Diversity of endophytic fungi associated with branches of *Berchemia discolor* in the Limpopo Province of South Africa

By

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DECLARATION

I, Cindy Ramokgano, declare that the dissertation, which I hereby submit for the degree *Master of Science* at the University of Pretoria, is my own independent work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature

Cindy Ramokgano

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PREFACE

Berchemia discolor (brown ivory, *Rhamnaceae*), is an indigenous multipurpose tree that plays a role in the socio-economic well-being of small-scale farmers and rural communities. The tree is of cultural importance in most countries in Africa, including South Africa. It is extensively valued, well-known for its medicinal properties, its use in the production of liquor, and its nutritional values.

Despite the ecological importance of *B. discolor*, and it being valued by rural communities and that it has pharmaceutical properties, virtually nothing is known regarding pathogens of this tree species. However, a few diseases such as rust and powdery mildew have been reported to affect *B. discolor* in other parts of the world. In South Africa, there are no known reports of fungi affecting the health of the species.

Due to the lack of information regarding diseases of *B. discolor* trees, the literature review of the dissertation gives a background on fungal diseases of trees in the family *Rhamnaceae*. A focus is placed on endophytic fungi that can remain latent and later induce disease symptoms when the trees are under stress. The literature review provides an overview of studies done on nonpathogenic and pathogenic endophytes that cause diseases in the *Rhamnaceae*. It also includes a discussion on the fungal diagnostics and techniques applied and used in identification and classification of fungal species.

Chapter two of this dissertation focuses on fungal diversity associated with *B. discolor* in the Mapungubwe National Park. It aimed at identifying the fungi found on both diseased and healthy branches of *B. discolor* trees found in the national park. It also establishes a foundation on the diversity of fungi associated with *B. discolor* trees in South Africa and other plants in the National Park. The isolated fungi were identified and characterised based on DNA sequence data.

Chapter three of this dissertation evaluates the diversity of *Botryosphaeriaceae* species associated with symptomatic and asymptomatic branches of *B. discolor* in agricultural and natural ecosystems from different collection sites in the Limpopo Province. The *Botryosphaeriaceae* are well-known fungi associated with native and non-native woody trees, plantations and forestry species worldwide. They are opportunistic pathogens known to affect both agricultural and indigenous trees worldwide. They cause symptoms such as cankers and branch dieback. The first aim

of the research presented in this Chapter was to identify isolates collected at sampling sites in Tshipise, Dambale and Mapungubwe National Park, that are all in the Limpopo Province. The identification was based on using multi-gene DNA sequence data. A second aim was to determine if there were overlapping fungal species from both asymptomatic and symptomatic branches in the agricultural and natural ecosystems. This was done to identify and distinguish nonpathogenic endophytes from the pathogenic endophytes within the *Botryosphaeriaceae*.

The work presented in this study provides foundational knowledge on the fungal diversity associated with *B. discolor*, and the host range expansion of various fungi obtained in this study including the *Botryosphaeriales* associated with *Rhamnaceae* plants. This information is vital as it gives insight into areas that were previously neglected and/or rarely considered for fungal surveys and samplings, such as the Mapungubwe National Park.

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CHAPTER 1

Literature review

Fungi associated with species in the family *Rhamnaceae*

1. Introduction

The *Rhamnaceae* (order *Rosales*) is a family of trees, shrubs, climbers and herbs comprising approximately 55 genera and 950 species (Christenhusz and Byng, 2016). The cosmopolitan members of the taxon are distributed in the tropics, subtropics and the warm temperate regions of the world (Richardson *et al.*, 2000). According to the "SANBI online checklist of Plants of southern Africa", nine genera and 161 species are native to southern Africa. Some of the notable genera include *Berchemia*, *Colubrina*, *Helinus*, *Lasiodiscus*, *Noltea*, *Phylica*, *Rhamnus*, *Scutia* and *Ziziphus*.

Various reports on species in the *Rhamnaceae* are related to their traditional medicinal properties. Their pharmacological uses in traditional medicine are due to healing properties associated with their secondary metabolites (Khoo *et al.*, 2016). It is noteworthy that most studies were focused on screening for the biological activities and chemical compounds from genera such as *Ziziphus*, *Berchemia* and *Rhamnus* (Alfred, 2019; Mongalo *et al*., 2020). For example, Samie *et al.* (2010) found acetone and hexane extracts from *B. discolor* to inhibit *Candida* species in patients infected with HIV/AIDS in South Africa. Green *et al.* (2010) found that the acetone extracts of this plant could be one of the important sources of mycobactericidal compounds against multidrug-resistant *Mycobacterium tuberculosis*. Rhamnose found in the leaves of *Rhamnus* and *Ziziphus* (Clifford *et al*., 2002; Ribeiro *et al*., 2021) was used in anti-aging facial moisturisers, and as a result has sparked interest in the cosmetic industry (Pageon *et al*., 2019). Not only do these plant secondary metabolites show a positive beneficial effect on human health, but also on agriculture production, and can thus contribute significantly to the economy (Pang *et al*., 2021).

The *Rhamnaceae* are rich in species that play an important role ecologically and that may have economic benefits in many countries. In natural ecosystems, trees species serve as shade, provide habitation for many faunas and play a role in nitrogen fixation (Debela *et al*., 2012). Some of the most notable commercialised genera with economic importance include *Ziziphus, Ceanothus* and *Colletia* (Richardson *et al*., 2000). Commercialised products of these plants are fruits, timber, sealing wax-making resins and ornamental cultivars. Extracts from these plants and trees are also used in the making of products such as dye and soap (Simpson, 2010) and used as ingredients for making biscuits (Habou et al., 2020 cited by Moussa *et al*., 2020). For example, the

production of dried fruits of *Z. jujuba* is the primary source of income for *ca.* 20 million farmers in China (Meng-jun *et al.*, 2015; Shahrajabian *et al.*, 2020). *Rhamnaceae* also contains other fruit tree species with commercial value that have lesser popularity, and are mostly used and sold in the rural community areas where they are readily available in the local markets (Nkosi *et al.*, 2020). Recently in South Africa, *B. discolor* and *Z. mucronata* were reported as some of the South African indigenous species that have potential for developing new food products such as jam, juice, wine, processed products, beverages, flavors, and spices (Van Wyk, 2011; Nkosi *et al.*, 2020).

The health of native and non-native trees is increasingly threatened by diseases caused by fungal pathogens (Roy *et al.*, 2014; Wingfield *et al.*, 2015). Some of the disease examples from South Africa include Armillaria root rot caused by *Armillaria* species. These species are important pathogens that are capable of causing serious root diseases of trees in natural forests and plantations. Some of the various hosts affected by *Armillaria* include *Protea* spp. (Coetzee *et al.*, 2003), non-native *Quercus* spp., *Pinus* spp. and *Eucalyptus* spp. (Coetzee *et al.*, 2001). Recently in South Africa, *Armillaria* was reported to be spreading in native woody plants growing at the foot of Table Mountain in Cape Town (Coetzee *et al.*, 2018). Another example is that of the South African pathogen *Chrysoporthe austroafricana*, which was initially reported as *Cryphonectria cubensis* (Gryzenhout *et al.*, 2004). This fungus is responsible for *Cryphonectria* canker and branch dieback of introduced *Eucalyptus* spp. (Gryzenhout *et al.*, 2004; Chungu *et al.*, 2019), *Tibouchina* spp. (Myburg *et al.*, 2002; Oliveira *et al.*, 2021) and also *Syzygium* spp. (Heath *et al.*, 2006; Jimu *et al.*, 2018) in different parts of the world. Such reports show that trees in natural ecosystems and plantations are greatly threatened by pathogens, especially when they are under stress. There is therefore a need to understand the association of these fungal pathogens and their host trees in their various ecosystems.

Fungal diseases of species within the *Rhamnaceae* have largely been ignored, except for those causing diseases on plants with economic importance. Most studies are from China due to the cultivation of species such as *Z. jujuba* (approximately 95% of *Z. jujuba* global cultivation) (Shahrajabian *et al.*, 2020). In South Africa, there are only two studies focused on *Z. mucronata*, firstly Maier *et al.* (2006) reported a smut fungus, *Coniodictyum chevalieri* on trees in the Kruger national park. Secondly, Thaphathi

(2020), identified several species belonging to six fungal families occuring on *Z. mucronata*. To date, there is little information regarding fungal endophytes and pathogens affecting tree species in the *Rhamnaceae* from South Africa. Furthermore, no published records of fungi associated with *Berchemia discolor*, either in agroecosystems or natural ecosystems of South Africa were available at the time of this review. However, Karani *et al.* (2022) reported several fungi causing cankers and dieback on this tree species in Kenya.

The importance of indigenous *B. discolor* and the protection of associated ecosystems from diseases, caused by fungal pathogens are of crucial importance. Natural ecosystems generally can only be effectively protected when their vital attributes, such as the flora and the fauna associated with them are known (Wingfield *et al.*, 2020; Xu *et al.*, 2020). Also, not only do indigenous trees play important roles in their native areas, but they have nutritional and medicinal attributes that are appreciated by the pharmaceutical industry and also by the people of various rural communities in Africa (Orwa *et al.*, 2009; Green *et al.*, 2010; Samie *et al.*, 2010; Debela *et al.*, 2012).

This review aims to provide a summary of fungi associated with *Berchemia* species worldwide. However, due to the lack of information, a discussion of fungi associated with tree species in the family *Rhamnaceae* is also presented. This includes information available on reported non-pathogenic and pathogenic fungal endophytes associated with the *Rhamnaceae*. Attention is also given to the different techniques used for fungal identification.

2. Endophytes

Endophytes are microorganisms, including fungi and bacteria, that spend all or a significant part of their life cycle in plant parts without causing any visual disease symptoms (Saikkonen *et al.*, 1998). For this review, the focus is only on fungal endophytes. These fungi are ubiquitous and can be found in various hosts colonising different plant tissue parts (Sieber, 2007). Fungal endophytes are differentiated into two groups; clavicipitaceous and nonclavicipitaceous endophytes. Clavicipitaceous endophytes are transmitted vertically (from parent to offspring) and nonclavicipitaceous endophytes are those transmitted horizontally (via spores)

(Rodriguez *et al.*, 2009). These two endophyte groups are distinguished based on their evolutionary relatedness, taxonomy, host range and how they function ecologically (Rodriguez *et al.*, 2009).

Endophytic fungi can colonise their hosts during different life stages of the plant. Numerous endophytes are seed-borne and are found existing inside plant tissues from the time when germination starts (Akutse *et al.*, 2017). Other endophytes can enter the plant during the early development stages through stomatal openings and lenticels (Carroll, 1888 cited by Burgess and Wingfield, 2002). Generally, they colonise the plant surfaces before entering the plant cell. From entry, the endophytes may start systematic colonisation of the entire plant, i.e., from roots to shoots, from shoots to flowers, and from flowers to fruits and seeds. They may also cause infections that are localised, either outside or inside the various plant organs (Brader *et al.*, 2017). According to Park *et al.* (2012), as soon as endophytes penetrate the plant cell, they grow thin hyphae within the cell walls and also in the spaces between the cells. Once they are established inside the cells, they may remain in a latent state, which may last until other factors such as environmental conditions that may trigger host stress, create favourable conditions for the fungus to cause disease. There are, however, fungal endophytes that continue within the colonised plant tissue without causing any harm (Faeth, 2002). The endophyte-plant tissue colonisation outcome is also affected by the type of the endophyte, that is, whether they are clavicipitaceous endophytes and/or a nonclavicipitaceous endophytes (Rodriguez *et al.*, 2009).

Endophyte communities in some plants can be dominated by species that are regarded as pathogens. According to Sieber (2007), the reason may be that the 'pathogens' have co-evolved with their hosts, therefore, the symptoms are very rare and can be limited to single localities and/or on a few branches of a single tree. The author also showed that it is possible for endophytes and pathogens of a single host tree to have a close relationship, and form monophyletic phylogenetic clades in phylogenetic studies (Figure 1) (Sieber, 2007). Most species in the *Botryosphaeriaceae* display the typical characteristics of endophytes with this nature and are often found to cause diseases in the same hosts under stress conditions, and can colonise various indigenous trees worldwide (Slippers and Wingfield, 2007; Jami *et al.*, 2017). When and if disease outbreaks occur, it is mostly enhanced by other

unknown external factors or by a rare virulent genotype of the endophyte/“pathogen”. Invasion of fungi, especially pathogens into new areas can also lead to outbreaks, this is because co-evolution between the invading fungi and tree species in the new areas would not have occurred, making trees susceptible with no resistance to the fungi (Sieber, 2007). The question remains concerning these fungi, to what extent do we consider them as true endophytes or as pathogens going through a period of latency?

2.1. Endophyte-host interaction

The endophyte-host interaction has not been studied adequately. Much of the information regarding the interaction is based on grass endophytes (see Sullivan and Faeth, 2008). One of the solutions to filling the knowledge gap requires conducting controlled inoculation experiments where the trees, endophytes, and the pathogen can be manipulated to provide insight into the evolutionary ecology of the endophyte-pathogen-host interaction (Busby *et al.*, 2013). Manipulated inoculation experiments under greenhouse conditions conducted by Rubini *et al.* (2005) showed that the endophyte *Gliocladium catenulatum* antagonised *Moniliophthora perniciosa* which is the cause of witches’ broom disease of *Theobroma cacao*. Blumenstein (2015) showed that antagonism by endophytes is reflected in the formation of a reaction barrier between the endophyte-pathogen. This was observed in three ways, firstly, when there was competition for substrate, secondly, by mycoparasitism and lastly, by a mutual intermingling growth of the fungal endophyte and pathogen *in vitro*. Such studies suggest that certain endophytes protect trees from being attacked by pathogens.

Several studies have shown that fungal leaf endophytes reduce pathogen symptom severity in some plants. Arnold and Herre (2003) found fungal endophytes in the genera *Colletotrichum*, *Xylaria,* and *Fusarium* to reduce the occurrence of leaf necrosis and leaf death caused by *Phytophthora* species in seedlings of *Theobroma cacao.* The occurrence of endophytic fungi on *T. cacao* was further shown to increase the host defense system against *Phytophthora palmivora* (Herre *et al.*, 2007). *Aureobasidium pullulans, Alternaria tenuissima, Neofusciccocum luteum, Fusarium* sp., *Monographella nivalis, Sordaria* sp. and *Penicillium crustosum* were isolated as

endophytes and were found to reduce growth of the Dutch elm disease pathogens (*Ophiostoma* species) *in vitro* (Blumenstein, 2015). The studies of Arnold and Herre (2003), Herre *et al.* (2007), and Blumenstein (2015) suggested that tree endophytes have a mutual relationship with their host and may demonstrate the dynamic coordination occurring inside a tree.

