	Shrubland	ITS	1	*Ophiosphaerella narmari*	Pleosporales/Phaeosphaeriaceae	98	KC841081
H. barbertoni	2017	Shrubland	ITS	16	Ascomycota clone	Ascomycota	99	EU490152
H. barbertoni	2017	Shrubland	ITS	1	*Dactylonectria pauciseptata*	Hypocreales/ Nectriaceae	99	MK602783
H. epipactidea	2018	Grassland	ITS	3	*Curvibasidium pallidicorallinum*	Microbotryales /Microbotryaceae	99	JX188149
H. epipactidea	2018	Grassland	ITS	4	*Curvibasidium cygneicollum*	Microbotryales /Microbotryaceae	97	KY102972
H. epipactidea	2018	Grassland	ITS	3	Ascomycota clone	Ascomycota	99	EU490049
H. epipactidea	2018	Grassland	ITS	38	*Cladosporium westerdijkieae*	Capnodiales/Cladosporiaceae	99	MF473314
H. epipactidea	2018	Grassland	ITS	9	*Alternaria infectoria*	Pleosporales /Pleosporaceae	99	MK828116
H. epipactidea	2018	Grassland	ITS	1	*Epicoccum* sp.	Pleosporales/ Didymellaceae	99	MG976379

Table 2 continued: List of sequences obtained by cloning PCR-amplified fungal DNA from both *H. barbertoni* and *H. epipactidea*. ITS, LSU and SSU gene regions were amplified, cloned into a sequencing vector, and sequenced. Included in the table: Orchid species, habitat, number of cloned inserts obtained and BLASTn search results (Closest related taxa, classification, similarity and GenBank accession number**)**

Orchid species	Collection date	Habitat	Gene region	# Clones	Closest related taxa	Classification	% Similarity	Accession no
H. epipactidea	2018	Grassland	ITS	1	*Rhodotorula glutinis*	Sporidiobolales/Sporidiobolaceae	99	KJ463904
H. epipactidea	2018	Grassland	ITS	1	*Westerdykella multispora*	Pleosporales/Sporormiaceae	95	MH872199
H. epipactidea	2018	Grassland	ITS	2	*Epicoccum nigrum*	Pleosporales/ Didymellaceae	99	MF281326
H. epipactidea	2018	Grassland	ITS	1	*Cladosporium* sp.	Capnodiales/Cladosporiaceae	98	JQ951582
H. epipactidea	2018	Grassland	ITS	2	*Cladosporium pseudocladosporioides*	Capnodiales/Cladosporiaceae	98	MF473222
H. epipactidea	2018	Grassland	ITS	1	*Alternaria* sp.	Pleosporales /Pleosporaceae	98	MH102088
H. epipactidea	2018	Grassland	ITS	3	Uncultured *Davidiella*	Capnodiales/Davidiellaceae	99	HG935280
H. epipactidea	2018	Grassland	ITS	1	*Uncultured Psiloglonium*	Hysteriales, /Hysteriaceae	97	HG935427
H. epipactidea	2018	Grassland	ITS	1	*Epicoccum nigrum*	Pleosporales/ Didymellaceae	98	KU837875
H. epipactidea	2018	Grassland	ITS	1	Uncultured soil fungus	-	99	JQ666321
H. epipactidea	2018	Grassland	ITS	1	Uncultured *Davidiella*	Capnodiales/Davidiellaceae	98	HG935274
H. epipactidea	2018	Grassland	ITS	1	Uncultured fungus	-	99	EU869182
H. epipactidea	2018	Grassland	ITS	14	Uncultured *Tulasnellaceae*	Cantharellales/ Tulasnellaceae	96	JX649082
H. epipactidea	2018	Grassland	ITS	1	Uncultured *Tulasnellaceae*	Cantharellales/ Tulasnellaceae	96	HM230650
H. epipactidea	2018	Grassland	ITS	1	Uncultured *Tulasnellaceae*	Cantharellales/ Tulasnellaceae	97	GQ241863

Table 3: Table showing the variations of fungal genera occurring in three individual plants of *Habenaria barbertoni* and three *Habenaria epipactidea*. Green bars represent fungi found in all samples and pink bars represent those found in all three *H. barbertoni* samples. No fungal taxa were consistently shared between all samples of *H. epipactidea*. Most of the fungal genera were only found in one or two but not all orchid samples

Fungal taxa (OTU)	***H. barbertoni* (1)**	***H. barbertoni* (2)**	***H. barbertoni* (3)**	***H. epipactidea* (1)**	***H. epipactidea* (2)**	***H. epipactidea* (3)**
Alternaria sp.	•	•	•	•	•	•
Ascochyta sp.	•	•	•	•	•	•
Atractiella sp.	•	•	•			
Aureobasidium sp.	•	•	•	•	•	•
Biappendiculispora sp.					•	
Bifiguratus sp.				•		
Boeremia sp.	•	•		•	•	
Calonectria sp.		•			•	
Cladosporium sp.		•				•
Colletotrichum sp.	•	•	•	•	•	•
Curvibasidium sp.	•	•	•		•	•
Curvularia sp.		•				
Cylindrocladium sp.					•	
Daldinia sp.		•			•	
Dictyosporium sp.		•				
Didymella sp.	•		•	•	•	•
Epicoccum sp.	•		•	•	•	•

Table 3 continued: Table showing the variations of fungal genera occurring in three individual plants of *Habenaria barbertoni* and three *Habenaria epipactidea*. Green bars represent fungi found in all samples and pink bars represent those found in all three *H. barbertoni* samples. No fungal taxa were consistently shared between all samples of *H. epipactidea*. Most of the fungal genera were only found in one or two but not all orchid samples

Fungal taxa (OTU)	***H. barbertoni* (1)**	***H. barbertoni* (2)**	***H. barbertoni* (3)**	***H. epipactidea* (1)**	***H. epipactidea* (2)**	***H. epipactidea* (3)**
Epulorhiza sp.					•	
Fusarium sp.	•		•	•	•	•
Genolevuria sp.		•		•		
Gibberella sp.	•			•		
Hendersonia sp.	•	•				
Heterophoma sp.	•	•		•		
Humicola sp.	•	•	•			
Ilyonectria sp.	•	•				•
Leucosporidium sp.					•	
Mortierella sp.	•	•	•	•	•	•
Neoascochyta sp.	•					
Neodidymelliopsis sp.					•	
Nigrospora sp.	•	•				
Nothophoma sp.		•				
Papiliotrema sp.	•	•				
Paraconiothyrium sp.		•	•			
Periconia sp.				•		

Table 3 continued: Table showing the variations of fungal genera occurring in three individual plants of *Habenaria barbertoni* and three *Habenaria epipactidea*. Green bars represent fungi found in all samples and pink bars represent those found in all three *H. barbertoni* samples. No fungal taxa were consistently shared between all samples of *H. epipactidea*. Most of the fungal genera were only found in one or two but not all orchid samples

Fungal taxa (OTU)	***H. barbertoni* (1)**	***H. barbertoni* (2)**	***H. barbertoni* (3)**	***H. epipactidea* (1)**	***H. epipactidea* (2)**	***H. epipactidea* (3)**
Phaeomycocentrospora sp.					•	
Phaeosphaeria sp.				•		
Phoma sp.	•	•	•	•	•	•
Pyrenochaeta sp.	•	•				
Pyrenochaetopsis sp.		•				
Rhodosporidiobolus sp.	•					
Rhodotorula sp.	•	•	•		•	•
Saitozyma sp.	•	•	•			•
Serendipita sp.	•			•		
Setophaeosphaeria sp.			•			
Setophoma sp.			•			
Sigarispora sp.	•	•				•
Solicoccozyma sp.		•			•	
Stagonosporopsis sp.		•				•
Teichospora sp.	•					
Tulasnella sp.	•	•	•	•	•	•
Vishniacozyma sp.						•

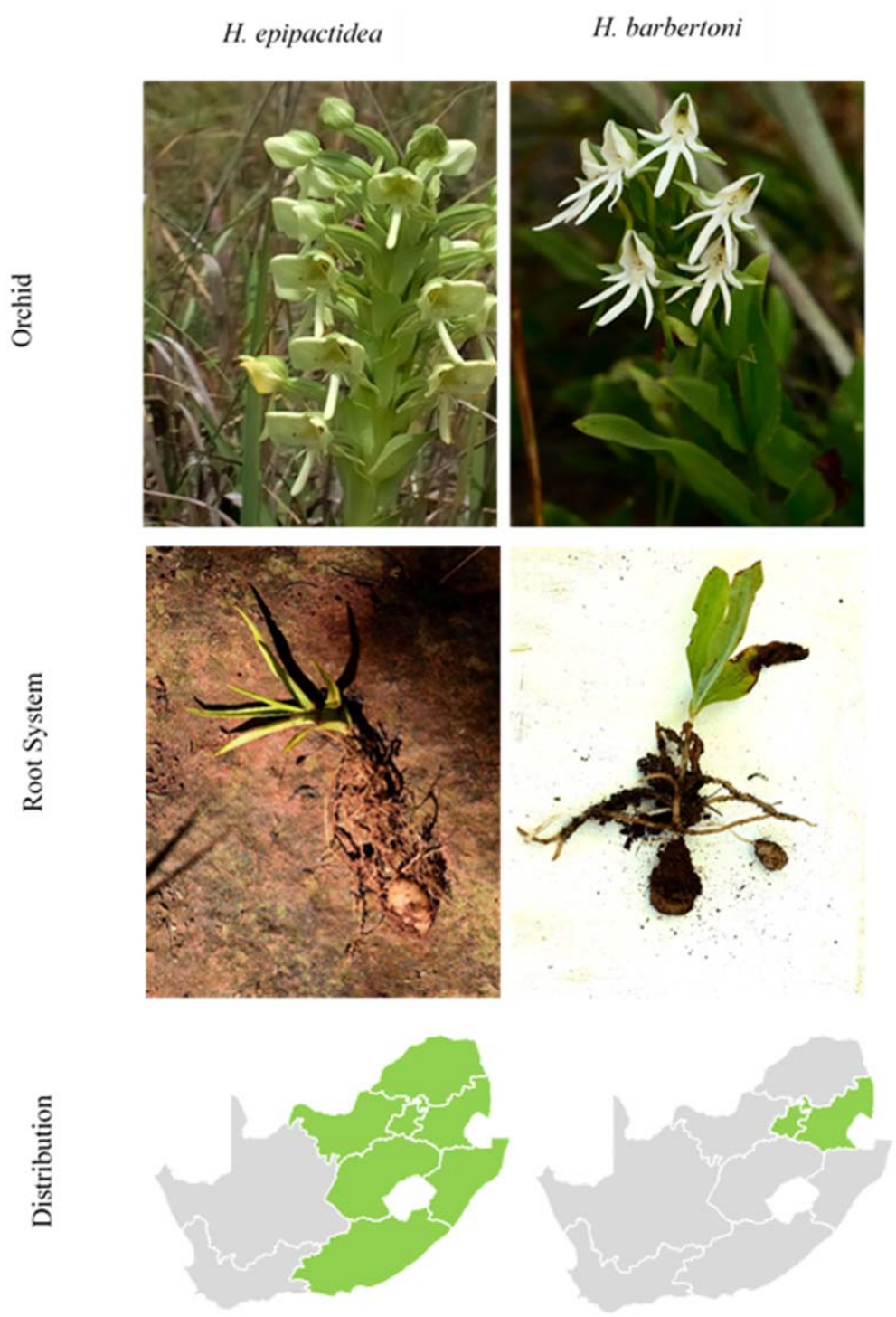

Figure 1. The orchids *Habenaria epipactidea,* and *Habenaria barbertoni* chosen for this study, their respective root structures and current distribution across the country according to the SANBI Red list (SANBI, 2020). *Habenaria epipactidea* has much longer roots compared to *H. barbertoni*. The conservation status of *H. epipactidea* is of least concern whereas *H. barbertoni* is an endangered orchid. Orchid pictures by Gerrit van Ede.

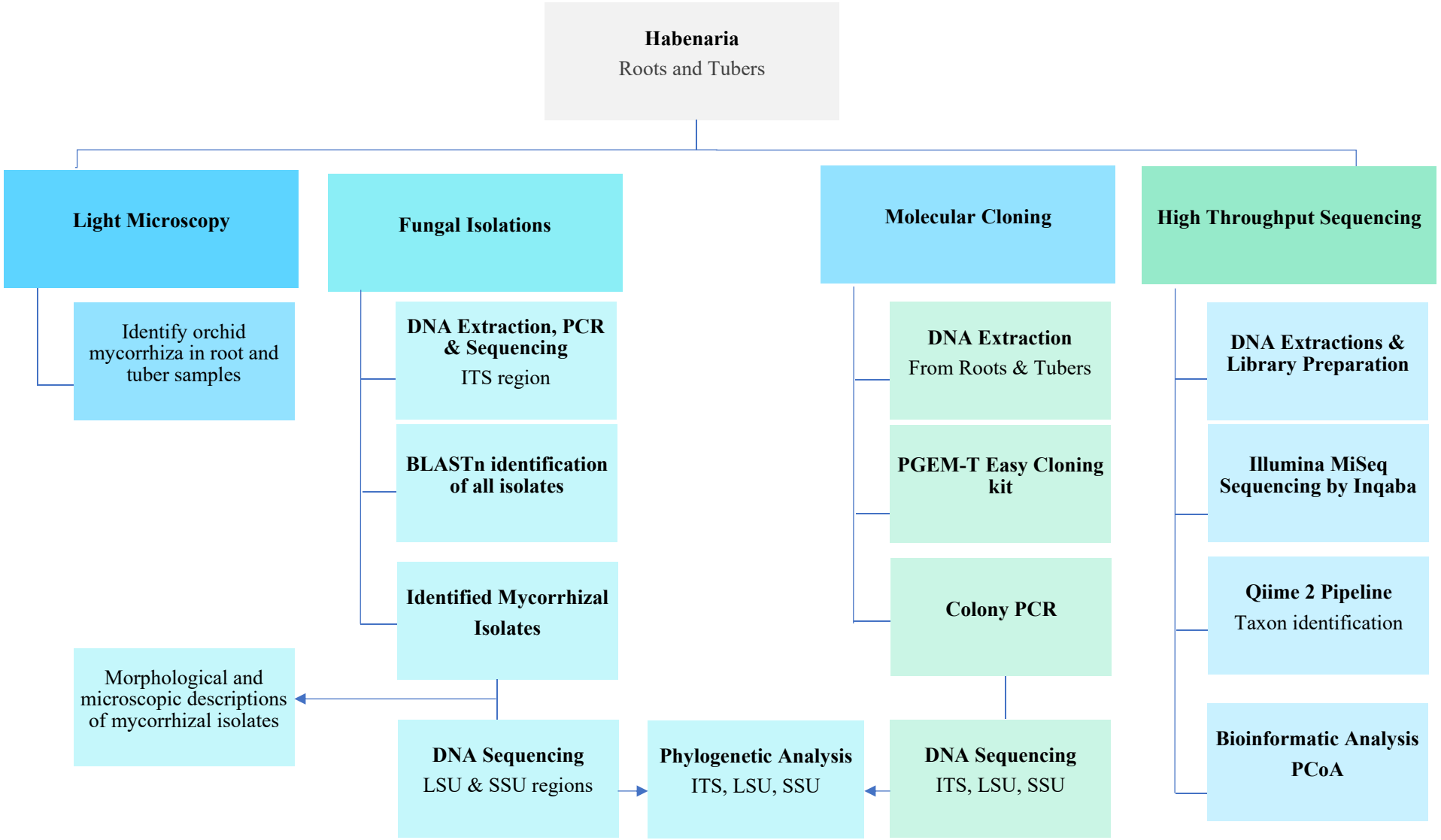

Figure 2. Flow diagram outlining the techniques used to identify and catalogue the mycorrhizal fungi associated with *H. barbertoni* and *H. epipactidea*.

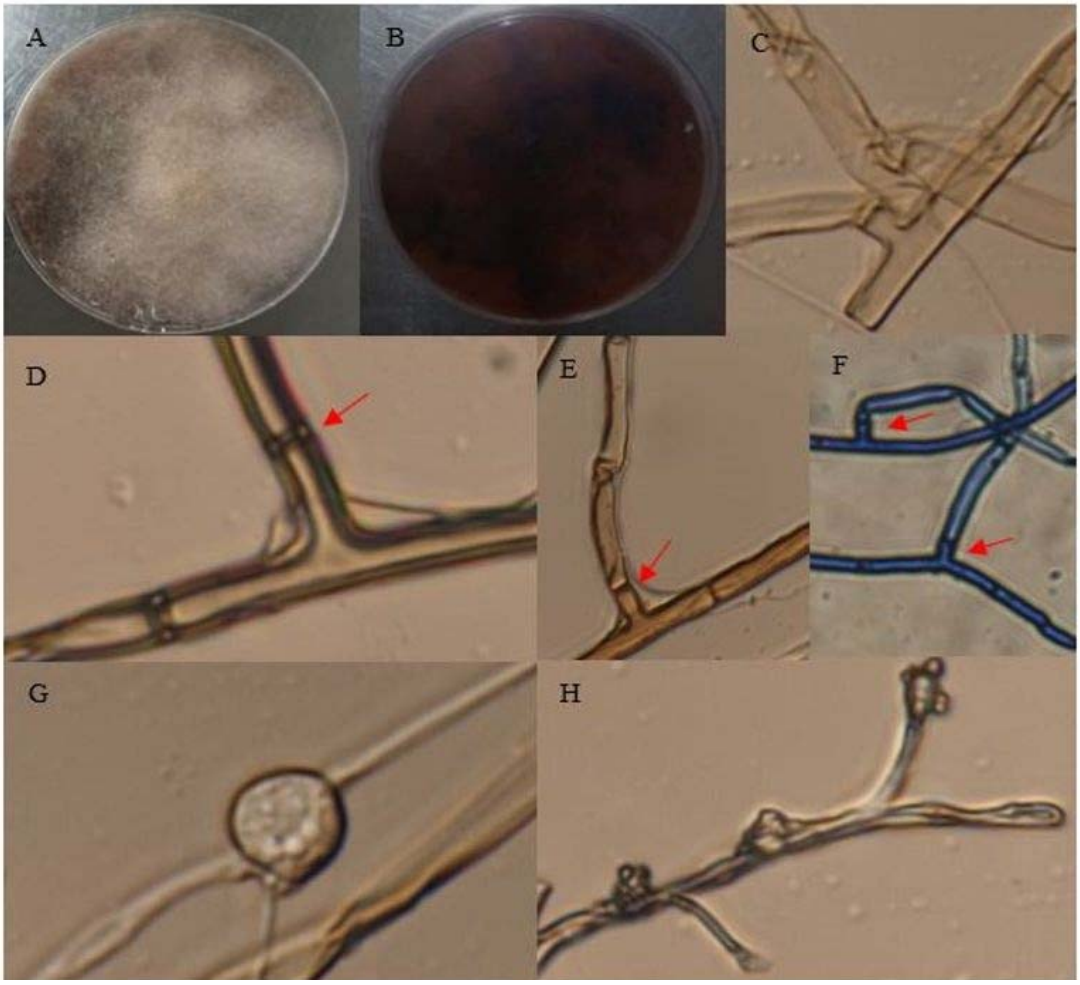

Figure 3. Microscopic descriptive images showing fungal isolates identified using the BLASTn search tool as possible orchid mycorrhizal fungi. (A) and (B) *Ceratobasidium* sp. isolate which has a brown colony colour and white-brown aerial mycelium. Microscopic features common fungi in the *Rhizoctonia* complex are also shown. (C) Hyphal knot; (D-F) septate hyphae branching at 90º angles; (G) chlamydospore in between hyphae and hyphal encrustations.