Findings of the above-mentioned studies alone are not conclusive in showing that fungi always establish a mutual relationship with their plant host. This is especially true because a huge portion of the microbial communities in plant species may represent commensal microorganisms. Although many fungi display particular niche associations with the host plants, their functions in these niches and their interaction with their plant hosts are unknown (Brader *et al.*, 2017). Yet others are regarded as opportunistic pathogens. Batista *et al.* (2021) likened host-pathogen interactions to a chess game, where, for a specific outcome to be obtained, numerous actions are probable from each organism. It is also known that reproducing the same interactions under controlled conditions may not always be a representation of what happens in nature, while pathogenicity trials do not depict to the full the pathogenic and resistance mechanisms (Félix *et al.*, 2017). More studies have to be conducted to further understand these fungal endophytes and pathogen interactions within a host plant.

3. Non-pathogenic and pathogenic fungal endophytes occurring on species in the *Rhamnaceae*

This literature review adopts the concept set up by Hardoim *et al.* (2015) and Brader *et al.* (2017) that endophytes should be defined by their ecological niche and not function (Hardoim *et al.*, 2015; Brader *et al.*, 2017). In this regard then, endophytes can be either pathogens or non-pathogens (see Brader *et al.*, 2017 for discussion), with both colonising plant tissues and spending all or a significant part of their life cycle in the plant without causing any visual disease symptoms (Saikkonen *et al.*, 1998). The term non-pathogenic endophytes, as is used here, refers to the group of endophytes that after colonisation and development never cause disease symptoms, while pathogenic endophytes refer to those that, after colonisation, may induce disease symptoms (Hardoim *et al.*, 2015; Brader *et al.*, 2017).

3.1. Non-pathogenic fungal endophytes occurring on the *Rhamnaceae*

Very little attention has been given to non-pathogenic fungal endophytes associated with trees of the *Rhamnaceae*. To date, only three studies reported the diversity of these endophytic fungi associated with trees in the family. The first published report that considered trees in the *Rhamnaceae* dealt with *Ziziphus spina-christi* and *Z. hajanensis* from Oman (EL-Nagerabi *et al.*, 2013). The authors found 52 fungi belonging to 21 genera and 29 groups with sterile mycelia being associated with these tree species. Of the identifiable genera, the dominating taxa included *Alternaria*, *Aspergillus*, *Rhizopus*, *Cladosporium*, *Drechslera*, *Curvularia*, *Fusarium*, *Hansfordia*, *Anguillospora*, *Bactrodemium*, *Catenuaria*, *Dendryphiella*, *Helminthosporium*, *Ulocladium*, *Penicillium*, *Alysidium*, and *Trichocladium* (see EL-Nagerabi *et al.*, 2013). Another study in southern India reported *Xylaria* species and *Nemania* species from *Ziziphus* species (Govinda Rajulu *et al.*, 2013). A more recent study from India, reported *Phomopsis* species from healthy leaves of *Z. jujuba* and *Z. xylopyrus* (Suryanarayanan *et al.*, 2018). To date, there are no other published reports of non-pathogenic endophytes occurring on other species within the *Rhamnaceae*. Although the *Rhamnaceae* are distributed worldwide, these reports represent a small percentage of the endophytes that can be associated with a single host tree within the family.

Earlier studies have indicated that tree species in the same family are frequently dominated by fungal endophytes that are evolutionary closely related (Sieber, 2007; Ma *et al.*, 2013; Brader *et al.*, 2017). Sieber (2007) showed in his review paper that there is a link between host and endophyte relationships; i.e., endophytes occurring on closely related plant species (belonging to the same family) are more similar than those occurring on plant hosts belonging to different families. Therefore, it is reasonable to postulate that the reported endophytes isolated from the *Ziziphus* species discussed above may have related fungi colonising other species in the *Rhamnaceae*, such as *Berchemia discolor*.

3.2. Pathogenic endophytes of *Rhamnaceae* trees

Several studies have identified pathogenic endophytes associated with the *Rhamnaceae* that, after colonisation, cause disease symptoms and can be harmful to the overall productivity of the trees. Some of these fungi have an extended latency period in their life cycle and are well-known to cause disease symptoms when the plant host experiences stressful conditions. These include fungi in the families *Botryosphaeriaceae*, *Diaporthaceae* and *Nectriaceae* on *Rhamnaceae* species. An overview of some of the disease-causing fungi on the *Rhamnaceae* is shown in Table 1 and are discussed below.

3.2.1. *Botryosphaeriaceae*

The *Botryosphaeriaceae* have been reported as pathogens of over 100 host species, and are associated with numerous disease symptoms. Some of the observed symptoms on infected plants include fruit rots, gummosis and bark discolorations, twig and stem cankers, branch-dieback, leaf spots, sooty cankers, damping-off of seedlings, blue staining, post-harvest diseases, crown thinning, abortion of seed capsules and in severe cases it can result in plant mortality (Slippers and Wingfield, 2007; Jami *et al.*, 2017; Slippers *et al.*, 2017). Most of the diseases caused by the *Botryosphaeriaceae* are expressed when the trees are exposed to stressful conditions (Smith *et al.*, 1996; Slippers and Wingfield, 2007). Good examples are the studies of Davis *et al.* (2002) and Garcia *et al.* (2017) on *Botryosphaeriaceae* occurring *Ceanothus crassifolius* under drought stress.

Botryosphaeriaceae comprises species that are ecologically and evolutionary diverse, and are commonly isolated from woody plant hosts including ornamental, forestry and agricultural trees (Slippers and Wingfield, 2007; Slippers *et al.*, 2017). Their worldwide host range can also be explained by their propensity for changes in host associations, i.e., host jump (movement between distant/ unrelated hosts), host shift (movement between phylogenetically related host) and host expansion (Pillay *et al.*, 2013; Jami *et al.*, 2014; Mehl *et al.*, 2017; Batista *et al.*, 2021). Few species in the *Botryosphaeriaceae* have been reported on *Rhamnaceae* trees in different parts of the world, providing valuable information in terms of the fungi's frequencies and host range. These include China, Italy and California, occurring on different *Rhamnaceae*

species (see Table 1) (Dissanayake *et al.*, 2016; Dissanayake *et al.*, 2017; Zhu *et al.*, 2018).

During this review, there were no published reports of pathogenic *Botryosphaeriaceae* on *Berchemia* species. However, Zhu *et al.* (2018) found five genera causing diseases on *Z. jujuba* (Table 1). *Dothiorella* species *(D. sarmentorum* and *D. rhamni*) were reported by Dissanayake *et al.* (2016) and Dissanayake *et al.* (2017), occurring on branches and twigs of *Paliurus spina-christi* and *Rhamnus alaternus*. The genus *Botryosphaeria* includes pathogenic species known to cause canker and dieback of trees in various parts of the world (Slippers and Wingfield, 2007). Davis *et al.* (2002) reported *B. dothidea* on branches of *Ceanothus crassifolius*. The same species was documented on diseased *Z. jujube* (Zhu *et al.*, 2018).

Several studies focused on the diversity of the *Botryosphaeriaceae* on various indigenous host trees in South Africa. These included tree species of *Vachellia* (previously *Acacia*) (Jami *et al.*, 2012; 2013, 2014; 2015), *Adansonia* (Pavlic *et al.*, 2008; Sakalidis *et al.*, 2011; Cruywagen *et al.*, 2017), *Sclerocarya* (Mehl *et al.*, 2017), and as well as the *Rhizophora mangle* (Osorio *et al.,* 2017). Although there are no published reports of the *Botryosphaeriaceae* on trees of the *Rhamnaceae* in South Africa, it will not come as a surprise if they are isolated from *B. discolor* trees, given the ability of *Botryosphaeriaceae* to change from related and unrelated hosts (Mehl *et al.*, 2017).

3.2.2. *Diaporthaceae*

The family *Diaporthaceae* includes several genera containing well-known endophytes and pathogens (Senanayake *et al.*, 2017) of which some were reported on *Rhamnaceae.* For example, Danggomen *et al.* (2013) identified *Phomopsis liquidambaris* and *Diaporthe phaseolorum* in diseased and healthy plant tissues of *Colubrina asiatica.* Recently, *Diaporthe eres w*as reported from dead branches of *Rhamnus alpinus* in Italy (Dissanayake *et al.*, 2017) and caused cankers on *Z. jujuba* in China (Zhang *et al.*, 2018). Symptoms caused by these pathogens include leaf yellowing, wilting, leaf drop and fruit rots, shoot dieback and stem cankers (Gomes *et al.*, 2013; Machingambi *et al.*, 2015).

3.2.3. *Nectriaceae*

Within *Nectriaceae, Fusarium* represents one of the most important genera of plant pathogens, with some being considered amongst the top 10 most important plant pathogenic fungi globally (Dean *et al.*, 2012). Symptoms associated with these fungal pathogens include wilting of leaves, gum exudations on tree parts and twig dieback (Kapoor *et al.*, 2004). Few of the fungi have been reported to cause disease on species of *Rhamnaceae*. Brown (1964) and Mirzaee *et al.* (2011) respectively, reported members of the *Fusarium solani* species complex (*sensu* Geiser *et al.*, 2021) as causal agents of stem canker on *Maesopsis eminii* and branch dieback of *Z. jujube* in Iran. Members of the *Fusarium oxysporum* species complex (*sensu* Geiser *et al.*, 2021) have been reported as causing disease on *Z. jujuba* in China (Gao *et al.*, 2012). The latter included symptoms such as brown discoloration of stems, stunted growth, wilted leaves and deaths of approximately 60% of the plants. More recently in Namibia, *Fusarium* species were reported on diseased *Ziziphus mucronata* (Luchen *et al.*, 2017).

3.2.4. Information gap on fungi occurring on the *Rhamnaceae* in South Africa

The majority of the published information regarding the fungal diseases occurring on *Rhamnaceae* is generally from cultivated/commercialised species. An account of some of the disease-causing fungi on the *Rhamnaceae* is shown in Table 1. For example, it was indicated by Mirzaee (2014) that cultivated species such as *Z. jujuba* are affected by more than 30 pathogens including fungi, bacteria, and phytoplasma. Some of the disease symptoms caused by these pathogens include basal and root rot leaf spots, fruit rots, stem canker, stem dry rot, and twig blight (Dhileepan, 2017).

In South Africa, fungi associated with the *Rhamnaceae* have largely been neglected. Only two published reports, both recording fungi associated with *Z. mucronata*. The first report is of a rare smut fungus *Coniodictyum chevalieri* (Ustilaginomycotina, *Exobasidiales*; *Cryptobasidiaceae*) on fruits, leaves, and branches of *Ziziphus mucronata* (Maier *et al.*, 2006). The fungal species causes a severe decline in the vigor, flower, and fruit production of the plants (Maier *et al.*, 2006). This fungus has only been recorded on *Z. mucronata*, and so far never on any other species within the

Rhamnaceae, and therefore may be host-specific. Secondly, Thaphathi (2020) studied the diversity of fungal pathogens associated with *Z. mucronata* in the Limpopo Province. Identifying pathogens belonging to several families and in various genera namely, *Alternaria, Botryosphaeria*, *Cytospora*, *Didymella*, *Diplodia*, *Dothiorella*, *Fusarium* and *Neofusicoccum*, with all of these isolated from branches with dieback.

4. Brown ivory (*Berchemia discolor*)

Brown ivory is a semi-deciduous tree that can grow up to 20 m in height, depending on ecological conditions (El Tahir, 2001). The species is prevalent in arid and semi-arid bushveld and rocky terrain. It is widely distributed in various countries of southern Africa but also occurs more to the north in Sudan and Ethiopia (Figure 2A). In South Africa, *B. discolor* is found in various provinces including northern Kwa-Zulu-Natal, Mpumalanga and Limpopo (Venter and Venter, 2002; Orwa *et al.*, 2009; Els, 2010).

Berchemia discolor is highly regarded by people in various communities because of its dietary and medicinal attributes. The fruits of *B. discolor* are rich in vitamin C and potassium (Figure 2B) (El Tahir, 2001; Rampedi, 2010). The fruits are also used in numerous foodstuffs such as flavoured porridge and also to make non-alcoholic and alcoholic beverages (Rampedi, 2010). The nutritional compositions of the fruits together with the 6.1% protein content of the fruit pulp show that the fruit trees have unexploited dietary food potentials, and can benefit various rural communities with poor diets in South Africa (El Tahir, 2001). The leaves, the inner and outer bark of the trees have ethnomedicinal uses for conditions such as flu and colds, stomach ache, high blood pressure, ulcers, liver problems, skin itching, nose bleed and also calf weakness (Cheikhyoussef and Embashu, 2013). Extracts from the leaves and roots have aphrodisiac properties (Lovett *et al.*, 2006). Overall, however, all previous studies on this species focused on its ethnomedicinal and pharmacological attributes (El Tahir, 2001; Green *et al.*, 2010; Debela *et al.*, 2012) and not on fungal diseases affecting the health of *B. discolor.*

4.1. Fungi occurring on *Berchemia* species

There is a lack of information regarding fungi associated with *Berchemia* species. This is because either the studies conducted on fungi occurring on the species are not published, or because the topic has been neglected. For example, information regarding fungi occurring on *Berchemia* species found on the fungal databases (https://nt.ars-grin.gov/fungaldatabases) of the United States Department of Agriculture (USDA) fungal databases listed 30 fungal species from 24 *Berchemia* species in different parts of the world (Table 1). Of these, some are well-known pathogens of economic importance, for example, *Botryosphaeria dothidea* that is a pathogen of woody plants and commercialised trees (Slippers and Wingfield, 2007; Slippers *et al.*, 2017), and that was isolated from *B. scandens* in Georgia. Another well-known pathogen is *Puccinia conorata,* known as a virulent pathogen on oats in Canada (Menzies e*t al*., 2019).

For *B. zeyerhi*, information was found on the Pacific Island Ecosystems at Risk (PIER) website (http://www.hear.org/pier/wra/pacific/berchemia_zeyheri_html wra.htm). It listed the fungus *Septoria rhamni-catharticae* var. *rhamni-saxatilis* as occurring on this plant in South Africa. The USDA website (https://nt.ars-grin.gov/fungaldatabases), listed *Oidium* species that were isolated from *B. discolor* in South Africa Rhodesia, and Zimbabwe (Rothwell, 1975, 1982). In most cases, the provided information did not indicate the symptoms from which the fungi were obtained. In other words, information regarding the type of plant tissue, the disease status of the tree, and the severity of the infections are lacking. This is important because various fungal species relate to host plants differently, i.e., they can be pathogenic to one and nonpathogenic endophyte to another host.

Recently, Karani *et al.* (2022) identified several fungi causing cankers and dieback on *B. discolor* trees in Kenya. The authors identified pathogens belonging to *Alternaria, Curvularia, Colletotrichum Dothiorella, Diaporthe*, *Fusarium*, *Nigrospora* and *Neopestalotiopsis.* Although the species were identified from cankers and dieback symptoms, they were shown to be less virulent during pathogenicity trials. To date, the report by Karani *et al.* (2022) is the first report to deal with fungi causing cankers and dieback on *B. discolor* trees in southern Africa.