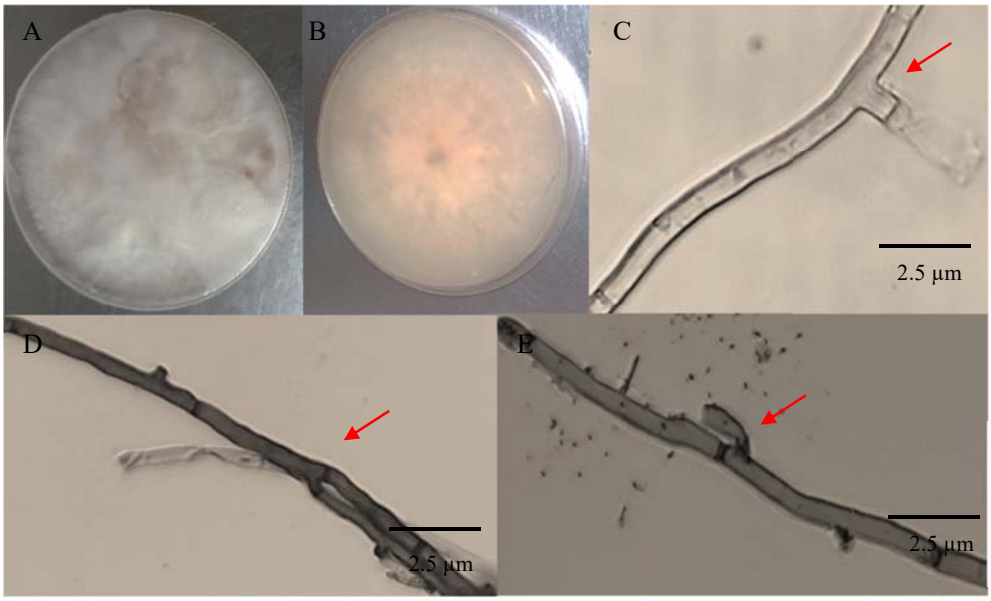

Figure 4. Microscopic descriptive images showing fungal isolate identified using the BLASTn search tool as possible orchid mycorrhizal fungi. (A) and (B) *Coprinellus micaceus* isolate showing white cottony mycelium with well-developed white-yellow aerial mycelium. Microscopic features of this isolate were observed as (C-E) septate hyphae with new clamp connections forming (indicated by the arrows).

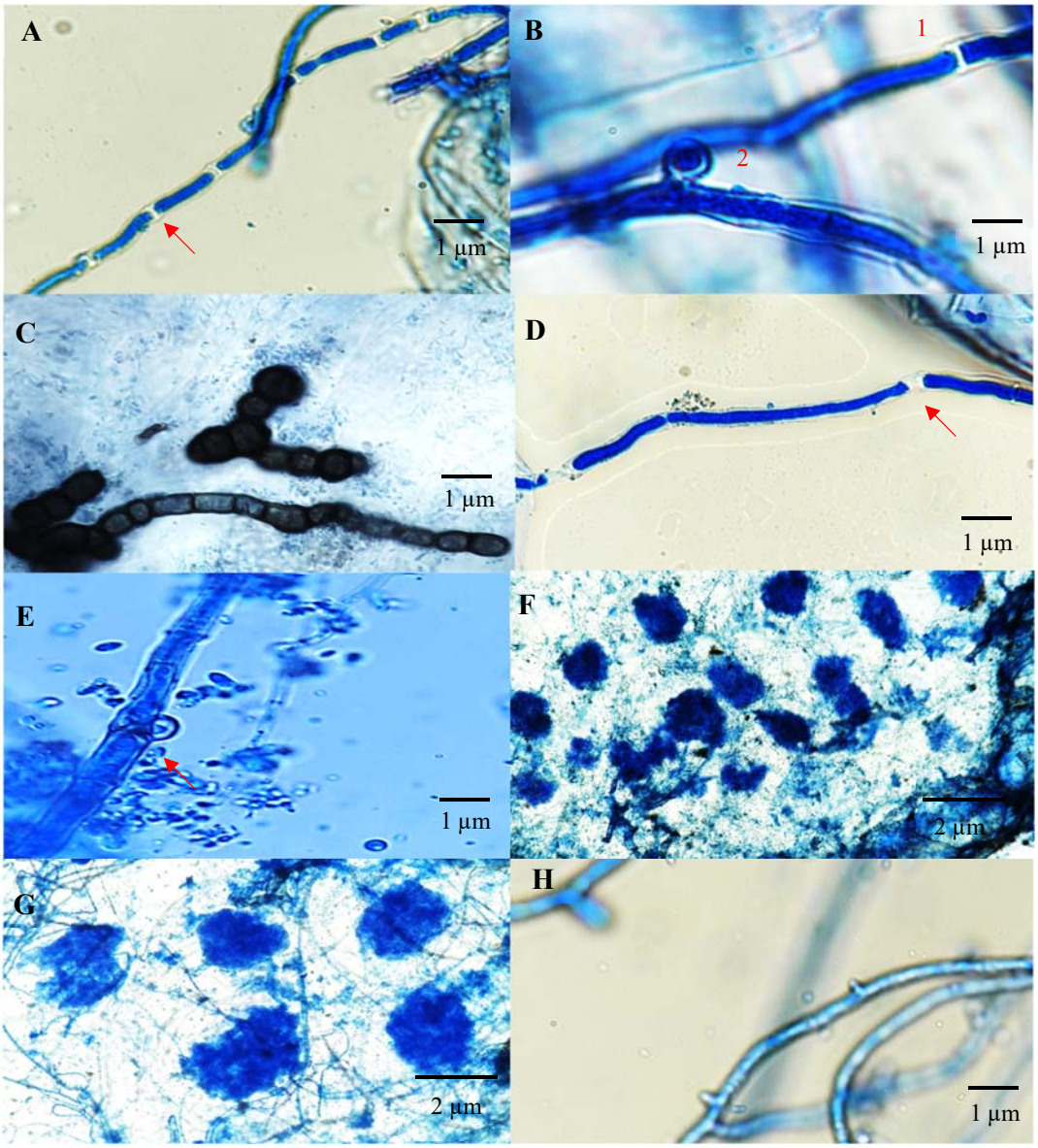

Figure 5. Light microscope images showing characteristic orchid mycorrhizal (Basidiomycota) structures observed in roots and tubers of *H. barbertoni* (A-D) and *H. epipactidea* (E-H). (A) Fungal hyphae with dolipore septa within a *H. barbertoni* root. (B1) Fungal hyphae with dolipore septa and (B2) septate hyphae with a basidiospore in *H. barbertoni* roots. (C) Monilioid structures within orchid tubers. (D) Fungal hyphae with dolipore septa extending in between cell walls of root cortical cells of the *H. barbertoni* tuber. (E) Septate fungal hyphae with a clamp connection within *H. epipactidea* root indicated with an arrow. (F) Hyphal coils (pelotons) within the *H. epipactidea* root and (G) tuber hyphal coils (pelotons) with fungal hyphae extending from each peloton. (H) Fungal hyphae forming new clamp connections in *H. epipactidea* roots.

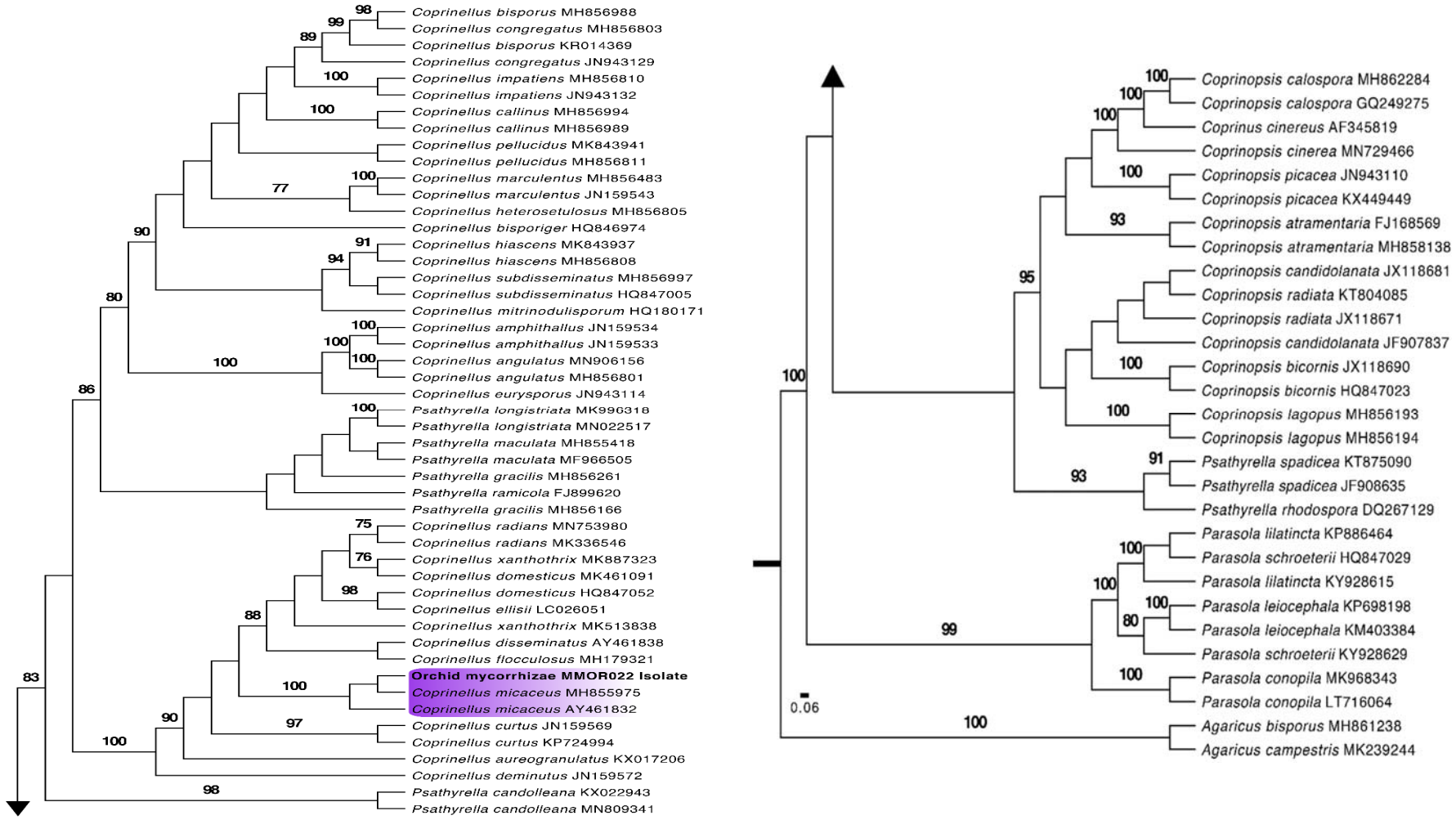

Figure 6. Multigene phylogenetic analysis of ITS-LSU sequences from the family Psathyrellaceae. Orchid mycorrhizae MMOR022 (purple block) represents a new sequence obtained in this study from *H. barbertoni*. The MMOR002 isolate was closely related to *Coprinellus micaceus* fungi in the family Psathyrellaceae. This maximum likelihood tree was generated with RAxML using ITS and LSU sequences. Bootstrap values shown at nodes are based on 1000 replicates, values below 70% are not shown. GenBank accession numbers of sequences are included where available. Scale bar indicates number of nucleotide substitutions per site.

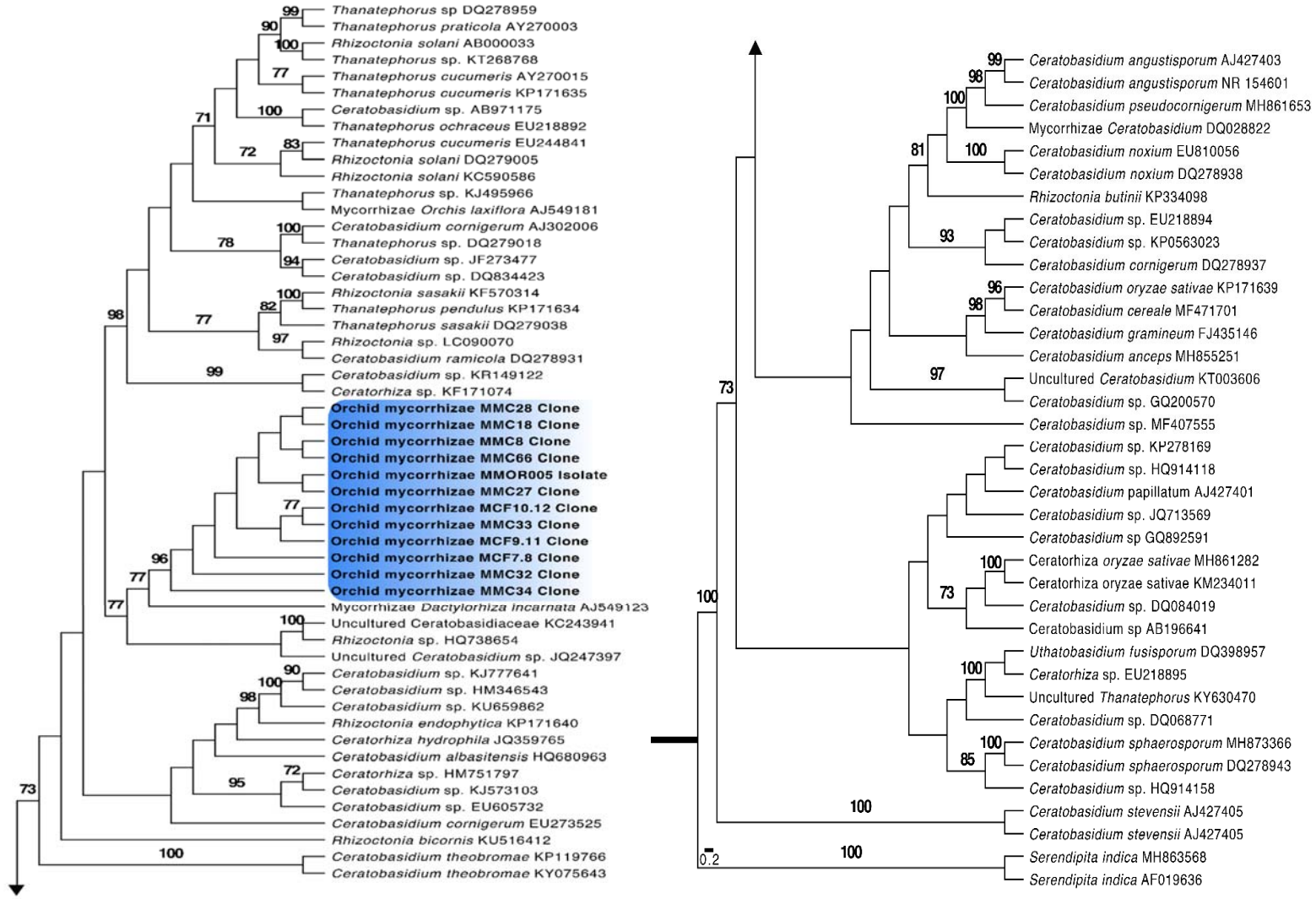

Figure 7. Phylogenetic analysis of ITS sequences from the family Ceratobasidiaceae. Orchid mycorrhizae MMC (blue block) represent new sequences obtained in this study from *H. barbertoni*. Orchid mycorrhizae MMOR 005 represents the ITS sequence of the isolate obtained from this study. All the MMC sequences group closely to a sequence of a mycorrhizal fungus associated with the *Dactylorhiza incarnata* orchid. This maximum likelihood tree was generated with RAxML using ITS sequences. Bootstrap values shown at branch points are based on 1000 replicates and values below 70 were removed. GenBank accession numbers of ITS sequences are included where available. Scale bar indicates number of nucleotide substitutions per site.

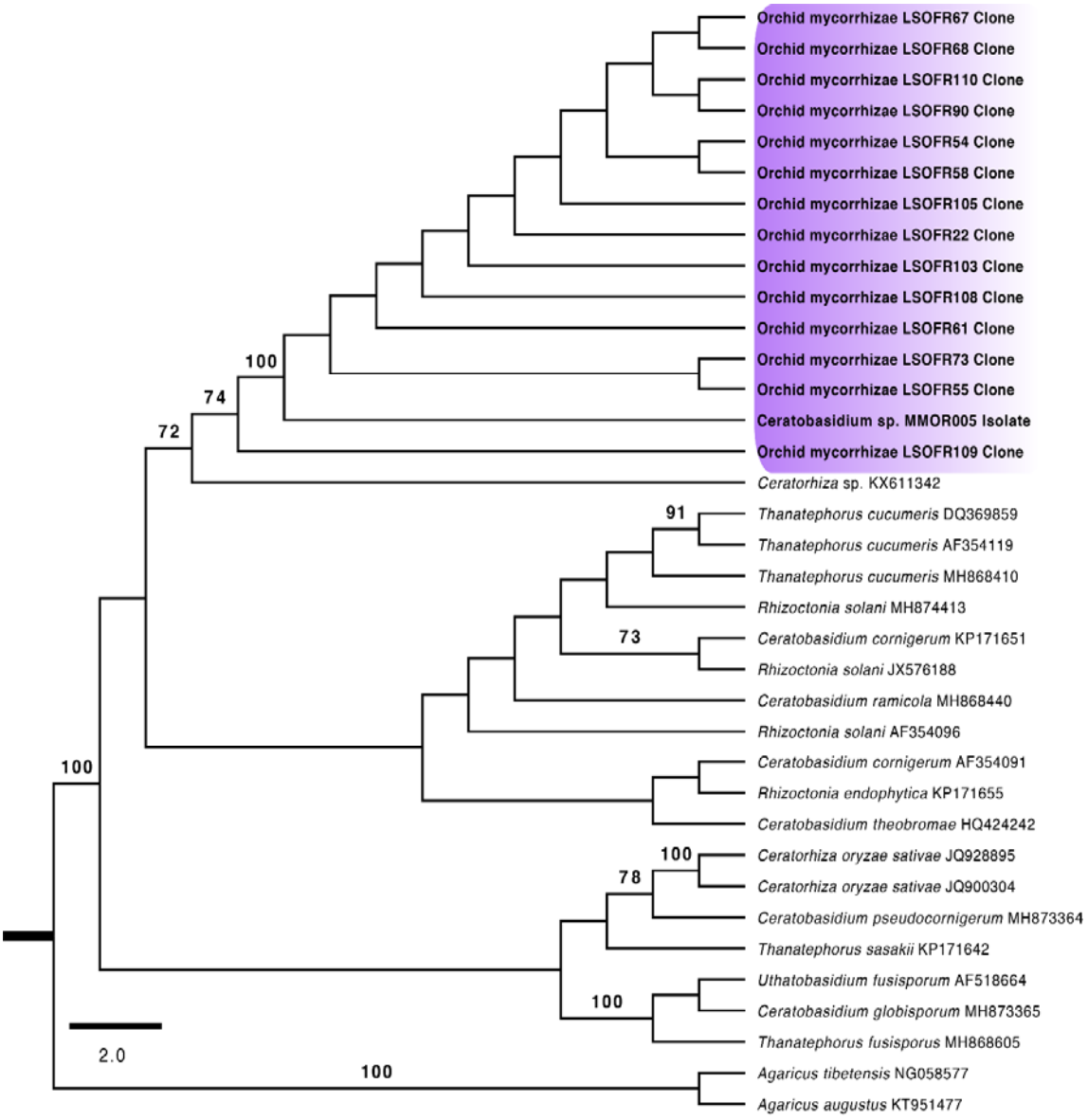

Figure 8. Phylogenetic analysis of LSU sequences from the family Ceratobasidiaceae. Orchid mycorrhizae MMC (purple block) represent new sequences obtained in this study from *H. barbertoni.* Orchid mycorrhizae MMOR 005 represents the LSU sequence of the isolate obtained from this study. The group of sequences obtained in this study were distantly related to *Ceratorhiza* (KX611342) species. This maximum likelihood tree was generated with RAxML using ITS sequences. Bootstrap values shown at branch points are based on 1000 replicates and values below 70 were removed. GenBank accession numbers of LSU sequences are included where available. Scale bar indicates number of nucleotide substitutions per site.