Currently, there are no records of non-pathogenic endophytes associated with *Berchemia* species. However, a recent study on saprophytes associated with the leaves of *B. floribunda* was conducted in Thailand (Promputtha *et al.*, 2019). The study identified 40 fungal taxa residing in the phylum Ascomycota. Among these, *Cladosporium oxysporum, Lasiodiplodia theobromae,* and undescribed species of *Nectria, Verticillium* and *Mycosphaerella* were identified. Although some of the fungi reported are unlikely to have significant impacts on tree health, species such as *Lasiodiplodia theobromae* is a well-known pathogen and endophyte of several economically important plants (Salvatore *et al.*, 2020). This fungus is also one of the most pathogenic members of the *Botryosphaeriaceae* with a wide host and geographic range. In South Africa, it is reported as an endophyte and pathogen of *Mangifera indica*, and in both South Africa and Kenya as a pathogen of *Sclerocarya birrea* and *Adansonia digitata* (Sakalidis *et al.*, 2011; Mehl *et al.*, 2017; Sheillah *et al.*, 2020).

5. Methods used in fungal taxonomy and diagnosis

The classification and identification of fungal species have undergone significant changes over the past century. Initially, species or genera were delimited based on morphological and phenotypic characteristics. This led to multiple names given to a single species or, on the contrary, a single name was given for cryptic species that look the same but are genetically dissimilar (Taylor *et al.*, 2000). The use of DNA sequencing and application of phylogenetic inference have shown that the fungal diversity is underestimated when species recognition is based on the morphological species concept (Crous *et al.*, 2006).

Today, the most widely accepted method of fungal species classification and delineation rely on phylogenetic species recognition (i.e., a phylogenetic species concept) coupled with morphological information. In some cases, biological species recognition, in which species are recognised based on their ability to mate and produce viable offspring, is applied as further support for newly identified species. This approach is however not applicable to asexual organisms and also impractical on geographically isolated populations (Taylor *et al.*, 2000; Aldhebiani, 2018). The section below provides an overview of the most commonly used morphology-based and

molecular-based approaches for diagnosing and delineating species, as they are applicable on both asexual and sexual species.

5.1. Species recognition based on morphology

Morphology has been been used traditionally by mycologists to classify and identify fungi for decades. This involves the use of phenotypic characters such as spore-producing structures formed during sexual and or asexual reproduction, traits related to spore related traits (e.g., shape, size, color; thickness of the wall layers, their subtending hyphae, and conidiophore cell structure) and fruiting bodies (e.g., size, shape, and tissue type).

The use of morphological characteristics to identify fungi poses several challenges. For example, one often requires knowledge and expertise to identify fungi accurately to species level solely based on their phenotypic characters (Lutzoni *et al.*, 2004). Often, these characters can be misleading and be a problem even for a trained mycologist (Stenlid, 2002). Some fungi grow slowly and take long to sporulate or develop characteristics that are used in the identification. Additionally, many fungi cannot be cultured, or do not produce sexual structures that are typically used in taxonomic studies and may not be known from nature, therefore making descriptions based on morphology is not always reliable. To further exacerbate the issue, certain fungi look the same (e.g., *Dothiorella* and *Diplodia* species), yet when other methods are used they are shown to belong to distinct species (Phillips *et al.*, 2005). The opposite also happens, where strains look different due to different growth conditions, but in actuality belong to the same species (Phillips *et al.*, 2005). Morphological characterisation alone can thus in most cases not be used for diagnosing fungi (Taylor *et al.*, 2000). To overcome the limitations of morphological characterisation, molecular techniques with improved accuracy and reliability (Capote *et al.*, 2012) are used to define species boundaries (see below).

5.2. Molecular techniques for recognising fungal species

Molecular-based taxonomy has unlocked our understanding of the diversity of fungi. As a result, there has been a substantial increase in the number of fungi described and described worldwide (Hawksworth, 2001; Crous *et al.*, 2013; 2014; 2015; Hawksworth and Lücking, 2017; Crous *et al.*, 2019). Molecular-based methods for species recognition have numerous advantages when compared to morphology-based methods (Table 2). For example, they are often rapid and can be applied without sexual structures, and can be used even when fungi cannot be cultured.

A number of methods have been introduced during the last three decades for the delineation of fungal species. These methods can generally be grouped into two; PCR-based methods without the need for DNA sequencing and those involving DNA sequencing of various genomic regions or genes. Examples of the former include RFLPs (Restriction fragment length polymorphisms), RAPDs (Random amplified Polymorphic DNAs), AFLPs (Amplified Fragment Length Polymorphisms), qPCR (Quantitative real-time PCR) and LAMP (Loop-Mediated Isothermal Amplification). RFLPs were mainly used for studying diversity of species (Jeng *et al.*, 1991), but were also applied for identifying species. For example, Van der Walt (2008) identified 10 *Botryosphaeriaceae* species using PCR-RFLP groupings in South Africa. Diguta *et al.* (2011) also used RFLP markers to delineate 119 filamentous fungi from vineyards in Burgundy. RAPDs were also applied for diversity studies (Pipe *et al.*, 1995; Capote *et al.*, 2012), but several studies used it for species diagnosis. Examples of this include the use of RAPD cluster analysis on *Macrophomina phaseolina* isolates (Das *et al.*, 2008), molecular characterisations of *Pestalotiopsis* species (Joshi *et al.*, 2009), *Fusarium oxysporium* f. sp. *melongenae* (Baysal *et al.*, 2010) and the identification and characterisation of *Diaporthe vaccinia* (Michalecka *et al.*, 2017). The principle of RFLP and RAPD were combined to develop AFLPs (Singh *et al.*, 2013) which were subsequently used to quickly generate large numbers of marker fragments for any microorganism without prior knowledge of its genomic sequence (Singh *et al.*, 2013). Like the two other methods, AFLP was used to study and characterise genetic variation within species (e.g., Belabid *et al.*, 2004) but it has also been applied to diagnose or recognise species. For example, Skrede *et al.* (2012) used AFLP markers

and DNA loci to compare their ability to delimit cryptic hybrid species in the genus *Coniophora*.

The Loop-Mediated Isothermal Amplification (LAMP) and qPCR methods can both be designed to be species-specific. Both are highly sensitive and specific and have allowed for the easy detection of fungal pathogens directly from samples extracted from infected symptomatic and asymptomatic tissues (Luchi *et al.*, 2020). For example, using qPCR, Cook *et al.* (2009) compared and quantified the endophyte *Undifilum oxytropis* found in locoweeds. *Fusarium culmorum* known to cause Fusarium head blight in cereals (Osborne and Stein, 2007) was successfully quantified and validated against other *Fusarium* species using qPCR (Bilska *et al.*, 2018). The LAMP technique was recently used by Li *et al.* (2019) for fast detection of the Rice blast disease in the early stages caused by *Magnaporthe oryzae.* The technique was also found to detect DNA concentrations as low as 0.001ng/µl of the postharvest pathogen *Neofabraea perennans*, which is responsible for the Bull's eye rot (BER) in apple and pears (Enicks *et al.*, 2020). LAMP has the advantage of allowing rapid diagnosis for testing pathogens in field conditions (Luchi *et al.*, 2020).

DNA sequence comparisons of multiple genes, or even genome sequence comparisons, superseded earlier PCR-based methods and now provide robust information to delineate and diagnose species. Numerous gene or genomic regions are used in DNA-based sequence approaches and in phylogenetic analyses to identify and delineate fungal species. These regions include the genes and regions associated with the nuclear ribosomal RNA (rRNA) cistron and various protein-coding regions. Those associated with the rRNA cistron include two internal transcribed spacers (ITS1 and ITS2) and the intergenic spacer (IGS), as well as genes encoding the 5.8S rRNA and the large and small ribosomal subunits (i.e., LSU and SSU) (James *et al.*, 2001; Schoch *et al.*, 2012; Fidler *et al.*, 2017). Commonly used protein-coding, genes include those for β-tubulin (Fidler *et al.*, 2017), calmodulin (Mulè *et al.*, 2004), translational elongation factor 1-α (Stielow *et al.*, 2015; Luo *et al.*, 2019), DNA-directed RNA polymerase II largest (*rbp11*) and second-largest (*rpb2*) subunits (Matheny, 2005; Luo *et al.*, 2019), DNA topoisomerase I (*TOP1*) (Stielow *et al.*, 2015), phosphoglycerate kinase (*PGK*) (Stielow *et al.*, 2015), and MCM7 and TSR1 (Tretter *et al.*, 2013).

Among these, ITS1 and ITS2 together with the conserved 5.8S gene of the nuclear RNA cistron (collectively often referred to as the "ITS region") was one of the first regions to be used for differentiating species based on DNA sequences (Walker and Doolittle, 1982; White *et al.*, 1990). The ITS region is also accepted as the universal barcode locus for fungi as it was shown to amplify consistently nearly across all fungal lineages, and in most cases, it is conserved within a species but variable between most species (Schoch *et al.*, 2012). It is, however, known that individual copies of the region might differ in their sequence, and the region might vary within a species or be conserved between recently diverged species (White *et al.*, 1990; Schoch *et al.*, 2012).

To complement ITS-based diagnostics, contemporary taxonomic studies typically include the sequences for several protein-coding genes. The data for these loci are then used together to recognise species boundaries based on Genealogical concordance phylogenetic species recognition (GCPSR) (Taylor *et al.*, 2000). In essence, a species is recognised based on the Genealogical concordance of multiple genes using phylogenetic analyses. Genetic isolation and species limits are recognised at the point where nodes among gene trees go from being congruent to incongruent (Figure 3). Application of the GCPSR has greatly assisted in the classification of fungi and led to the discovery of many new species, especially cryptic species. For example, Laurence *et al.* (2014) determined species boundaries within the *F. oxysporum* species complex with eight protein-coding loci. By making use of ITS and four protein-coding genes, Cruywagen and co-workers (2017) diagnosed *Lasiodiplodia* isolates to species level and even identified putative hybrid species. Furthermore, the use of more than one gene region on the Botryosphaeriales has assisted with synonymising genera and species that were considered distinct (Batista *et al.*, 2021; Zhang *et al.*, 2021). These gene regions are also applicable for delineating other fungi. Good examples of the successful application of the GCPSR include the identification of species within various species complexes including within the *Colletotrichum* species complex (Liu *et al.*, 2016), and the cryptic species within the species complex of *Wallemia sebi* (Jančič *et al.*, 2015).

Phylogenomics is an approach that will undoubtedly revolutionise future taxonomic studies on fungi. This approach makes use of the phylogenetic analyses of thousands of genes extracted from the whole genome sequence of organisms (Fitzpatrick *et al.*,

2006; Zhang *et al.*, 2017; Chethana *et al.*, 2021). Phylogenomics improves phylogenetics accuracy by maximising phylogenetic informativeness by the inclusion of a large number of gene sequences (Zhang *et al.*, 2017). This method is gaining increased attention and it will shed more light on the biology, classification, diversity, and taxonomy of fungi. For example, the use of genome sequencing has created platforms to answer some of the most intriguing questions such as the biology, reproductive strategies, and endophytic nature of the *Botryosphaeriaceae* (Lopes *et al.*, 2017; Marsberg *et al.*, 2017; Garcia *et al.*, 2021).

6. Conclusions

Berchemia discolor is distributed across vast areas of southern Africa, and play an important role ecologically, economically, and are important to various rural communities in Africa. Published studies have paid considerable attention to medicinal properties and nutritional value of the species, but the fungi associated with it have been largely neglected. As a result, this review focused on fungi associated with the *Rhamnaceae* worldwide. In South Africa, *B. discolor* trees are more prevalent especially in Limpopo Province, and virtually, the diseases of the species have been ignored.

This study will give further insight and understanding of the diversity and distribution of potential fungal pathogens that might threaten *Rhamnaceae* trees. It will also be increasing the knowledge of the biodiversity of native fungi and their roles in native tree health. As indicated by Sieber (2007), trees of the same family are most likely dominated by fungal endophytes that are evolutionary closely related. Therefore, the relatives of the identified fungi from other *Rhamnaceae* species may be found colonising *B. discolor.* This dissertation thus aims to contribute and gain knowledge of fungal endophytes associated with *B. discolor.* The first objective was to determine the diversity of fungi in association with *B. discolor* trees in Mapungubwe National Park. The second objective was to identify and determine similarities of the *Botryosphaeriaceae* isolated from asymptomatic and symptomatic branches of *B. discolor* in agricultural and natural ecosystems.