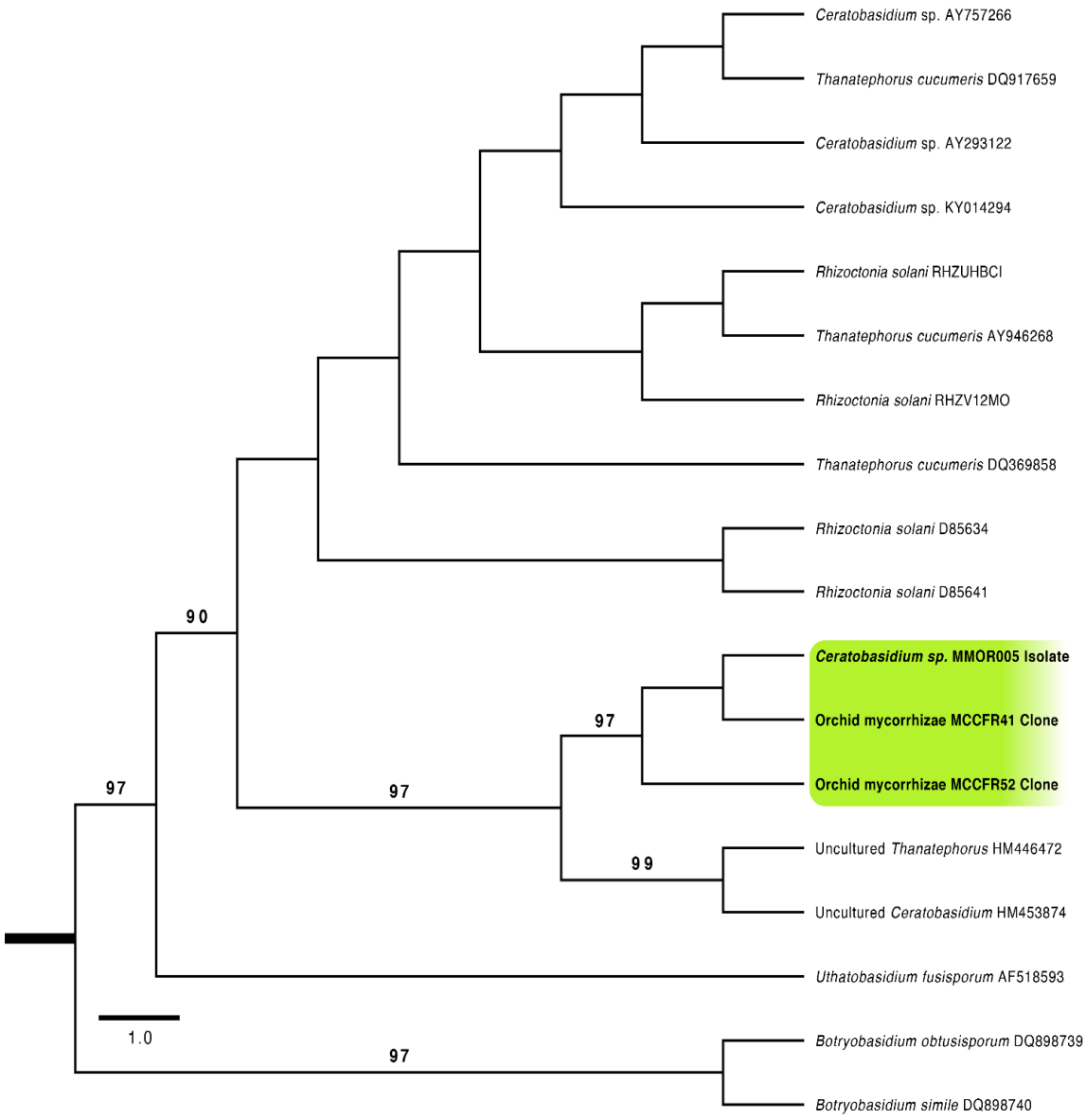

Figure 9. Phylogenetic analysis of SSU sequences from the family Ceratobasidiaceae. Orchid mycorrhizae MMC (green block) represent new sequences obtained in this study from *H. barbertoni*. Orchid mycorrhizae MMOR 005 represents the SSU sequence of the isolate obtained from this study. All the sequences from this study were distantly related to uncultured species of *Ceratobasidium* and *Thanatephorus*. This maximum likelihood tree was generated with RAxML using SSU sequences. Bootstrap values shown at branch points are based on 1000 replicates and values below 70 were removed. GenBank accession numbers of SSU sequences are included where available. Scale bar indicates number of nucleotide substitutions per site.

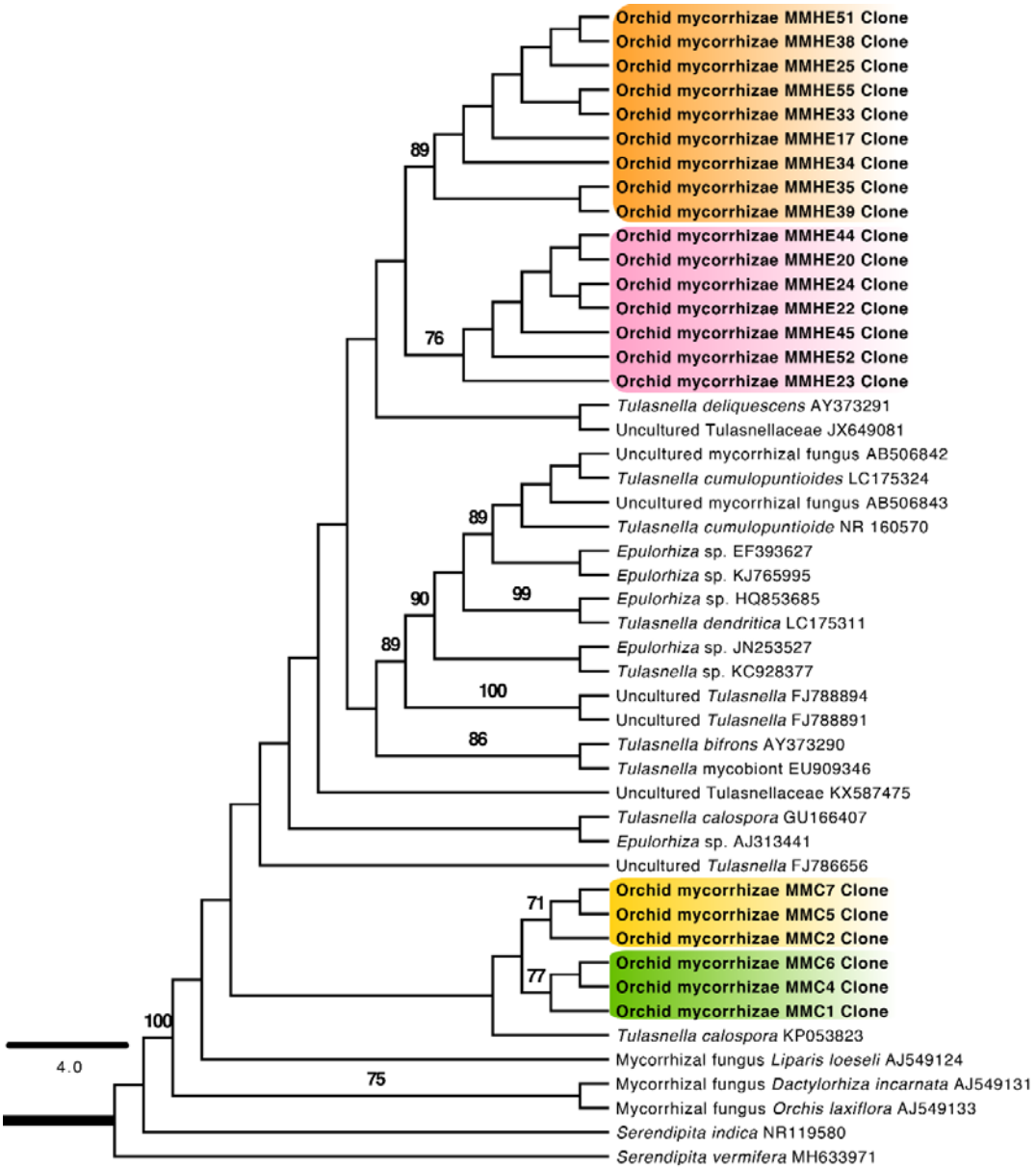

Figure 10. Phylogenetic analysis of ITS sequences from the family Tulasnellaceae. Orchid mycorrhizae MMC (orange and pink blocks) represent new sequences obtained in this study from *H. barbertoni* and orchid mycorrhizae MMHE (yellow and orange blocks) represent new sequences obtained in this study from *H. epipactidea*. Both MMC and MMHE sequences group closely to a number of uncultured species from the Tulasnellaceae. This maximum likelihood tree was generated with RAxML using ITS sequences. Bootstrap values shown at branch points are based on 1000 replicates and values below 70 were removed. GenBank accession numbers of ITS sequences are included where available. Scale bar indicates number of nucleotide substitutions per site.

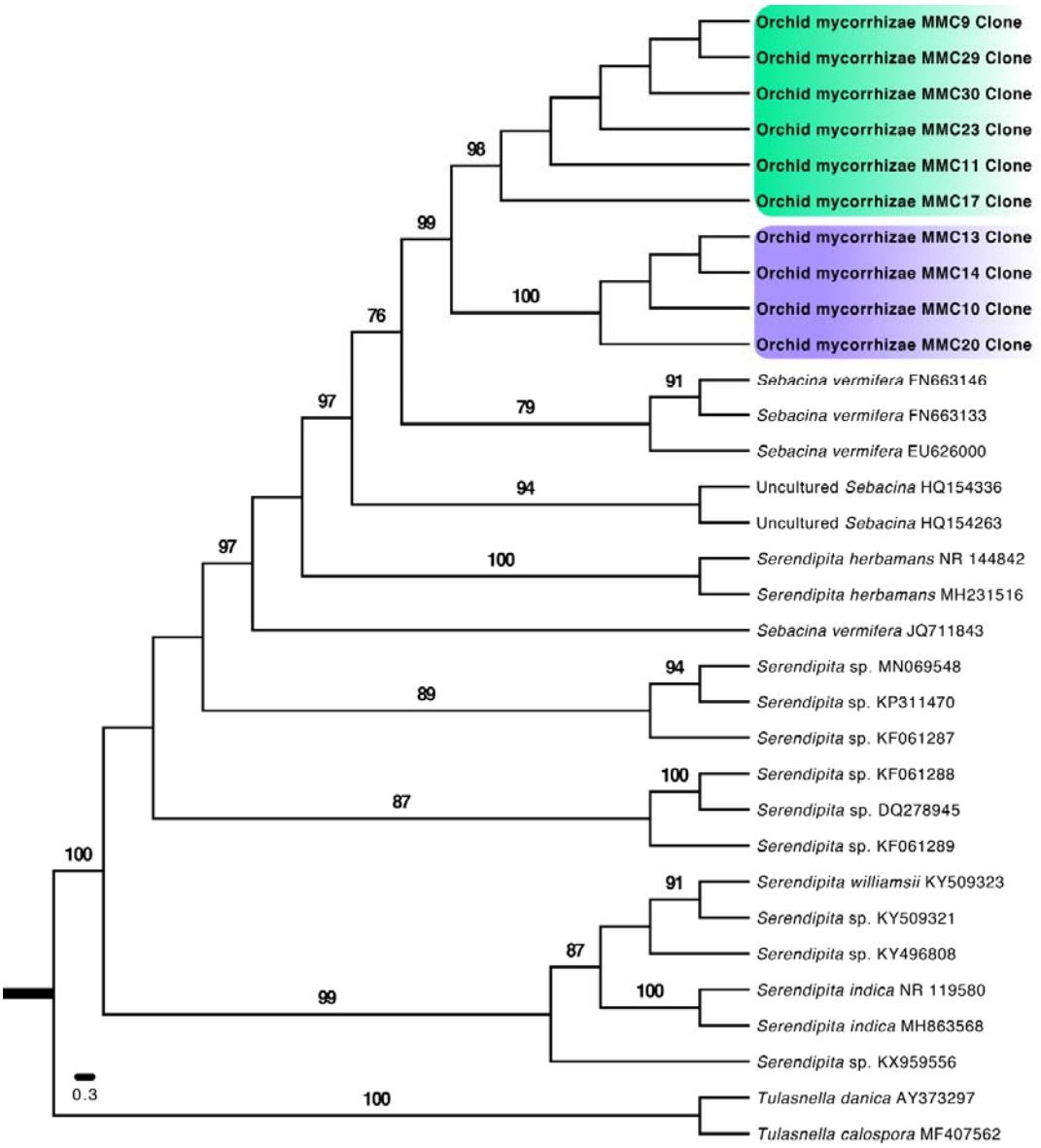

Figure 11. Phylogenetic analysis of ITS sequences from the family Serendipitaceae. Orchid mycorrhizae MMC (green and purple block) represent new sequences obtained in this study from *H. barbertoni.* All the MMC sequences group closely to *Sebacina vermifera*. This maximum likelihood tree was generated with RAxML using ITS sequences. Bootstrap values shown at branch points are based on 1000 replicates and values below 70 were removed. GenBank accession numbers of ITS sequences are included where available. Scale bar indicates number of nucleotide substitutions per site.

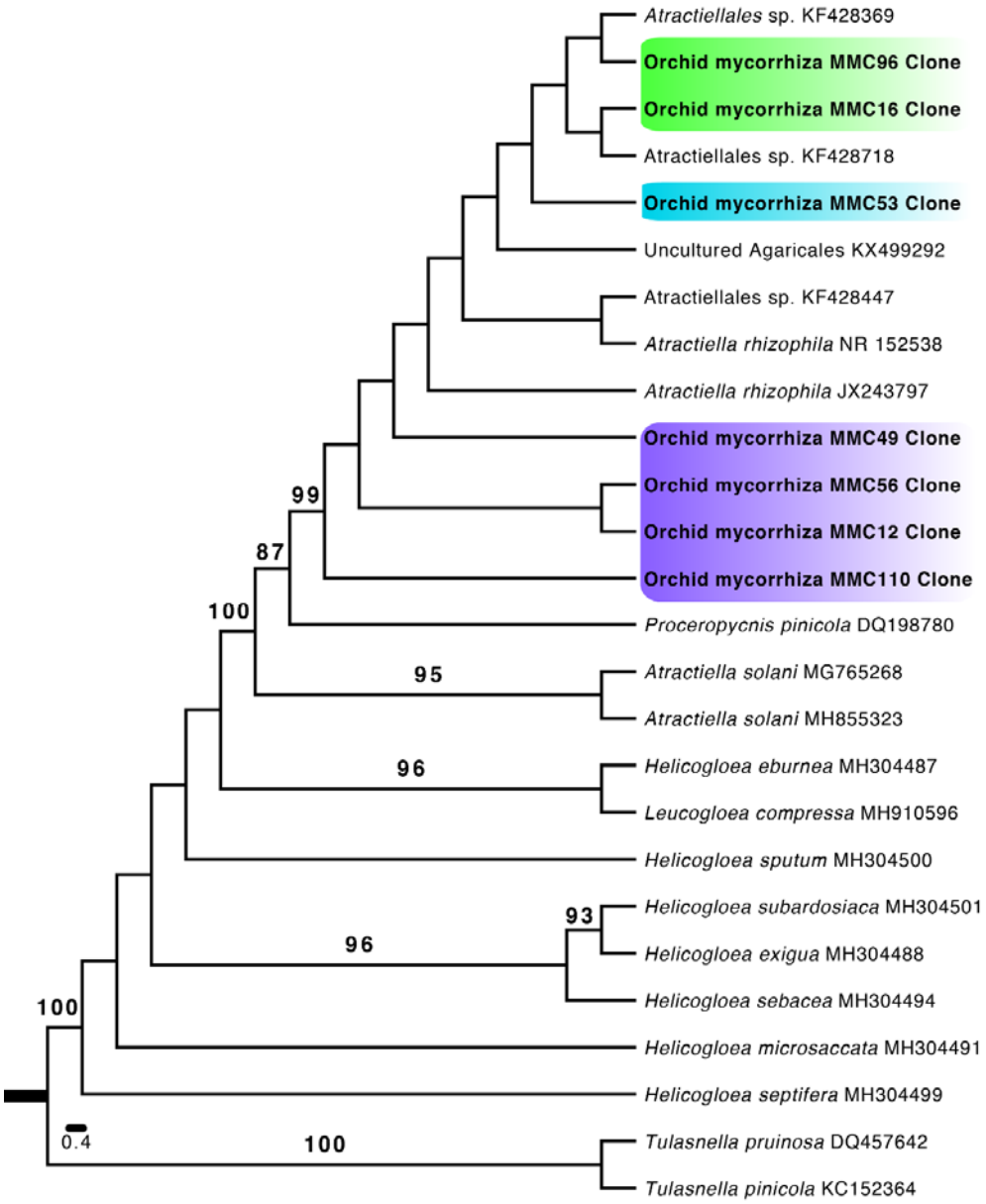

Figure 12. Phylogenetic analysis of ITS sequences from the family Hoehnelomycetaceae. Orchid mycorrhizae MMC (green, blue, and purple blocks) represent new sequences obtained in this study from *H. barbertoni.* All the MMC sequences group closely to sequences of *Atractiellales* sp. and *Atractiella rhizophila*. This maximum likelihood tree was generated with RAxML using ITS sequences. Bootstrap values shown at branch points are based on 1000 replicates and values below 70 were removed. GenBank accession numbers of ITS sequences are included where available. Scale bar indicates number of nucleotide substitutions per site.

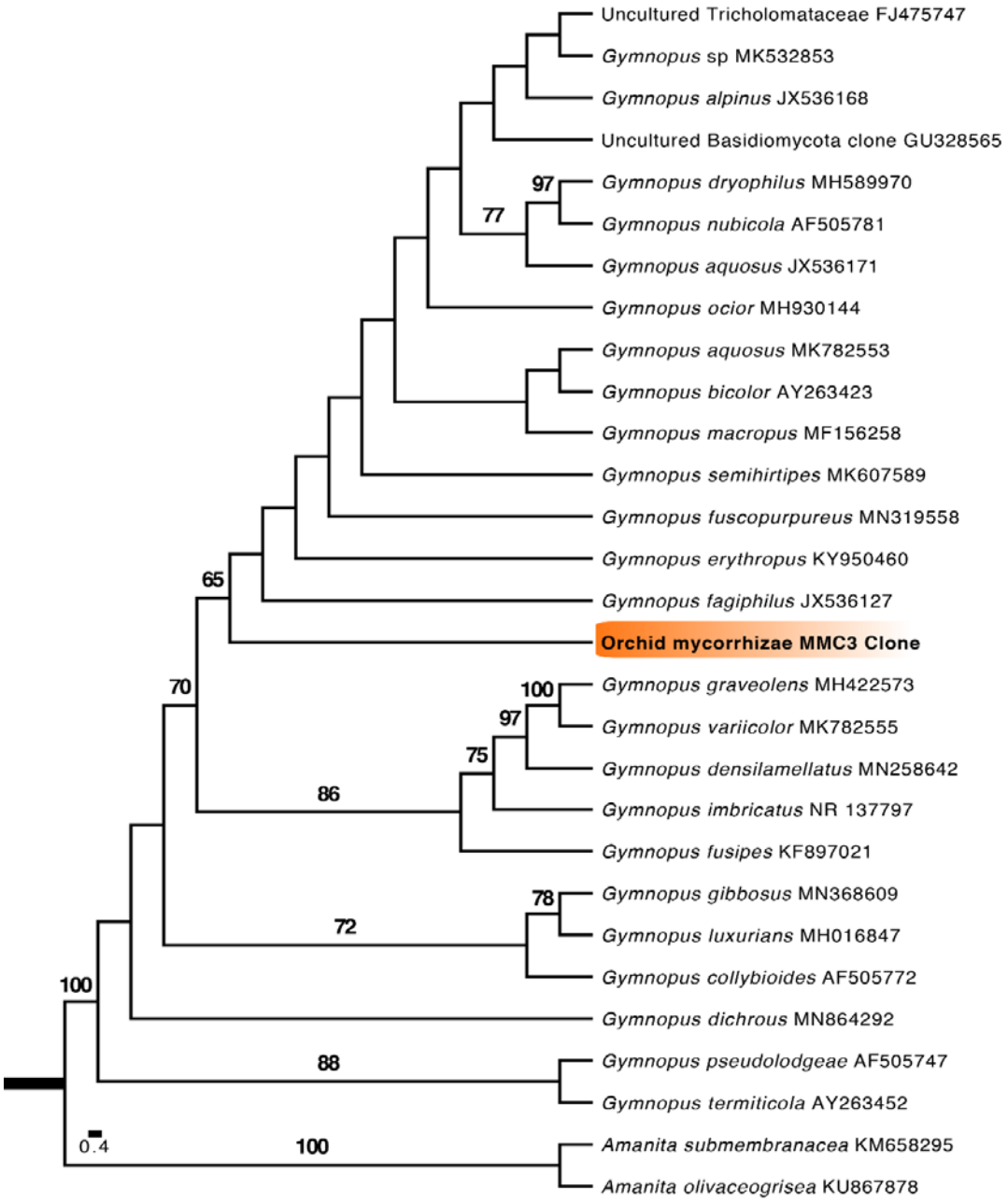

Figure 13. Phylogenetic analysis of ITS sequences from the genus *Gymnopus* from the family Omphalotaceae. Orchid mycorrhizae MMC (orange block) represents a new sequence obtained in this study from *H. barbertoni.* The sequence Orchid mycorrhiza MMC3.clone obtained in this study did not match 100% with any of the reference sequences. This maximum likelihood tree was generated with RAxML using ITS sequences. Bootstrap values shown at branch points are based on 1000 replicates and values below 60 were removed. GenBank accession numbers of ITS sequences are included where available. Scale bar indicates number of nucleotide substitutions per site.