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8. Tables

Table 1. Pathogens of *Rhamnaceae* trees

Family	Fungus	Host	Disease Caused	Location	References
	Aplosporella ginkgonis	*Ziziphus jujuba*	Canker and dieback	Dongling Mountain of China	Zhu *et al.* (2018)
	Ap. javeedii	*Z. jujuba*	Canker and dieback	Dongling Mountain of China	Zhu *et al.* (2018)
	Botryosphaeria dothidea	*Z. jujuba*	Canker and dieback	Dongling Mountain of China	Zhu *et al.* (2018)
	B. dothidea	*Ceanothus crassifolius*	Dead plant tissue	California	Davis *et al.* (2002)
Botryosphaeriaceae	*B. dothidea*	*Berchemia scandens*	N/A	Georgia	https://nt.ars-grin.gov/fungaldatabases
	B. obtusa	*Z. jujuba*	Thick rotten disease	China	Zhu *et al.* (2018)
	Diplodia zizyphiae	*Z. jujuba*	Blight	India	Chauksay *et al.*, cited by Dhileepan (2017)
	Dothiorella rhamni	*Rhamnus alaternus*	Dead branches	Italy	Dissanayake *et al.* (2017)
	Dothiorella sp.	*B. discolor*	Cankers and dieback	Lower Eastern Kenya	Karani *et al.* (2022)

Table 1. (**Continued)** Pathogens of *Rhamnaceae* trees

Family	Fungus	Host	Disease Caused	Location	References
	D. sarmentorum	*Paliurus spina-christi*	Diseased branches and twigs	Italy	Dissanayake *et al.* (2016)
	Lasiodiplodia theobromae	*B. floribunda*	Dead leaves	Thailand	Promputtha *et al.* (2019)
	Phaeobotryon rhoinum	*Z. jujuba*	Canker and dieback	Dongling Mountain of China	Zhu *et al.* (2018)
	Ph. rhois	*Z. jujuba*	Canker and dieback	Dongling Mountain of China	Zhu *et al.* (2018)
	Botryosphaeria sp.	*Z. mucronata*	Branches with dieback	South Africa	Thaphathi (2020)
Botryosphaeriaceae	*Dothiorella* sp.	*Z. mucronata*	Branches with dieback	South Africa	Thaphathi (2020)
	Diplodia sp.	*Z. mucronata*	Branches with dieback	South Africa	Thaphathi (2020)
	Neofusicoccum sp.	*Z. mucronata*	Branches with dieback	South Africa	Thaphathi (2020)
	Botryosphaeria sp.	*Z. mucronata*	Branches with dieback	South Africa	Thaphathi (2020)
	Dothiorella sp.	*Z. mucronata*	Branches with dieback	South Africa	Thaphathi (2020)

Table 1. (**Continued)** Pathogens of *Rhamnaceae* trees

Family	fungus	host	Disease caused	location	References
Cryptobasidiaceae	*Coniodictyum chevalieri*	*Z. mucronata*	Smuts	South Africa	Maier *et al.* (2006)
Corynesporascaceae	*Corynespora ziziphae*	*Ziziphus* sp.	Leaf spot	India	Jain *et al.* (2002)
Pleomassariaceae	*Asteromassaria pulchra*	*B. racemosa*	N/A	Japan	https://nt.ars-grin.gov/fungaldatabases)
Pythiaceae	*Phytophthora tentaculata*	*Frangula californica*	Root rot, stem cankers, death of trees	California	Rooney-Latham *et al.* (2015)
Mycosphaerellaceae	*Pestalotia funera*	*Z. mauritiana*	Twig blight	India	Dolly and Razdan (2010)
	Pseudocercospora rhamnaceicola	*Z. vulgaris*	Foliar blight	Taiwan	Hsieh and Goh (1990)
Pucciniaceae	*Puccinia coronata*	*Rh. frangula*	Crown rust	N/A	Abtahi and Nourani (2017)
Phakopsoraceae	*Phakopsora zizyphi-vulgaris*	*Z. mauritiana, Z. Jujuba, Z. mucronata*	Leaf rust	China, India, South Africa	Berndt and Wood (2012), Dhileepan (2017) personal observation

Table 1. (**Continued)** Pathogens of *Rhamnaceae* trees

Family	Fungus	Host	Disease Caused	Location	References
Tubeufiaceae	*Helicomyces* sp.	*B. floribunda*	Dead Leaves	Thailand	Promputtha *et al.* (2019)
Dematiaceae	*Berkleasmium phyllostachydis*	*B. floribunda*	Dead Leaves	Thailand	Promputtha *et al.* (2019)
Dictyosporiaceae	*Dictyosporium sacchari*	*B. floribunda*	Dead Leaves	Thailand	Promputtha *et al.* (2019)
Cladosporiaceae	*Cladosporium zizyphi*	*Z. jujuba*	Leaf spot	Australia	Dhileepan (2017) personal observation
Pleosporaceae	*Curvularia pseudoclavata*	*B. discolor*	Cankers and dieback	Lower Eastern Kenya	Karani *et al.* (2022)
Cladosporiaceae	*Cladosporium oxysporum*	*B. floribunda*	Dead Leaves	Thailand	Promputtha et al. (2019)
Pleosporaceae	*Helminthosporium massarinum*	*B. volubillis*	Wood	South Carolina	https://nt.ars-grin.gov/fungaldatabases
	Alternaria sp.	*Z. mucronata*	Branches with dieback	South Africa	Thaphathi (2020)
	Alternaria sp.	*B. discolor*	Cankers and dieback	Lower Eastern Kenya	Karani *et al.* (2022)

Table 1. (**Continued)** Pathogens of *Rhamnaceae* trees

Family	Fungus	Host	Disease caused	Location	References
	Volutella sp.	*B. floribunda*	Dead Leaves	Thailand	Promputtha *et al.* (2019)
	Fusarium sp.	*Z. mucronata*	Branches with dieback	South Africa	Thaphathi (2020)
	Nectria sp.	*B. floribunda*	Dead Leaves	Thailand	Promputtha *et al.* (2019)
Nectriaceae	*Cy. floridanum*	*B. floribunda*	Dead Leaves	Thailand	Promputtha *et al.* (2019)
	F. equiseti	*B. discolor*	Cankers and dieback	Lower Eastern Kenya	Karani *et al.* (2022)
	F. chlamydosporum	*B. discolor*	Cankers and dieback	Lower Eastern Kenya	Karani *et al.* (2022)
	F. equiseti	*B. discolor*	Cankers and dieback	Lower Eastern Kenya	Karani *et al.* (2022)
Plectosphaerellaceae	*Verticillium* sp.	*B. floribunda*	Dead Leaves	Thailand	Promputtha *et al.* (2019)
Incertae sedis.	*Periconia lateralis*	*B. floribunda*	Dead Leaves	Thailand	Promputtha *et al.* (2019)
Incertae sedis.	*Beltrania rhombica*	*B. floribunda*	Dead Leaves	Thailand	Promputtha *et al.* (2019)

Table 1. **(Continued)** Pathogens of *Rhamnaceae* trees

Family	Fungus	Host	Disease caused	Location	References
Sclerotiniaceae	*Grovesinia pyramidalis*	*B. racemosa*	N/A	Japan	https://nt.ars-grin.gov/fungaldatabases
Gloniaceae	*Glonium curtisii*	*B. scandens*	Dead limbs	Louisiana, USA	"
Hymenochaetaceae	*Inocutis jamaicensis*	*Scutia buxifolia*	White fibrillar, stringy wood rots	N/A	https://nt.ars-grin.gov/fungaldatabases)
Mycosphaerellaceae	*Mycosphaerella* sp.	*B. floribunda*	Dead Leaves	Thailand	Promputtha *et al.* (2019)
Chaetosphaeriaceae	*Dictyochaeta tropicalis*	*B. floribunda*	Dead Leaves	Thailand	Promputtha *et al.* (2019)
Mycosphaerellaceae	*Cercospora* sp.	*B. racemosa*	N/A	Hong Kong	https://nt.ars-grin.gov/fungaldatabases
Diatrypaceae	*Diatrypella favacea*	*B. scandens*	N/A	Georgia	"
	Diatrypella quercina	*B. scandens*	N/A	Georgia	"

Table 1. **(Continued)** Pathogens of *Rhamnaceae* trees

Family	Fungus	Host	Disease caused	Location	References
Diporthaceae	*Diaporthe* sp.	*Z. mucronata*	Branches with dieback	South Africa	Thaphathi (2020)
	Dia. ganjae	*B. discolor*	Cankers and dieback	Lower Eastern Kenya	Karani *et al.* (2022)
Cytosporaceae	*Cytospora* sp.	*Z. mucronata*	Branches with dieback	South Africa	Thaphathi (2020)
Gloniaceae	*Glonium curtisii*	*B. scandens*	Dead limbs	Louisiana, USA	"https://nt.ars-grin.gov/fungaldatabases
Didimosphaeriaceae	*Didymella* sp.	*Z. mucronata*	Branches with dieback	South Africa	Thaphathi (2020)
Erysiphaceae	*Erysiphe berchemiae*	*Berchemia* spp.	N/A	Japan	"https://nt.ars-grin.gov/fungaldatabases
Diatrypaceae	*Eutypella berchemiae*	*B. scandens*	N/A	Georgia	"
Trichosphaeriaceae	*Nigrospora sphaerica*	*B. discolor*	Cankers and dieback	Lower Eastern Kenya	Karani *et al.* (2022)

Table 1. (**Continued)** Pathogens of *Rhamnaceae* trees

Family	Fungus	Host	Disease caused	Location	References
Glomerellaceae	*Colletotrichum gloeosporioides*	*B. discolor*	Cankers and dieback	Lower Eastern Kenya	Karani *et al.* (2022)
Xylariaceae	*Biscogniauxia capnodes var. rumpens*	*Berchemia* sp.	N/A	Louisiana	https://nt.ars-grin.gov/fungaldatabases)
Sporocadaceae	*Neopestalotiopsis* sp.	*B. discolor*	Cankers and dieback	Lower Eastern Kenya	Karani *et al.* (2022)

Table 2. Molecular diagnostic techniques used in fungal identification

Techniques	Advantages	Disadvantages	References
RFLP (Restriction fragment length polymorphisms)	It is inexpensive and does not require advanced instruments. Easily designed achieved using public available programs.	Time-consuming. Not suitable for high-throughput analyses.	Jeng *et al.* (1991), Garcia *et al.* (2004), Rasmussen (2012)
RAPD (Random Amplified Polymo rphic DNA)	Is fast and inexpensive. Does not require extensive knowledge of DNA sequence of the target organism.	It has poor reproducibility. Cannot differentiate non-homologous co-migrating bands.	Garcia *et al.* (2004), Capote *et al.* (2012)
AFLP (Amplified Fragment Length Polymorphisms)	Does not require prior knowledge of the genomic sequence. Requires only small amounts of starting template and has better results as compared to RFLP and RAPD	It is labour-intensive and time-consuming and expensive	Garcia *et al.* (2004), Singh *et al.* (2013)
qPCR (Quantitative real-time PCR)	Automated and no need for post-amplification analyses	Cost and complexity due to simultaneous thermal cycling and fluorescence detection	Luchi *et al.* (2020), Bilska *et al.* (2018)

Table 2. (Continued) Molecular diagnostic techniques used in fungal identification

Techniques	Advantages	Disadvantages	References
LAMP (Loop Mediated Isothermal Amplification)	Affordable, specific, highly and sensitive Rapid diagnostic test of fungi in both laboratory and field conditions. It is a simple screening essay, can amplify 10^9 copies of DNA within an hour	The reaction mechanism is a bit complicated	Li *et al.* (2019); Luchi *et al.* (2020)
GCPSR (Genealogical concordance phylogenetic species recognition)	The concept can be explored within an explicit hypothetical framework A large number of characters can be analysed	Unreliable phylogenies and wrong classifications due to erroneous reference sequence data from misidentified or contaminated samples in GenBank and other public databases It is challenging to determine species boundaries among conflicting gene trees, due to recombination within a clonal population	Taylor *et al.* (2000), Jančič *et al.* (2015), Liu *et al.* (2016), Hilário *et al.* (2021)

Table 2. (Continued) Molecular diagnostic techniques used in fungal identification

Techniques	Advantages	Disadvantages	References
Phylogenomics	because genomic data limit the impact caused by individual genes on the topology of the phylogenetic tree and result in a phylogeny that truly represents the entire genome It gives good and high statistical support at higher classification level than at species level classification	It is hard to obtain high quality and pure uncontaminated DNA for whole-genome sequencing of unculturable fungi and most AM fungi. Can be expensive to generate sequence alignments and phylogenies. Systematic error may still occur as a result of non-proportional alignments of concatenated genes	Fitzpatrick *et al.* (2006); Zhang *et al.* (2017),Young and Gillung (2020) Chethana *et al.* (2021),

9. Figures

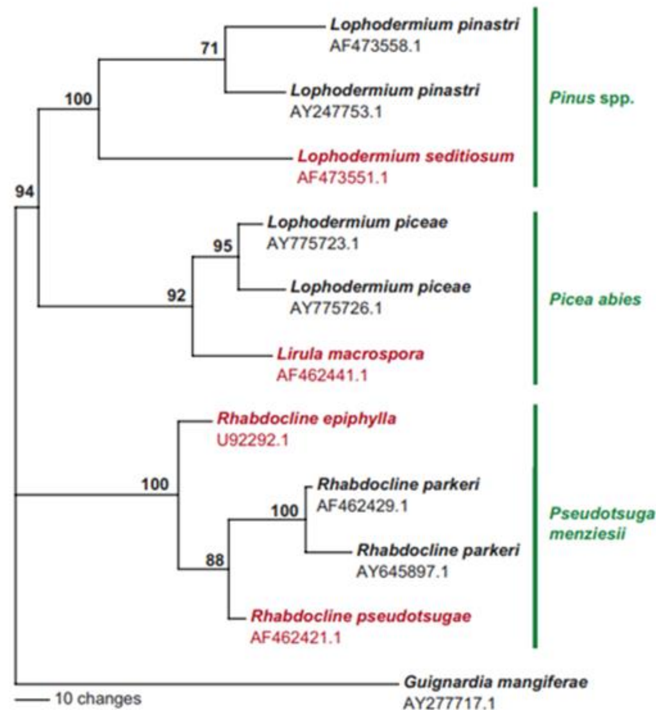

Figure 1. Maximum parsimony phylogeny based on DNA sequences of the ITS DNA sequences of *Rhytismataceae* fungal endophytes (in black) and their closely related pathogenic relatives (in red). The bootstrap supports are shown above the branches. The phylogeny was rooted with *Guignardia mangiferae* (phylogenetic tree published by Sieber, 2007).

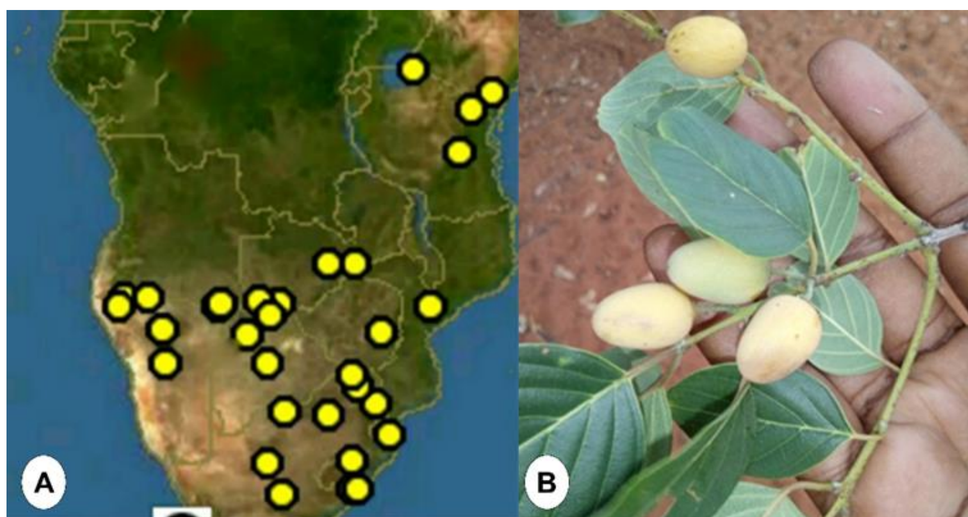

Figure 2. *Berchemia discolor* trees in southern Africa. A) The native areas of *Berchemia* spp. shown in yellow. B) Fruits and leaves of the *Berchemia discolor.*

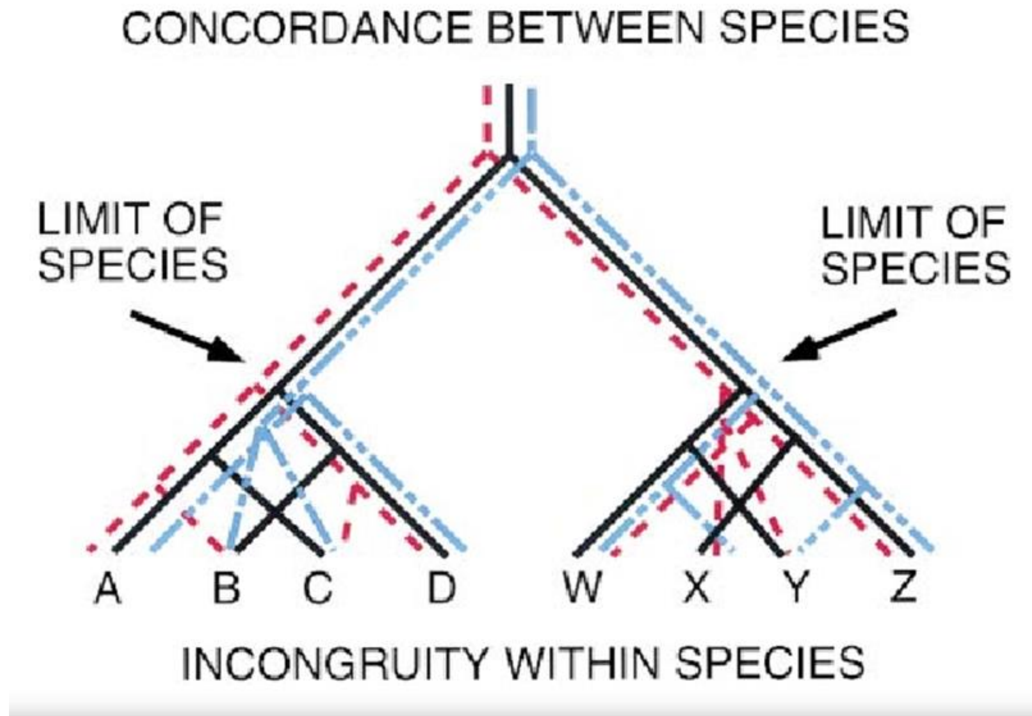

Figure 3. Species diagnosis based on the GCPSR. Schematic diagram showing concurrent analyses of three gene genealogies, displaying the shift from congruency to incongruency amongst branches representing species (taken from Taylor *et al.*, 2000).