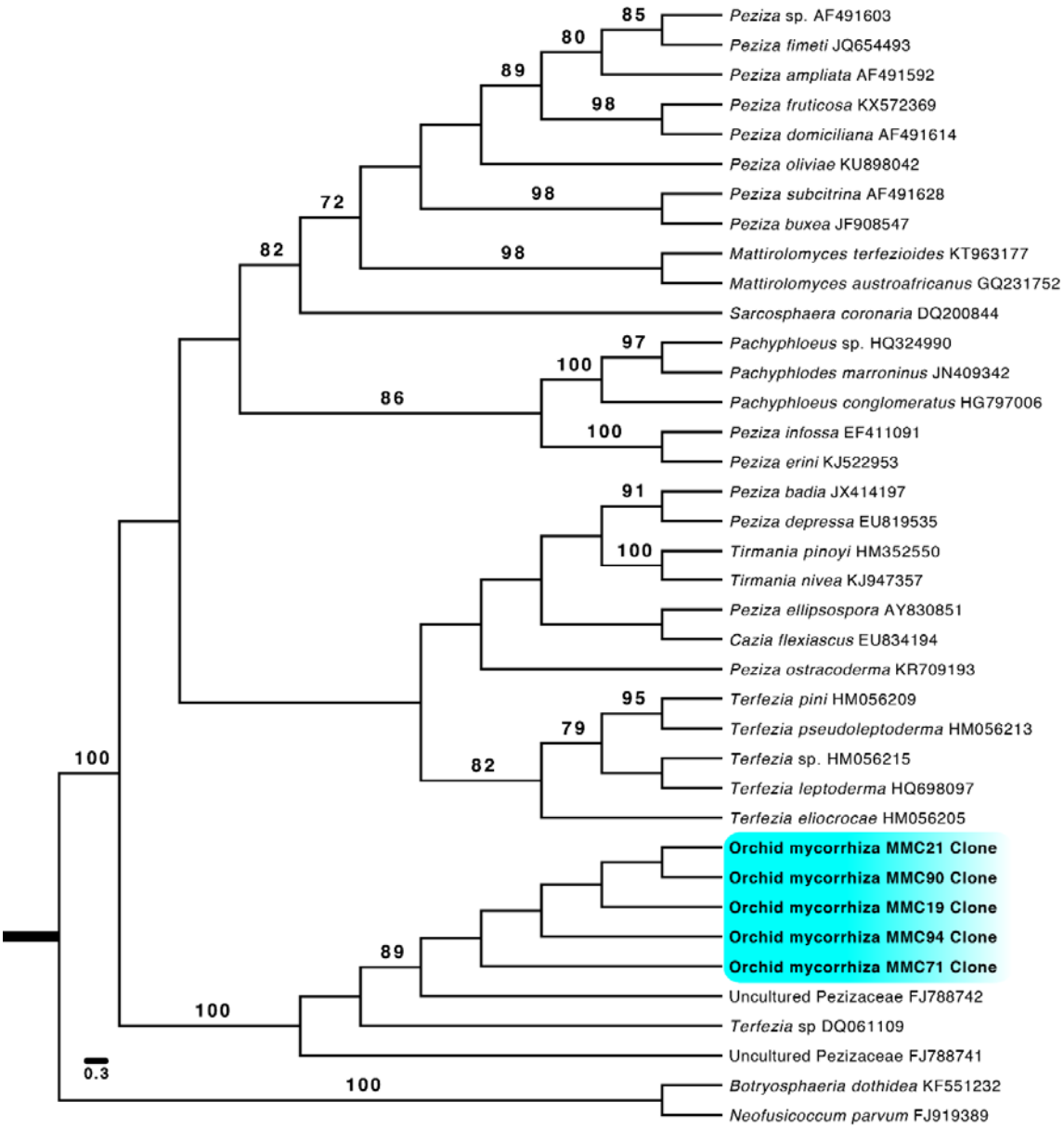

Figure 14. Phylogenetic analysis of ITS sequences from the family Pezizaceae. Orchid mycorrhizae MMC (blue block) represent new sequences obtained in this study from *H. barbertoni.* All the MMC sequences group closely to an uncultured species from the Pezizaceae. This maximum likelihood tree was generated with RAxML using ITS sequences. Bootstrap values shown at branch points are based on 1000 replicates and values below 70 were removed. GenBank accession numbers of ITS sequences are included where available. Scale bar indicates number of nucleotide substitutions per site.

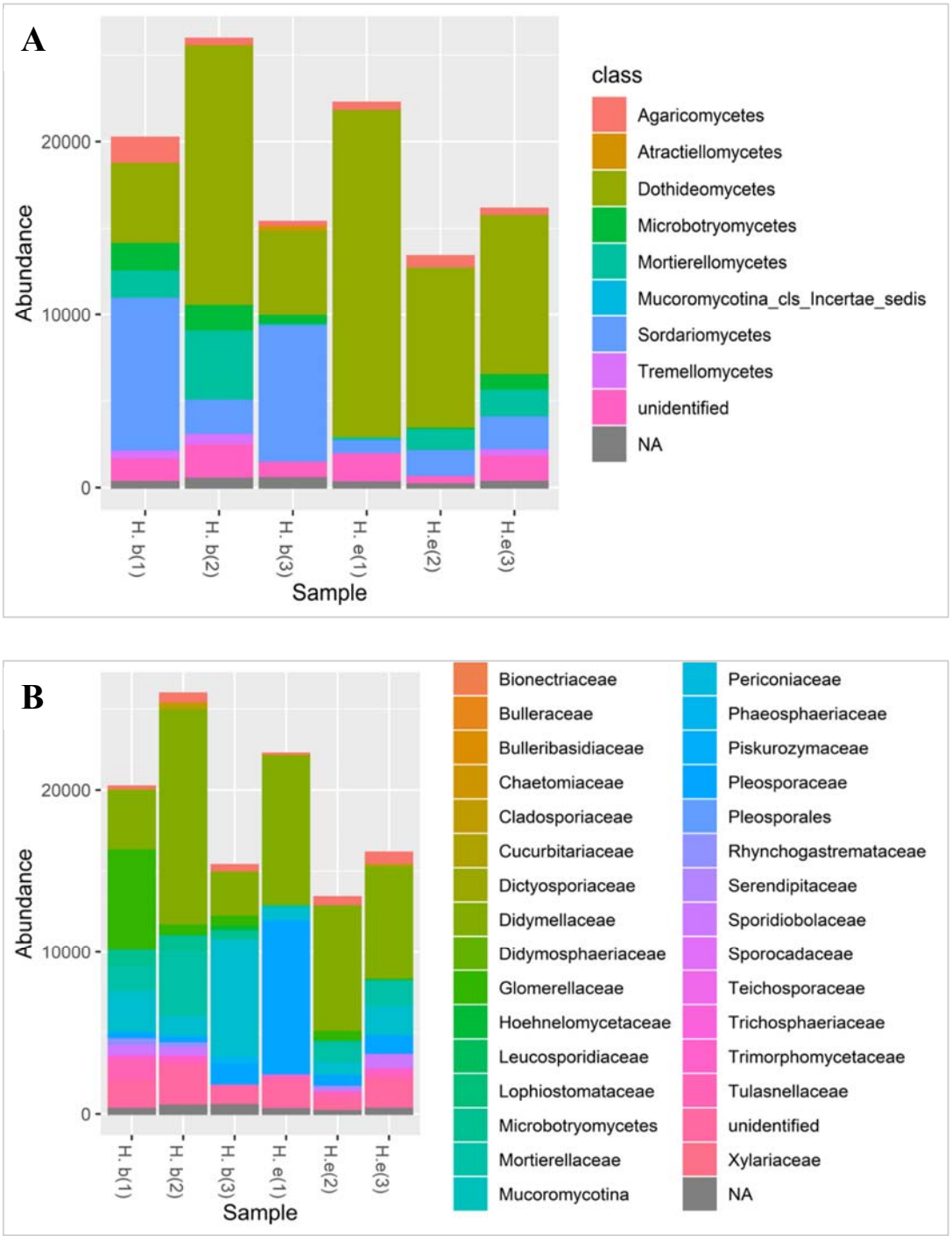

Figure 15. Stacked bar plot showing the relative abundance of fungal taxa in *Habenaria* orchid roots and tubers based on ITS illumina sequence data. Only two taxonomic levels are shown (A) Class and (B) Families. Three samples of *H. barbertoni* plants are denoted, H. b (1,2,3) while *H. epipactidea* samples are denoted as H. e (1,2,3). Orchid mycorrhizal taxa associating with these orchids are Tulasnellaceae, Serendipitaceae and Hoehnelomycetaceae with varied abundances in each sample.