CHAPTER 2

Fungi associated with *Berchemia discolor* trees in Mapungubwe National Park

ABSTRACT

Trees of *Berchemia discolor* (*Rhamnaceae*) in Mapungubwe National Park (MNP) were observed having severe symptoms of branch-dieback. Various fungi have been reported being associated with dieback symptoms and other diseases of woody tree species worldwide. However, there is a lack of information regarding the fungi associated with *B. discolor* in South Africa. The aim of this study was thus to identify potential endophytes and fungal pathogens that are associated with branch-dieback of *B. discolor* in the Mapungubwe National Park. Fungi collected from healthy and infected branches were identified based on their Internal Transcribed Spacer (ITS) region sequences. Ten families were identified, namely, *Botryospaheriaceae*, *Diaporthaceae*, *Nectriaceae*, *Didymellaceae*, *Sporocadaceae*, *Pleosporaceae*, *Diatrypaceae*, *Cytosporaceae*, *Didymosphaeriaceae,* and *Davidiellaceae*. The predominant genera isolated as endophytes and/or as pathogens of *B. discolor* from both asymptomatic and symptomatic branches were *Dothiorella*, *Lasiodiplodia,* and *Alternaria,* respectively. This study is the first to record fungal endophytes and potential pathogens from *B. discolor* in Mapungubwe National Park. The results represented set the foundation for future work to elucidate the fungal diversity in the Mapungubwe National Park and also fungi associated with *B. discolor*.

Keywords: *Berchemia discolor*, endophyte diversity, ITS, Mapungubwe National Park.

1. Introduction

Fungal pathogens of plants usually attack their vascular tissues, while disrupting carbohydrate, mineral, and water flow within the plant, thereby interfering with the general growth and health of the plant (Horst, 1990). All plants, including trees, are affected by fungal pathogens and eventually, in severe cases, may die. Dieback caused by fungi is known to cause devastating losses. In forestry, as an example, an estimated R 9.5 million ($708 995,22) loss per year was experienced due to the well-known pine fungus *Diplodia sapinea* (= *Sphaeropsis sapinea*) in South Africa (Zwolinski *et al.*, 1990). However, indigenous tree losses cannot be estimated by monetary means. It is possible for an ecosystem shift to occur because of a wiping out of a tree species in a particular system, impacting biodiversity, thus to some degree also the functionality of the biodiversity in the ecosystem.

Many of the fungal pathogens reported as causing diseases of indigenous trees can lead to detrimental losses of these trees in natural ecosystems. For example, the widespread mortality of *Fraxinus excelsior* caused by the ash dieback fungus *Hymenoscyphus fraxineus,* is threatening the existence of the indigenous tree species from European landscapes (Burokiene *et al.*, 2015). In Africa, members of the *Botryosphaeriaceae* were reported to cause stem cankers and major dieback of *Grevillea robusta* in East Africa (Njuguna, 2011), and are also associated, for example, with diseased *Sclerocarya birrea* trees in South Africa (Mehl *et al.*, 2017). Although several recent publications dealt with fungal pathogens and endophytes (Njuguna, 2011; Jami *et al.*, 2014; 2017; 2018; Cruywagen *et al.*, 2015; 2017), the diversity of fungi, specifically those causing disease on indigenous trees, remain understudied. This study thus aimed to expand our knowledge of fungi occurring on indigenous trees by focussing on *B. discolor*, an indigenous species in South Africa, and elsewhere in Africa.

Berchemia discolor (brown ivory) plays a role in the socio-economic well-being of small-scale farmers and rural communities (Cheikhyoussef and Embashu, 2013). In rural communities, the trees are planted as shade or for ornamental purposes, timber for producing decorative products and furniture, and for construction materials (Orwa *et al.*, 2009; Cheikhyoussef and Embashu, 2013). The leaves and the bark from the trees are commonly used for traditional medicine (Lovett *et al.*, 2006; Green *et al.*,

2010). The edible fruits are rich in carbohydrates, calcium, sodium, iron, magnesium, and potassium, and can be used for the production of beverages sold for income (Lusepani, 1999; Debela *et al.*, 2012; Misihairabgwi and Cheikhyoussef, 2017).

Berchemia discolor trees are widely distributed in Africa and were reported in countries including Angola, Arabian Peninsula, Botswana, Ethiopia, Eritrea, Kenya, Madagascar, Malawi, Mozambique, Namibia, Republic of Zambia, Somalia, Sudan, Swaziland, Tanzania, Uganda, Yemen, and Zimbabwe (Orwa *et al.*, 2009). In South Africa, they are found in the Limpopo Province (Orwa *et al.*, 2009), including the Mapungubwe National Park. This park is located in the Shashe-Limpopo Province area of the Vhembe district and is one of the natural reserves in South Africa with landscapes that have no human alteration (Huffman, 2000). Within the park, a few *B. discolor* trees are scattered. At the time of the study, all of the trees had shown severe symptoms of branch-dieback possibly caused by fungi.

Virtually nothing is known regarding the fungal diversity associated with trees growing in the Mapungubwe National Park. Important to tree health, the diseases and the associated fungal pathogens that are affecting trees in the Mapungubwe National Park are mostly not known. This kind of information is important as natural ecosystems can only be effectively protected if their key attributes, including the understanding of the vegetation and the fungi associated with them, are known. We focused specifically on this tree species because it is one of the *Rhamnaceae* tree species with little information regarding its associated fungi, and it was one of the tree species severely affected by branch-dieback in the Mapungubwe National park. Not only is it found in abundance in other parts of the Limpopo province, but it is one of the most valued trees with plenty of beneficial attributes. Therefore, the aim of this study was to identify the diversity of the fungi associated with healthy-looking and diseased branches of *B. discolor* in the MNP.

2. Materials and methods

2.1. Sample collection and isolations

During August 2017, 13 *B. discolor* trees were surveyed in Mapungubwe National Park (SANParks permit KUNE1442), which is situated between 22°15′0″S and 29°12′0″E.

One healthy branch and one branch showing symptoms of dieback were collected per tree during sampling. The branches were approximately 10-12 mm in diameter and approximately 20 cm in length. In total, from the 13 trees, 25 branches were collected of which 13 were from asymptomatic and 12 were branches with dieback. The collected samples were kept in paper bags and processed at the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. Isolations of fungal endophytes and pathogens were done as soon as possible after collection.

Endophyte isolations from all healthy branches (Figure 1 A) were made after surface-disinfection of branch tissue in 10 % hydrogen peroxide for 2 min, and then rinsing three times (1 min each) in sterile distilled water. Branches were then cut into 12 thin disc-pieces (0.5 cm) and plated onto malt extract agar (MEA 2 % malt extract and 1.5% agar, Biolab, Midrand, South Africa) amended with streptomycin (0.1g/L). Isolations of potential pathogens were done from all dieback-branches after debarking and exposing parts of the vascular tissue between the necrotic and healthy parts of the branch (Figure 1 B). Discs were cut and plated onto MEA amended with streptomycin.

The inoculated MEA plates were incubated at 25 ℃ for seven days and checked daily for fungal growth. Pure cultures were obtained by transferring hyphal-tips of the fungi to fresh MEA plates. In total, 69 pure fungal isolates displaying various colony morphological characteristics were obtained (Figure 2 A-D) and considered for further molecular studies. The pure cultures were deposited in FABI’s CMW Culture Collection.

2.2. DNA extractions, PCR amplification and sequencing

Mycelium from isolates grown for 7 to 14 days were scraped from the surfaces of the medium, and freeze-dried. DNA extractions were done using a modified method described by Brunner *et al.* (2001). Briefly, dried mycelia were transferred into homogenising tubes with extraction buffer (100mM Tris-HCl, pH=8; 2M NaCl; 25mM EDTA; 2% CTAB), supplemented with 2% (w/v) polyvinylpyrrolidone (PVP), 2% (v/v) β-mercaptoethanol, 500 mg/ L spermadine and a ceramic ball. The mycelium was homogenised using the Fast-Prep™ (FP 120 homogenising system) at speed of 4.0m/sec for 20 seconds twice. The homogenising tubes were then placed in a hot

water bath at 65℃ for an hour (for the disruption of cell walls). The tubes were centrifuged at 13 362 x g for 30 minutes and the supernatant were transferred to 2 ml Eppendorf tubes. For the separation of the DNA from excess protein and polysaccharides, a volume of 650 µl chloroform: isoamyl alcohol (24:1) was added, vortexed, and centrifuged at 13 362 x g at 4℃ for 20 minutes. This step was repeated. The supernatant was transferred to new tubes (2 ml), to which 1000 µl of isopropanol was added. DNA was precipitated overnight at -20℃, centrifuged at 13 362 x g for 30 minutes to obtain a DNA pellet. Pellets were washed twice with 250 µl of 70% room temperature ethanol. The DNA pellets were then dried in a Vacuspin and re-suspended in 60 µl sterile SABAX water (SABAX: Adcock Ingram, Bryanston, S.A.). DNA concentrations were determined using a NanoDrop® ND-1000 Spectrophotometer V3.7.1 (Thermo Fisher Scientific, USA), adjusted to a working concentration of 60 ng/µl with SABAX water for use in PCR reactions.

PCR amplification of the complete ITS-1 and ITS-2 regions, including the 5.8S gene, of the ribosomal DNA were performed using primer pairs ITS-1 (5' TCC GTA GGT GAA CCT TGC GG 3') and ITS-4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White *et al.*, 1990). PCR was performed in a 25 µl final volume. The master mix consisted of 0.2 µM of each primer, 80-100 ng DNA template, 0.5 U MyTaq™ DNA polymerase (Bioline, London, UK), 5 µl 5x Mytaq reaction buffer containing dNTPs, $MgCl_2$, stabilisers, and enhancers (Bioline GmbH, Luckenwalde, Germany). Sterile SABAX water was added to adjust the PCR mix to a volume of 25 µl.

The PCR protocol used was as follows: 94℃ for 3 min, 35 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s, elongation at 72℃ for 1 min, and a final elongation cycle at 72℃ for 7 min. The PCR products were visualised on a 1% agarose gel stained with Gel Red (Biotium, Hayward, US) in a 1% TAE buffer solution and visualised under UV light. PCR product sizes were estimated using a 100 bp marker under UV light. Eight µl of Exosap (Mixture of Exonuclease I and FastAP Alkaline Phosphate) (Thermo Fisher Scientific Inc. Waltham, MA, USA) was used to clean the PCR products. The purified PCR products were sequenced in both directions using the same primer pairs used in PCR reactions.

Sequence reactions in both directions were performed with an ABI PRISM ™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Rlmer Applied Biosystems

Inc., Foster City, California), following the manufacturer's instructions. The PCR sequencing conditions were as follows: 96 ℃ for 10 s followed by 25 cycles of 96 ℃ for 10 s, 55 ℃ for 10 s, and 60 ℃ for 4 min. Sodium acetate mixture containing 2 µl NaAc (3.2 M, pH 5.2), 8 µl SABAX, and 50 µl absolute alcohol was used to clean the final products. The products were dried in an Eppendorf 5301 vacuum concentrator, at 30 ℃. Sequencing was done on an ABI PRISM 3100™ automated DNA Sequencer at the sequencing facility of the University of Pretoria. Sequencing in forward (5' to 3' direction) was done on all isolates. Sequencing of the ITS region was subsequently done in both direction for selected isolates based on the preliminary phylogenetic analyses of the forward ITS-1 sequences, and based on the branch type the fungi were isolated from.

DNA sequences of the isolates were assembled using CLC Main Workbench v.8. and manually checked for errors. BLASTn searches were conducted on the NCBI (https://blast.ncbi.nlm.nih.gov) database using the assembled sequences (Table 1) (Altschul *et al.*, 1990). To infer species identities, an additional dataset was created using the sequences with the highest sequence similarity downloaded from GenBank and used in the phylogenetic analyses. The outgroups for the dataset were *Peziza oliviae* (OSC 148300) and *Peziza Nordica* (FH 00304781). The dataset was aligned using MAFFT (https://mafft.cbrc.jp/alignment/server/) version 7 (Katoh and Standley, 2013). All the reference taxa used in phylogenetic analyses are listed in Table 1. Maximum Likelihood (ML) phylogenetic and Bayesian inference trees were inferred using the aligned ITS dataset.

Maximum Likelihood searches for the best-scoring tree were done with RaxMLHPC v.8 (Stamatakis, 2014), using the fixed General Time Reversal (GTR) nucleotide model and bootstrap support (BS) was estimated with 1000 pseudoreplicates. Bayesian inference was performed with Mr. Bayes v3.2 (Huelsenbeck and Ronquist, 2001) using the best nucleotide substitution model (i.e. transition model with gamma and invariable sites; TIM+G+I), determined using jModelTest. The analysis was run for six million generations, with four runs consisting of four chains heated at the default temperature, and tree sampling every 100 generations. A 25% burn-in was used to summarise a consensus from 45, 000 trees and to determine posterior probability (PP) values at the nodes. Figtreev1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) was used to view Bayesian trees, while ML trees were viewed with MEGA 7 (Kumar *et al.*, 2016).

3. Results

3.1. Fungal isolates and their culture morphology

A total of 69 isolates were obtained (48 from asymptomatic branches and 21 from branches with dieback). They differed widely in colony morphology, with some having black-grey aerial mycelia, variously coloured aerial mycelia, with white aerial mycelia and mycelia growing inside the media (Figure 2).

3.2. DNA sequence similarity with sequences on GenBank and phylogenetic analyses

ITS sequences in the forward direction were obtained for all 69 isolates. These sequences were used for preliminary identification of the isolates using BLASTn searches against sequences on GenBank. Sequences that had high similarity with the isolates from this study were used as reference sequences for further analyses (Table 1). The results revealed that the isolates belonged to species in the classes, *Dothidiomycetes* and *Sordariomycetes* (Table 2, Table 3). The fungi were distributed across six orders namely, *Botryosphaeriales* (1 family), *Capnodiales* (1 family), *Diaporthales* (2 families), *Hypocreales* (1 family), *Pleosporales* (3 families) and *Xylariales* (2 families) (Table 3). The most prevalent families were the *Botryosphaeriaceae* that included the genera *Dothiorella* and *Lasiodiplodia*, followed by the *Pleosporaceae* represented by a single genus *Alternaria*. The most abundant genera were *Dothiorella* (25 isolates), *Lasiodiplodia* (9 isolates) and *Alternaria* (6 isolates) (Figure 3).

The selection of representative isolates for phylogenetic analysis was done by constructing a Neighbor-joining tree using the one-direction sequence dataset of the isolates in this study. The sequences from GenBank which had a high sequence similarity identity (95-100%) to the isolates in this study are shown in Table 2. A total of 48 isolates were selected and sequenced for the reverse direction (using primer ITS-4), and were then used in the final phylogenetic analysis of the ITS dataset (Figure 4).

The final ITS sequence dataset consisted of 142 sequences, of which 48 sequences represented isolates in this study. The remaining 94 sequences were obtained from GenBank and belonged to different species within the nine families that were closely related to the isolates in this study (Table 1). In this dataset, 70 of the sequences were from *Botryosphaeriaceae*, 15 sequences from *Sporocadaceae*, 10 sequences from *Didymellaceae*, 10 sequences from *Pleosporaceae*, nine sequences from *Nectriaceae*, seven sequences from *Diaporthaceae*, six sequences from *Cladosporiaceae*, five sequences from *Valsaceae*, five sequences from *Didymosphaeriaceae* and three sequences from *Diatrypaceae* (Table 1). The ITS dataset was rooted to *Peziza oliviae* (OSC 148300) and *P. nordica* (FH 00304781). The final ITS sequence alignment were 570 bp in length.

Both the ML tree and the Bayesian inference tree obtained for the ITS dataset had similar topologies and grouped the isolates into 10 clades, representative of *Diatrypaceae* (1 isolate), *Nectriaceae* (2 isolates), *Valsaceae* (2 isolates), *Didymosphaeriaceae* (2 isolates), *Cladosporiaceae* (2 isolates), *Pleosporaceae* (3 isolates), *Diaporthaceae* (3 isolates), *Didymellaceae* (3 isolates), *Sporocadaceae* (6 isolates) and *Botryosphaeriaceae* (23 isolates) (Figure 4).

All isolates obtained in this study resided within 17 genera within various families (see Figure 4). In sections 3.2.1 to 3.2.6 below, the results obtained for the clades in each of the six orders are described. For this purpose, their phylogenetic placement and statistical support, as well as their relatedness to known taxa were emphasised.

3.2.1. *Botryosphaeriales*

a) *Botryosphaeriaceae*

In the phylogenetic analyses of the ITS dataset, the *Botryosphaeriaceae* was represented by a broad and highly supported monophyletic group consisting of five clades (Groups A- E) (Figure 4). Group A represents the genus *Lasiodiplodia,* with seven isolates obtained from asymptomatic branches only. Isolates BMA12.2, BMA8.5 and BMA8.12 grouped closely with *L. pseudotheobromae, L. theobromae* and *L. jatrophicola* [bootstrap (BS) = 89%, posterior probability (PP) = 0.99], BMA11.5 grouped in a clade with *Lasiosiplodia margaritacea* with 71% bootstrap support (Figure

4), while BMA3.4, BMA10.1, and BMA10.9 grouped with *L. crassispora* (= *L. pyriformis*) with high branch bootstrap support (BS = 98%, PP = 1.00). The isolates also grouped closely with other *Lasiodiplodia* species and thus the identification of the isolates could only be made at the genus level.

Group B isolates (BMA5.5, BMA5.8 and BMA5.9) grouped closely with *Alanphillipsia* species (BS = 70%; PP = 0.98). The isolates were obtained from asymptomatic branches only. Although the BLASTn search results show that the isolates BMA5.5, BMA5.8 and BMA5.9 have a 96% similarity with *A. aloeigena* (Table 2), the isolates are regarded as *Alanphillipsia* spp. based on the phylogenetic analyses.

Isolate BMD11.3 in Group C was placed sister to *Oblongocollomyces variabilis.* The placement was not supported by statistical analyses in the ML or Bayesian trees. The BLASTn results showed that the isolate has 98% sequence similarity with an unidentified *Botryosphaeriaceae* sp. This isolate was obtained from branches with dieback.

Isolate BMD13.3 in Group D, obtained from a dieback branch had a 98% sequence similarity with strains of *B. dothidea on* GenBank (Table 2). The ITS phylogenetic tree, however, grouped it with six other *Botryosphaeria* species (BS = 98% and PP = 1.0) that formed a polytomy (Figure 4). The isolate could therefore only be identified to genus level as a species of *Botryosphaeria*.

Isolates residing within the genus *Dothiorella* (Group E) were frequently isolated from both asymptomatic and symptomatic branches of *B. discolor*. BLASTn search results (Table 2) confirmed membership to *Dothiorella*, and that the isolates have high sequence similarity to *D. longicollis* (99%), *D. mangifericola* (= *D. rosulata*) (99-100%) and *D. dulcispinae* (= *D. oblonga*) (99%), respectively (Table 2). Phylogenetic analyses grouped isolate BMA2.2 with *D. brevicollis* [BS = 67%, (PP values < 0.95 are not shown here or elsewhere in the text)], isolates BMD8.1, BMA11.10, BMA11.2, BMA7.1, BMA10.5, BMA11.6, BMA11.9, BMD10.5 (BS = 80% only) grouped with *D. diospyricola.* Two isolates BMA9.1 and BMA8.4 formed a clade with five other *Dothiorella* spp. (BS = 89%, PP = 0.97), but were placed closest to *D. mangifericola* (= *D. rosulata*) with support of 71% BS and PP of 0.93 (Figure 4). Isolate BMA13.3 grouped separately from the other *Botryosphaeriaceae* genera (BS = 97%). Due to

the uncertainty of the identity of the isolates they were only identified to the genus level (Figure 4).

3.2.2. *Pleosporales*

a) *Didymosphaeriaceae*

Three isolates (BMA1.3, BMA2.6, and BMA5.4) in Group F formed a monophyletic group sister to a clade comprising of sequences of *Paracamarosporium fagi* (BS = 100%, PP = 1.0) (Figure 4). Similar to the BLASTn results, which show that the isolates are also similar to *Pa. fagi* (97-98%) in Table 2. These isolates were only found on asymptomatic branches of *B. discolor*.

b) *Didymellaceae*

The *Didymellaceae* clade comprised two sister clades representative of two genera, *Didymella* and *Epicoccum* (BS = 77%, PP = 0.97) (Figure 4). The *Didymella* clade (Group G) included two isolates from branches with dieback (BMD1.1 and BMD5.1). The isolates grouped with *Di. keratinophila*, *Di. coffee-arabicae*, *Didymella* sp. 1 and *Di. glomerata* (BS = 75%, PP = 0.95). The BLASTn results showed that isolate BMD1.1 is most similar to *Di. glomerata,* while BMD5.1 is most similar to *Di. coffeae-arabicae* (Table 2). The *Epicoccum* clade (Group H) included one isolate (BMA5.2) from an asymptomatic branch, and grouped with *Epicoccum* sp. (BS = 95% only) and supported the BLASTn results that the DNA sequence is most similar to an *Epicoccum* sp. (Table 2).

c) *Pleosporaceae*

Three isolates in Group I resided within the genus *Alternaria*. BLASTn results showed the isolates in this group had high sequence similarity with sequences of *A. tenuissima* (BMA7.2), *Alternaria alternata* (BMA3.2), and *Alternaria porri* (BMD9.3) (Table 2). The clade included two isolates (BMA7.2 and BMA3.2) asymptomatic tissue and one isolate obtained from a branch with dieback (BMD9.3) (Figure 4). The isolates formed a monophyletic clade with *Al. mali, Al. tenuissima, Al. arborescenes*, *Al. brassicicola, Al. alternata,* and an undescribed *Alternaria* sp. The clade was well supported (BS = 100%; PP = 1.0).

3.2.3. *Capnodiales*

a) *Cladosporiaceae*

BLASTn results for isolates belonging to Group J (BMA5.6 and BMD3.4) had high sequence similarity to *Cladosporium cladosporioides* (100%) (Table 2) on GenBank. Phylogenetic analysis also placed the isolates together with *Cl. cladosporioides*, *Cl. angustisporum*, *Cl. oxysporum,* and an undescribed *Cladosporium* sp. with the strong support (BS = 100% and PP = 1.0) (Figure 4). The isolates and species in this group formed a polytomy and could therefore not be identified to species level. These isolates were collected from both asymptomatic and symptomatic branches.

3.2.4. *Diaporthales*

a) *Valsaceae*

Two isolates (BMD8.3 and BMD4.2) resided in Group K that also included sequences of *C. acaciae* and *Cytospora* spp. (BS = 94%, PP = 1.0) (Figure 4). BLASTn searches showed that two isolates have high sequence similarity to *C. acacia* and a *Cytospora* sp. (Table 2) with an identity percentage of 98-100. The *Cytospora* isolates were only obtained from branches with dieback and were regarded as *Cytospora* spp. Because the two isolates and the sequences formed two groups, it might be that they represent different species. Isolate BMD8.3 was closely related to *C. acacia,* while BMD4.2 was closely related to an undescribed *Cytospora* sp. and may belong to this species.

b) *Diaporthaceae*

Isolates BMD10.2, BMD2.2 and BMD2.1 formed a monophyletic group within a clade (Group L) with strong support (BS = 99%; PP = 1.0) and that included *Diaporthe anacardii* and *Diaporthe* spp. (Figure 4). The BLASTn results showed that the isolates have sequence similarities to *Diaporthe* spp. ranging between 97% and 98%. These isolates may represent a potentially new species within the genus *Diaporthe* (= *Phomopsis*). Furthermore, the sequence of the strain *Dia. anacardii* grouped separate from the isolates in this study and *Diaporthe* sequences, it may either be due to misidentification of the strain or the ITS had poor resolution for this sequence.

3.2.5. *Hypocreales*

a) *Nectriaceae*

The isolates grouping within *Nectriaceae* (Group M) were represented by two isolates (BMD13.1 and BMD7.2) from branches with dieback. The two isolates had high sequence similarity to an unidentified *Fusarium* sp. on GenBank (Table 2). The phylogenetic tree placed the two isolates within the genus *Fusarium* (Figure 4). Isolate BMD13.1 grouped with *F. equiseti* (BS = 99%, PP = 1.0). Isolate BMD7.2 grouped with *F. chlamydosporum* (BS = 99% and PP = 1.00). Based on the phylogenetic results, isolate BMD13.1 may thus belong to *F. equiseti* and isolate BMD7.2 may belong to *F. chlamydosporum*.

3.2.6. *Xylariales*

a) *Diatrypaceae*

Group N represented members of the *Diatrypaceae* and comprised one genus *Eutypella* from the family (Figure 4). Isolate BMD1.3 grouped with *Eutypella microtheca* (BS = 100%; PP = 1.0), reflecting the results from BLASTn searches (Table 2). Based on these results, this isolate was considered being *Eu. microtheca.*

b) *Sporocadaceae*

The *Sporocadaceae* clade (Groups O-Q) formed three sub-clades respectively, representing the genera, *Hymenopleella* (Group O), *Pestalotiopsis* (Group P) and *Neopestalotiopsis* (Group Q) (Figure 4). The BLASTn results showed that isolates in Groups O-Q have high sequence similarity with sequences of isolates belonging to *N. foedans, H. austroafricana*, and *P. bitilica* (Table 2). Isolate BMA2.1 grouped within Group O and was closely related to *H. austroafricana, H. hippophaes* and *H. hippophaeicola* (BS = 80%). Isolate BMD3.3 belonged to Group P and clustered with *Pe. neglecta, Pe. biciliata* and *Pe. microspora* (BS = 100%, PP = 1.0). Isolates BMA5.3, BMA5.1, BMA12.1 grouped within Group Q and clustered with sequences belonging to *Neopestalotiopsis* sp., *Pe. microspora* and *N. foedans* (BS = 100%, PP = 1.0). None of the isolates could be identified to species level based on the phylogenetic analyses of the ITS region.

4. Discussion

This study presents a first attempt to characterise the diversity of fungi associated with branches on *B. discolor* trees in Mapungubwe National Park. Using a phylogenetic approach based on sequences from the ITS region various taxa within the phylum Ascomycota were identified. These included five genera within *Botryosphaeriaceae,* three within *Sporocadaceae,* two within *Didymellaceae* and individual genera within the families *Cladosporiaceae, Valsaceae, Didymosphaeriaceae, Diaporthaceae, Diatrypaceae, Netriaceae* and *Pleosporaceae.* The genera *Dothiorella*, *Alternaria,* and *Lasiodiplodia* were frequently isolated from branches of *B. discolor*. The other genera identified included *Diaporthe, Cytospora, Nepestalotiopsis, Hymenopleella, Cladosporium, Fusarium, Paracamarosporium, Didymella, Pestalotiopsis, Eutypella, Botryosphaeria, Oblongocollomyces* and *Epicoccum.*

Ten fungal families obtained in this study fall within the phylum *Ascomycota*. Fungi in this phylum were also the focus of various studies that considered their association with various South African indigenous trees (Crous *et al.*, 2006; Damm *et al.*, 2007; Bensch *et al.*, 2012; Crous *et al.*, 2015; Cruywagen *et al.*, 2015; Jami *et al.*, 2017; Osorio *et al.*, 2017). Some of the prevalent orders isolated from several plant organs include the *Botryosphaeriales*, *Xylareales*, *Hypocreales* and *Pleosporales*, of which were also present in this study.