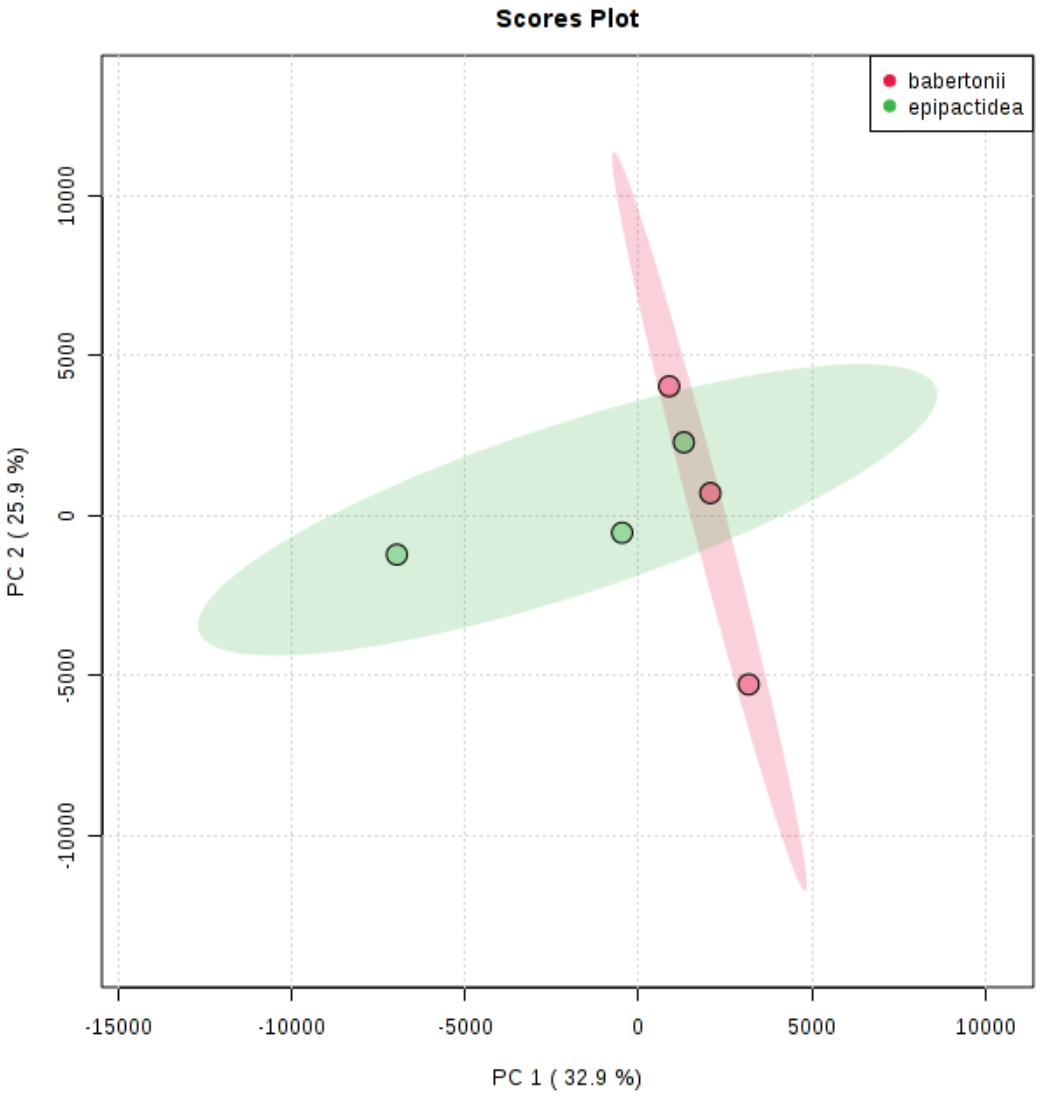

Figure 16. Principal coordinate analysis (PCoA) of the fungal taxa (ITS sequences) in *H. barbertoni* and *H. epipactidea* roots and tuber samples. Red circles show the three *H. barbertoni* plants and the green circles show the three *H. epipactidea* plants. The shaded area shows a 95% confidence interval. A clear separation and some overlap in fungal communities are observed between the two-orchid species.

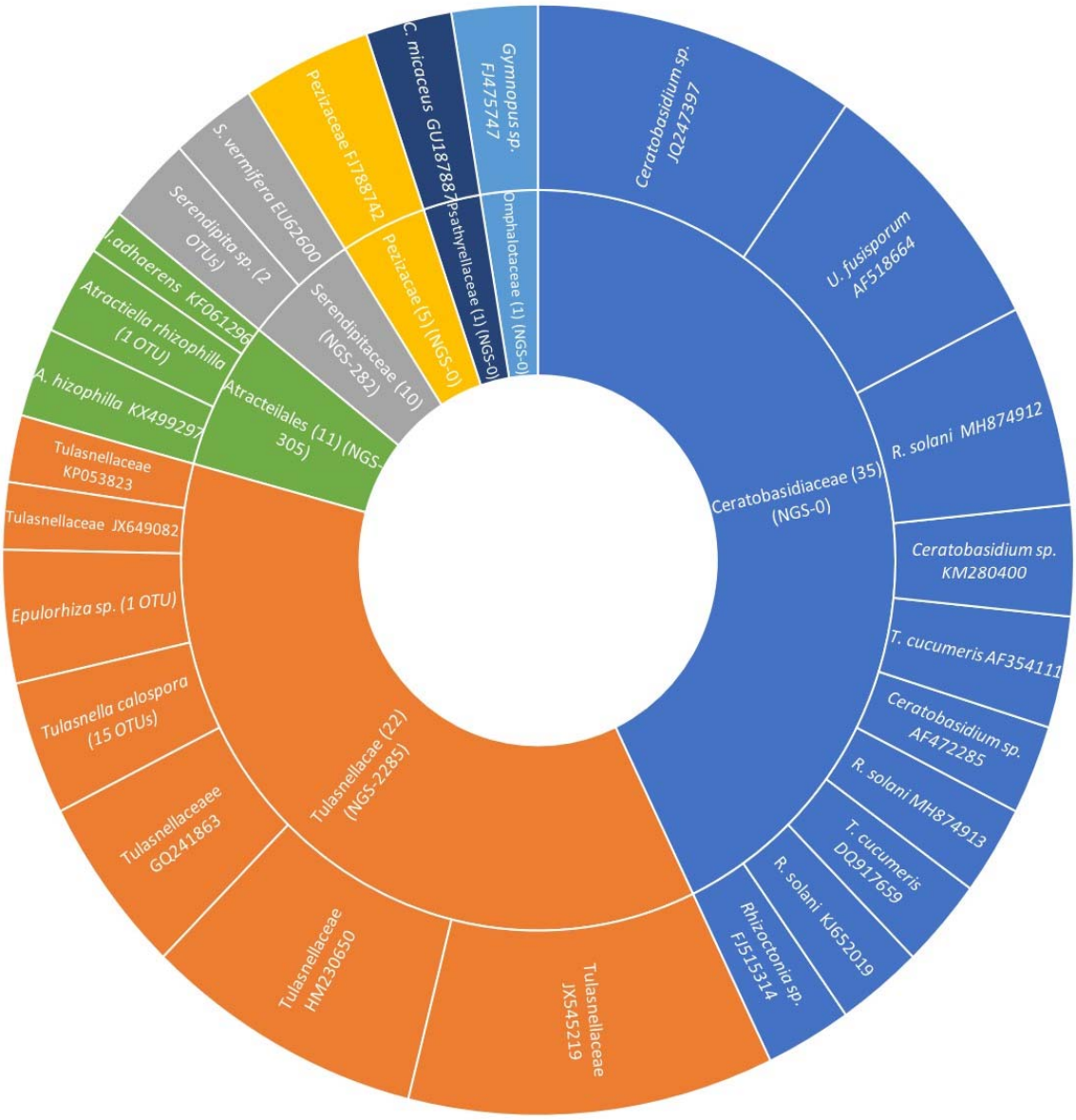

Figure 17. Summary diagram showing all the fungal species identified as orchid mycorrhizae associated with orchids of the genus *Habenaria*. Three gene regions (ITS, LSU and SSU) were used in fungal isolations and cloning to obtain sequences of these fungal species from orchid roots and tubers. The number of sequences obtained for each orchid mycorrhizal family identified is shown in brackets next to the family name. Orchid mycorrhizae obtained from Illumina sequencing data have the number of OTUs next to the fungal taxon name. NGS (Next Generation Sequencing) in brackets next to family names, shows the number of sequences representing each family.

Summary

Mycorrhizal fungi are an important group of soil microbes that mainly assist in increasing the nutrient and water uptake of plants in nature. Orchids benefit from mycorrhizal symbiosis by obtaining essential nutrients needed for their germination and growth. Therefore, orchid mycorrhizae are actively being studied and used in orchid conservation measures. The overall aim of this research was to identify the mycorrhizal diversity associated with endemic and endangered orchids of Southern Africa. The first chapter of this dissertation provides a literature review that focuses on the importance of orchid mycorrhizal associations for the survival of orchids in natural habitats worldwide. This chapter further explores the various types of mycorrhizae known and how these associations compare to orchid mycorrhiza. Lastly, this chapter describes the advances of techniques currently being used to study the mycorrhizal diversity and applications of which in orchid conservation.

The second chapter in this research focuses on the fungal diversity found in the soil of a critically endangered orchid *Brachycorythis conica* subsp. *transvaalensis*. Here high-throughput sequencing is used to catalogue this diversity. An assortment of diverse fungi was recorded in this chapter including a few candidate orchid mycorrhizae including members of the Agaricales, Cantharellales, Pleosporales and Sebacinales. In the last chapter of this dissertation, the fungal symbionts of the *Habenaria barbertoni* and *Habenaria epipactidea* orchids are studied. Results from this chapter showed that orchids in the genus *Habenaria* associate with potential mycorrhizae represented by families from the Basidiomycota (Ceratobasidiaceae, Serendipitaceae, Hoehnelomycetaceae, Omphalotaceae and Tulasnellaceae) and Ascomycota (Pezizaceae). Moreover, results showed that while the *Habenaria* orchids had a few fungal associates in common, the fungal diversity varied between the species.

This research highlights the vast diversity of fungi including mycorrhizae present in South African soils. The results from this dissertation provide information that can be used in future studies on orchid mycorrhizae from South Africa and is the first step towards *ex situ* conservation strategies for endemic orchids from this country

One of the future directions that can be taken for the study of orchids in South Africa could be the use of *in situ* seed baiting techniques. The application of this technique could help in clearly identifying which of the orchid mycorrhizal fungi are responsible for seed germination and

protocorm development. Fungi obtained through this method could also be used in the development of orchid growth trials. The seed baiting together with growth trials can clearly distinguish between mycorrhizal fungi responsible for seed germination, maintaining orchid growth and those present in the soil, but which do not affect orchid development. In addition, next-generation sequencing from the roots of these orchids could help identify a larger diversity of mycorrhizae in symbiosis with the adult orchids. Sampling and studying of *Habenaria* species from other areas of South African can be useful in improving our understanding of the environmental factors that can influence mycorrhizal associations. Moreover, studying the mycorrhizal diversity of these orchids across different seasons and collecting weather data can assist in understanding the effect of climate on the shifts in mycorrhizal communities. While this study focused mainly on a small subset of terrestrial orchids, the mycorrhizal diversity of a vast majority of South African terrestrial orchids as well as epiphytes and lithophytes can still be explored. Knowledge of the diversity of mycorrhizal fungi associated with orchids is the first step in understanding the interactions between orchids and mycorrhizal fungi governing this symbiosis.