This study identified 17 genera from 10 families and contributes new knowledge of the fungal biodiversity of Mapungubwe National Park as a natural ecosystem. The results of this study illustrate that indigenous trees of South Africa are rich reservoirs for fungal biodiversity as suggested by Crous *et al.* (2006). Knowledge regarding the biodiversity of indigenous trees and plants is important as it facilitates the evaluation of whether fungi in an ecosystem are indigenous or alien to that ecosystem (Desprez-Loustau *et al.*, 2007). Moreover, fungi could be unique to a particular host tree, emphasising acquiring and documentation of such information. Wood (2017) considers that the knowledge of native fungi in the natural ecosystems of South Africa remains much less documented. The results of this study, therefore, provide additional information regarding the biodiversity of indigenous fungi in the natural ecosystems of South Africa. They also contribute to the National Park’s fungal biodiversity database associated with trees.

The identifications of the 48 isolates obtained in this study were based on ITS DNA sequence comparisons. This gene region is the most widely used marker for the barcoding of fungi, it is also considered to successfully distinguish the broadest range of fungi (Schoch *et al.*, 2012). Also, the calculations of the bootstrap (BS) and posterior probability (PP) values were useful tools to assess the confidence of the taxon groupings (Hillis and Bull, 1993; Erixon *et al.*, 2003). The higher the BS and PP values, the more reliable the phylogenetic clades are and, therefore, the relationship of the taxa (Hillis and Bull, 1993; Erixon *et al.*, 2003). Accordingly, it was therefore assumed that the relationships of the taxa obtained from *B. discolor* branches are validated, as they clustered together with their best-aligned reference sequences from the GenBank, with high BS and PP values.

In this study, the ITS region provided sufficient resolution to identify the collected fungi to the genus level. In this regard, the identified genera included, *Alanphillipsia*, *Alternaria, Botryosphaeria, Cladosporium, Cytospora, Diaporthe, Didymella, Dothiorella*, *Epicoccum, Eutypella, Fusarium, Hymenopleella, Lasiodiplodia*, *Nepestalotiopsis, Oblongocollomyces, Paracamarosporium* and *Pestalotiopsis*. The ITS sequences could not, however, be used to delineate the isolates to species level and as a result, they were clustering with multiple species in each clade. This can be resolved in future if reference sequences from gene regions which have been proven to delineate diverse fungi to species level are available for described taxa, such as those encoding translation elongation factor 1-a and beta-tubulin (Bensch *et al.*, 2012; Crous *et al.*, 2015; Chen *et al.*, 2017; Yang *et al.*, 2017; Sessa *et al.*, 2018; Liu *et al.*, 2019). Additionally, there is a possibility that the identified genera were not the only ones present, because some of the fungi found in the interior of plants are potentially unculturable and therefore did not grow on the MEA. As a result, the fungal diversity determined in this study from *B. discolor* is but a glimpse of the true fungal biodiversity within this tree species.

The *Botryosphaeriaceae* were the most dominant group with five genera isolated from asymptomatic and branches with dieback of *B. discolor*. The results of this study agree with the conclusion that many of the members of *Botryosphaeriaceae* persist as latent pathogens within their hosts, with no visual disease symptoms (Slippers and Wingfield, 2007; Jami *et al.*, 2013; Mehl *et al.*, 2017). The genus *Dothiorella* was the most frequently isolated and the species were obtained from asymptomatic and

symptomatic branches. Following in abundance was the genus *Lasiodiplodia,* and was only obtained from asymptomatic branches. It is known that the genus encompasses pathogens of various indigenous trees (Pavlic *et al.*, 2007; Sakalidis *et al.*, 2011; Jami *et al.*, 2013, 2014; 2017). The other genera with only one to two isolates included *Botryosphaeria* and *Oblongocollomyces* sp., which were found as pathogens from branches with dieback. *Alanphilipsia* species were only found as endophytes in asymptomatic branches. The genera in the family *Botryosphaeriaceae* have a wide plant host range including agricultural cops, plantation forest trees, and native and non-native trees worldwide (Slippers and Wingfield, 2007). The fungi are widely known to cause diseases such as cankers, branch-dieback, gummosis, bark discolorations, black rots, fruit rots on the trees (Slippers and Wingfield, 2007; Zlatković *et al.*, 2016; Jami *et al.*, 2017).

The *Botryosphaeriaceae* isolates included in this study grouped in many instances closely to multiple genera and had very low statistical support at the nodes on the phylogenetic trees. The ITS gene region used in this study could therefore not delineate the isolates to species level with strong confidence. Phillips *et al.* (2013), recommended that at least two loci (ITS and *TEF-1α*) should be considered in delineating species within the *Botryosphaericeae.* Following this recommendation. Better resolution and robust placement of the isolates will be obtained in future studies on these isolates.

The *Xylariales* were represented by four genera residing within two families, namely, three genera within: *Sporocadaceae Hymenopleella, Neopestalotiopsis* and *Pestalotiopsis* and the genus *Eutypella* within the *Diatrypaceae*. *Sporocadaceae* includes endophytic, plant pathogenic, and/ or saprobic fungi associated with a wide range of hosts (Liu *et al.*, 2019). Twelve isolates in this study resided within *Pestalotiopsis*, *Neopestalotiopsis,* and *Hymenopleella*. Pestalotiopsis-like fungi (*Pestalotiopsis* and *Neopestalotiopsis*) are considered as important phytopathogens and are reported to cause canker lesions, shoot dieback, leaf spots, needle blight, tip blight, grey blight, scabby canker, severe chlorosis, fruit rots, and various post-harvest diseases (Jayawardena *et al.*, 2015; Maharachchikumbura *et al.*, 2016). The *Pestalotiopsis* isolate collected in this study was found on branches with dieback only, while *Hymenopleella* isolates were found only on asymptomatic branches. Karani *et al.* (2022) isolated *Neopestalotiopsis* spp. from cankers and dieback branches of *B.*

discolor in Kenya. In this study, *Neopestalotiopsis* isolates were only from asymptomatic branches.

The genus *Hymenopleella* was recently revised by Jaklitsch *et al.* (2016), but is still under-studied and poorly understood. Therefore, there is not much information regarding the association of the species in the genus and their hosts. In this study the isolates belonging to this genus were obtained from asymptomatic branches, thus suggesting that they may be endophytes.

Only one isolate obtained from branches with dieback clustered with *Eutypella* (*Diatrypaceae*) species. The occurrence of the isolate on diseased branches was not a surprise as the genus is well-known to be associated with diseases of various hosts (Moyo *et al*., 2018a; Moyo *et al*., 2018b). In South Africa, it is associated with cankers on commercialised grapevines.

The order *Pleosporales* was represented by four genera belonging to three families, two genera within *Didymellaceae: Didymella* and *Epicoccum* and individual genera within *Pleosporaceae* (*Alternaria*) and *Didymosphaeriaceae* (*Paracamarosporium*). *Didymellaceae* is regarded as one of the most species-rich families within the kingdom fungi, the species are generally found in various ecosystems (Chen *et al*., 2017), occurring as endophytes and pathogens of several plants, including plants within the *Asteraceae*, *Fabaceae*, *Poaceae, Ranunculaceae*, *Rosaceae* and *Solanaceae* (Chen *et al*., 2017; Del Frari *et al*., 2019). Isolates grouping with *Didymella* species were obtained from branches with dieback whereas the isolate grouping with *Epicoccum* species was obtained from asymptomatic branches. Both genera are known to encompass species associated with various plants and may be considered as secondary weak pathogens. Two isolates resided within *Paracamarosporium*, a genus within the Family *Didymosphaeriaceae*. The family covers fungi with a cosmopolitan distribution, they are found inside plants and in the soil and are regarded as saprobes, endophytes, and/ or pathogens of various hosts worldwide (Ariyawansa *et al*., 2014; Gonçalves *et al*., 2019). The isolates in this study were obtained from asymptomatic branches and based on the phylogenetic tree, they are closely related to *P. fagi* which was first described from *Fagus sylvatica* in Germany (Crous *et al*., 2015).

Alternaria species are commonly isolated and are well–known from a wide range of hosts worldwide, including on *B. discolor* (Karani *et al*., 2022) and *Ziziphus* species

within the *Rhamnaceae* (EL-Nagerabi *et al.*, 2013). Their wide host range could be because they are generally found everywhere and they can grow under different temperatures (Lourenço Jr *et al.*, 2009). Isolates in this study grouping with the *Alternaria* species were obtained from asymptomatic and dieback branches of *B. discolor*.

Species in *Diaporthales* are responsible for various diseases of trees that result in severe damages (Marin-Felix *et al.*, 2017). Isolates in this study resided within two genera namely, *Cytospora* and *Diaporthe* (= *Phomopsis*) (Rossman *et al.*, 2015), and the isolates were all obtained from branches with dieback. This was not surprising as the two genera encompass well-known fungal pathogens that can occur on woody plants and economically important plants, causing diseases such as a severe branch or trunk disease, root and fruit rots, dieback, stem cankers, leaf spots, leaf and pod blights and seed decay on various hosts (Farr *et al.*, 1989; Adams *et al.*, 2006; Gomes *et al.*, 2013; Rossman *et al.*, 2015; Marin-Felix *et al.*, 2017; Guarnaccia *et al.*, 2018). Some examples of their host species include *Vachellia* sp., *Eucalyptus* sp., *Malus* sp., *Pinus* sp., *Populus* sp., *Prunus* sp. and *Vitis* sp. (Adams *et al.*, 2006).

Two isolates obtained from branches with dieback were closely related to *F. equiseti* and *F. chlamydosporum* which are also associated with dieback (Trabelsi *et al.*, 2017; Pandey *et al.*, 2019). These two species were also isolated from *B. discolor* trees in Kenya, and were found to cause cankers and dieback (Karani *et al.*, 2022). The genus *Fusarium* (*Nectriaceae*) includes many important plant pathogens, which have been included as part of the top 10 most important plant pathogenic fungal genera globally based on their perceived scientific and economic importance (Dean *et al.*, 2012).

The genus *Cladosporium* consists of species with a wide host range and they are well known throughout the world. They associate with their hosts as plant pathogens, causing diseases such as leaf spots and also as common endophytes of trees (Schubert and Braun, 2005; Paul and Yu, 2008; Bensch *et al.*, 2012). In this study, the species were obtained from both diseased and asymptomatic branches.

Five genera were solely isolated from asymptomatic branches, however, they occurred in very low numbers, ranging from one isolate to four isolates. These genera included *Alanphillipsia*, *Neopestalotiopsis*, *Hymenopleella*, *Paracamarosporium* and *Epicoccum*. The species that were identified from dieback branches included

Botryosphaeria sp., *Oblongocollomyces* sp., *Diaporthe* spp., *Cytospora* spp., *Fusarium* spp., *Eutypella* sp., *Pestalotiopsis* sp. and *Didymella* spp., which were also isolated in low numbers ranging from one isolate to four isolates per genus. Greater diversity of these fungi was observed on the branches with dieback, than in asymptomatic branches. Eight species were obtained as pathogens while five species were isolated as endophytes. The infrequent isolations of the fungi in their low numbers may suggest that they are not major contributors to the branch-dieback symptoms observed from *B. discolor* trees in the National Park. The species of *Diaporthe*, *Fusarium* and *Neopestalotiopsis* were previously isolated from cankers and dieback branches of *B. discolor* (Karani *et al*., 2022). However, results from pathogenicity trial results were not disclosed by the authors, inference about their virulence to *B. discolor* can therefore not be made. Nevertheless, the identification of these in the current study contribute greatly to our knowledge of the fungi associated with the trees, while giving us an idea of the fungi associated with trees in Mapungubwe National Park.

Genera such as *Alanphillipsia*, *Neopestalotiopsis*, *Hymenopleella*, *Paracamarosporium*, *Lasiodiplodia* and *Epicoccum* obtained from asymptomatic branches may be regarded as endophytes of *B. discolor* trees following the endophyte definition by Saikkonen *et al.* (1998) which states that "endophytes are fungi that spend all or at least a significant part of their life cycles colonising plant parts without causing any visual disease symptoms" (Saikkonen *et al.*, 1998). While the endophyte definition by Petrini (1991), which states that "endophytes are generally defined to include pathogens during their latency or quiescent stage, that is when visible symptoms are not yet produced" covers *Dothiorella*, *Alternaria* and *Cladosporium* spp. which were obtained from both asymptomatic and symptomatic branches. Other genera such as *Botryosphaeria* sp., *Oblongocollomyces* sp., *Diaporthe* spp., *Cytospora* spp., *Fusarium* spp., *Eutypella* sp., *Pestalotiopsis* sp. and *Didymella* spp., which were only obtained from branches with dieback may be regarded as pathogens of *B. discolor*, and may have been directly or indirectly involved in the dieback symptoms of *B. discolor* trees. Although these observed dieback symptoms were from branches infested by these fungi, precautions must be taken before concluding that they are the causal agents of disease. This is because similar symptoms can also be triggered by other abiotic stress factors such as drought stress observed by Davis *et*

al. (2002) on *Ceanothus crassifolius.* Future work should consider pathogenicity trials to confirm them being also pathogens of *B. discolor*.

More isolates were found from asymptomatic branches than from symptomatic branches. There could be several factors at play, for example, the number of the collected samples may have affected the isolation frequency and species richness of the ascomycetous fungi recorded in this study. Only twenty five branches (13 = asymptomatic, 12 = symptomatic) were obtained, this was because there were limited *B. discolor* trees in Mapungubwe National Park and also, there were difficulties with collecting branches as they were too high to reach. Due to the several encountered limitations with sampling, one can conduct a seasonal survey to check if there would be a difference in the species diversity over different seasons.

The total number of fungal isolates obtained from *B. discolor* branches may have been influenced by the tree and branch age, and the sampling season. As highlighted by Brglez *et al.* (2020), tree age and sampling season impact isolation frequency. According to Kowalski and Kehr (1992), the degree of fungal colonisation and distribution of species could also depend on the plant-host community and the branch diameter. Both Kowalski *et al.* (2016) and Brglez *et al.* (2020) agreed that the methods used for isolations do not yield a complete picture of the real number and frequency of species, and this might be the case in this study also. The total number of fungi detected can be influenced by abiotic, biotic, and experimental factors, such as the plant species, type and phase disposition of the plant organ, climatic and edaphic conditions, the isolation procedure followed, and the total number and size of samples (Sieber, 2007). Furthermore, some slow-growing fungi can be outgrown by the fast-growing fungi. As a result, the fungi reported in this study are those that grew faster than others during fungal isolations.

In this study, isolates that may be potentially new to science belonged to the families *Botryosphaeriaceae*, *Valsaceae, Diaporthaceae* and *Didymosphaeriaceae*. This is in line with the consistent increase and addition of newly discovered fungi from studies on indigenous trees. Although the majority of the fungi obtained in this study were also commonly isolated from trees as endophytes and/or pathogens of trees in earlier studies (Adams *et al.*, 2006; Hakizimana *et al.*, 2011; Sakalidis *et al.*, 2011; EL-Nagerabi *et al.*, 2013; Cruywagen *et al.*, 2015) the identification of them in this study

contributes to a better understanding of host diversity. Future studies should focus on identifying strains to species level and to then describe the potentially new species obtained in this study.

5. Conclusions

This study reports Ascomyceteous fungi within 17 genera from 13 *B. discolor* trees from the Mapungubwe National Park. All the fungi are new records for the plant trees and also for the. The results of this study set the foundation for our understanding of the fungal diversity in *B. discolor* and also of the Mapungubwe National Park. Furthermore, the results suggest that the fungi associated with branch-dieback of *B. discolor* may represent an assemblage of fungal pathogens and other factors. Access to these findings makes it possible to assist the government in developing initiatives and legislation to safeguard South African woody resources against opportunistic pathogens in the natural ecosystems, such as Mapungubwe National Park. These trees are few in Mapungubwe and might be threatened by the fungi identified in this study, that are possibly pathogens. A concerted effort can be put into place to plant more trees in the area and protect them. The indigenous trees in the ecosystems can effectively be protected when the threats are identified and research is done to understand the diseases caused by pathogens. The findings can be added to the database of the National Parks regarding fungal tree pathogens.

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7. Tables

Table 1. Reference fungal species and strains and their ITS DNA sequence GenBank accession numbers.

Identity	Strain number	Host	Location	GenBank accession number
				ITS
Alanphillipsia aloes	CPC 21298	*Aloe dichotoma*	Western Cape	NR_137122
A. aloeicola	CPC 23674	*Aloe* sp.	Western Cape	NR_137926
A. aloeigena	CPC 21286	*A. melanacantha*	Namakwaland	NR_137121
A. aloetica	CPC 21109	*Aloe* sp.	Eastern Cape	NR_137123
A. euphorbiae	CPC 21628	*Euphorbia* sp.	Western Cape	NR_137124
Alternaria sp.	FP-027-D2	*Fucus* sp. (seaweed)	Netherlands	MH102093
Al. tenuissima	Claq-8	*Coreopsis lanceolata*	China	MK575837
Al. tenuissima	Claq-7	*Co. lanceolata*	China	MK575836
Al. alternata	NH5	Unknown	Egypt	MN518330
Al. mali	AS-S-4	Unknown	China	MK632002
Al. brassicicola	MVR1	*Terminalia arjuna*	India	MK158222
Al. tenuissima	Sp 12	*Euryale ferox*	India	MH938072
Al. arborescens	ALT-14	*Citrus reticulata*	Pakistan	MH879771
Botryosphaeria agaves	MFLUCC 11-0125	*Agave* sp.	Chiang Rai	NR_111792
B. corticis	CBS 119047	*V. corymbosum*	USA	DQ299245
B. dothidea	CMW 8000	*Prunus* sp.	Switzerland	AY236949

Table 1. (Continued)

Reference fungal species and strains and their ITS DNA sequence GenBank accession numbers.

Identity	Strain number	Host	Location	GenBank accession number
				ITS
B. dothidea	CBS 100564	*Paeonia* sp.	Netherlands	KX464085
B. dothidea	RI 1	*Pyrus* sp.	China	MK014151
B. ramosum	CBS 122069	*E. camaldulensis*	Perth	EU144055
Botryosphaeriaceae sp.	CMW 29962	Unknown		HM176528
Cladosporium oxysporum	DAFE_SP16-8	*Lupinus* sp.	Italy	MK560167
Cl. angustisporum	CLAD 64	Unknown	South Africa	MK271396
Cladosporium sp.	GPS3-1	Unknown	South Korea	MN518420
Cl. cladosporioides	RM 239	*Brachiaria*	Uganda	MG664763
Cladosporium sp.	R97206_ITS	Unknown	USA	MK268136
Cytospora sp.	CR 200	*Conocarpus erectus*	Costa Rica	DQ996039
C. acaciae	CBS 468.69	Unknown	Spain	MH859354
Cytospora sp.	JSP 02 B 3.1	*Atta capiguara*	Brazil	KR093918
Diaporthe anacardii	CBS 720.97	*Anacardium occidentale*	Eastern Africa	NR_111841
Diaporthe sp.	CML 1930	*Tapirira guianensis*	Brazil	JN153072
Diaporthe sp.	CML 1316	*Coffea arabica*	Brazil	JN153056
Diaporthe sp.	CML 871	*Co. arabica*	Brazil	JN153054

Table 1. (Continued)

Reference fungal species and strains and their ITS DNA sequence GenBank accession numbers.

Identity	Strain number	Host	Location	GenBank accession number
				ITS
Didymella coffeae-arabicae	CBS 123380	Unknown	Ethiopia	MH863293
Di. keratinophila	UTHSC DI16-200	Unknown	USA	NR_158275
Di. keratinophila	NV-2016	Unknown		LT592938
Didymellaceae sp.	SL77_49a_C3	*Acer palmatum*	China	MN105581
Didymella sp.	MFLUCC 16-0489	*Eriobotrya japonica*	China	MG967669
Di. glomerata	NH 1152	Unknown	Japan:Osaka	LC375366
Dothiorella acaciacola	CBS 141295	*Acacia mearnsii*	France	KX228269
D. brevicollis	CMW 36463	*V. karroo*	Gauteng	NR 111703
D. capriamissi	CBS 121763	*V. erioloba*	Gauteng	EU101323.
D. casuarini	CMW 4855	*Casuarini* sp.	Australia	DQ846773
D. citricola	ICMP 16828	Unknown	New Zealand	EU673323
D. dulcispinae	CMW 36460	*V. karroo*	South Africa	NR 111702
D. diospyricola	CBS 145972	*Diospyros mespiliformis*	South Africa	MT587398
D. iberica	CBS 115041	*Quercus ilex*	Spain	NR 111165
D. longicollis	CBS 122068	*L. cunninghamii*	South Africa	NR 136999

Table 1. (Continued)

Reference fungal species and strains and their ITS DNA sequence GenBank accession numbers.

Identity	Strain number	Host	Location	GenBank accession number
				ITS
D. longicollis	CBS 122066	*Terminalia* sp.	Australia	EU144052
D. oblonga	CBS 121765	*A. mellifera*	South Africa	NR 137689
D. oblonga	BT-RVCE02	Unknown	India	MK959599
D. omnivora	M 38	*Vitis vinifera*	Hungary	KY672851
D. plurivora	IRAN 1557C	Unknown	Iran	KC898225
D. rosulata	CBS 121760	*V. karroo*	Namibia	NR 136991
D. nigra	CBS 121783	*A. mearnsii*	South Africa	EU101333
D. striata	ICMP 16819	*C. sinensis*	New Zealand	EU673320
D. thailandica	MFLUCC 11-0438	*Bambusa* sp.	Thailandica	NR 111794
D. tectonae	MFLUCC 12-0382	*Tectona grandis*	Thailand	KM396899
D. uruguayensis	UY 672	*Eucalyptus*	USA	EU080923
D. viticola	CBS 117009	*V. vinifera*	Spain	NR 111186
D. vinea-gemmae	B116-3	*V. vinifera*	Australia	KJ573644
D. westrale	WA10NO 01	*V. vinifera*	Australia	HM009376
Epicoccum sp.	LGMF 1628	*Stryphnodendron adstringens*	Brazil	MG976431

Table 1. (Continued)

Reference fungal species and strains and their ITS DNA sequence GenBank accession numbers.

Identity	Strain number	Host	Location	GenBank accession number
				ITS
E. pneumoniae	UTHSC DI16-338	Unknown	Spain	LT592959
Epicoccum sp.	DS953	*Andropogon gerardi*	USA	MK809036
Eutypella microtheca	CBS 128337	Unknown	Australia	MH864886
Eu. microtheca	STEU_8199	Unknown	South Africa	MF359643
Fusarium sp.	BAB-5082	Leaves	India	KT186135
F. equiseti	Anna6	*Solanum lycopersicum*	iraq	MN498032
F. equiseti	CHTAM 35	*Taxus globosa*	Mexico	JF773657
Fusarium sp.	MRC 35	Unknown	USA	MH582472
F. chlamydosporum	CA2I9F1	*Chrysomelidae* sp.	brazil	KX421422
F. chlamydosporum	AY 998	Unknown	Namibia	MG250447
Fusarium sp.	BAB-5082	Leaves	India	KT186135
Hymenopleella hippophaeicola	CBS 140410	*Hippophae rhamnoides*	Austria	NR_154078
H. hippophaes	CBS 320.71	Unknown	France	MH860144
H. austroafricana	CBS 144027	*Combretum hereroense*	Zambia	MH554119

Table 1. (Continued)

Reference fungal species and strains and their ITS DNA sequence GenBank accession numbers.

Identity	Strain number	Host	Location	GenBank accession number
				ITS
Lasiodiplodia citricola	IRAN 1522C	*Citrus* sp.	Iran	GU945354
L. crassispora	CMM 0283	*Vitis vinifera*	Brazil	KJ450853
L. gonubiensis	CMW 14077	*Syzygium cordatum*	South Africa	AY639595
L. gravistriata	CMM 4564	*Anacardium humile*	Brazil	KT250949
L. hormozganensis	IRAN 1500C	*Olea* sp.	IRAN	GU945355
L. macrospora	CMM 3833	*Jatropha curcas*	Brazil	NR 147349
L. mahajangana	CERC 1960	*Pistachio*	China	KP217058
L. mahajangana	BL 104	*Cytisus scoparius*	Tunisia	KJ638317
L. margaritacea	CBS 122519	*Adansonia gibbosa*	Australia	EU144050
L. margaritacea	CR-12	*Catharanthus roseus*	India	MH790209
L. mahajangana	CMW 27820	*Terminalia catappa*	Madagascar	FJ900597
L. theobromae	MCR-1	*Morinda citrifolia*	India	MN443736
L. theobromae	CBS 164.96	Fruit trees along coral reef coast	Papua New Guinea	NR 111174
L. theobromae	MCR-1	*Morinda citrifolia*	India	MN443736
L. pontae	IBL 12	Tropical fruit trees	Brazil	KT151794

Table 1. (Continued)

Reference fungal species and strains and their ITS DNA sequence GenBank accession numbers.

Identity	Strain number	Host	Location	GenBank accession number
				ITS
L. pseudotheobromae	CBS 129752	*A. mangium*	Venezuela	MH865368
L. pseudotheobromae	CBS 447.62	*Citrus aurantium*	Suriname	EF622081
L. pseudotheobromae	CBS 116459	*Gmelina arborea*	Costa Rica	NR_111264
L. pyriformis	CBS 121770	*Acacia mellifera*	Namibia	NR_136993
L. rubropurpurea	WAC 12535	*Eucalyptus grandis*	Australia	DQ103553
L. subglobosa	CMM 3872	*Jatropha curcas*	Brazil	KF234558
Neopestalotiopsis sp.	HCH-66	*Heliconia* sp.	Mexico	MK256884
Neopestalotiopsis sp.	HCH-37	*Heliconia* sp.	Mexico	MK256882
Oblongocollomyces variabilis	CBS 121774	*V. karroo*	Namibia	NR_136994
O. variabilis	CBS 121776	*Acacia mellifera*	South Africa	EU101326
Paracamarosporium fagi	CPC 31037	*Elaeagnus rhamnoides*	Germany	KY929154
Pa. fagi	CPC 24892	*Fagus sylvatica*	Netherlands	KR611887
Pe. biciliata	CBS 236.38	Grapevine wood	Italy	MH855953
Neopestalotiopsis foedans	63	*Nectandra lineatifolia*	Ecuador	MN421910
Pestalotiopsis microspora	BQ	Grapevine wood	USA	KR909210
Pe. microspora	31	*Phylloicus fenestratus*	Brazil	MK120574

Table 1. (Continued)

Reference fungal species and strains and their ITS DNA sequence GenBank accession numbers.

Identity	Culture code	Host	Location	GenBank accession number
				ITS
Peziza oliviae	OSC 148300	Unknown	USA	NR_148069
Pe. nordica	FH 00304781	Unknown	Central Norway	NR_148104
Phaeobotryon negundinis	MFLUCC 15-0436	Unknown	Russia:Rostov region	NR_155669
Ph. rhois	CFCC 89662	*Rhus typhina*	China	KM030584
Pseudofusicoccum adansoniae	WAC 12689	*Mangifera indica*	Australia	EF585534
Ps. kimberleyense	CBS 122061	*Ficus opposita*	Australia	EU144059
Ps. stromaticum	CBS 117448	Unknown	South Africa	KF766223
Sakireeta madreeya	CBS 532.76	Undetermined grass	India: Madras	KM108376
Sphaeropsis citrigena	ICMP 16812	*Citrus sinensis*	New Zealand	NR_119697
S. eucalypticola	MFLUCC 13-0701	*Tectona grandis*	Thailand	KM396907
S. porosa	STE-U 5046	Grapevines	South Africa	AY343378

Table. 2 BLASTn results of the fungal isolates obtained from branches of *Berchemia discolor* used for DNA sequencing

Isolate no	CMW no.	Closest NCBI match	GenBank accession no.	Reference code no.	Query cover/ ID %
BMA5_5	**55624**	*Alanphilipsia aloeigena*	NR_137121	CPC 21286	**100/96**
BMA5_9	**55589**	*A. aloeigena*	NR_137121	CPC 21286	**100/95**
BMA7_2	**55643**	*Alternaria alternata*	MN518330	NH5	**100/100**
BMD9_3	**55642**	*Al. alternata*	MN518330	NH5	**100/100**
BMA3_2	**55613**	*Al. alternata*	MN518330	NH5	**100/100**
BMD11_3	**55597**	*Botryosphaeriaceae* sp.	HM176528	CMW 29962	**100/99**
BMD13_3	**55916**	*Botryosphaeria dothidea*	MK014151	RI1	**100/99**
BMA5_6	**55635**	*Cladosporium* sp.	MN518420	GPS3-1	**99/100**
BMD3_4	**55634**	*Cladosporium* sp.	MN518420	GPS3-1	**100/100**
BMD4_2	**55636**	*Cytospora* sp.	DQ996039	CR200	**98/100**
BMD8_3	**55605**	*C. acaciae*	DQ996039	CR200	**100/98**
BMD1_1	**55917**	*Didymella glomerata*	LC375366	NH1152	**100/100**
BMD5_1	**55633**	*Didymellaceae* sp.	MN105581	SL77_49a_C3	**100/100**
BMA11_9	**55580**	*Dothiorella longicollis*	MH863172	CBS 122068	**100/99**
BMD10_5	**55616**	*D. longicollis*	MH863172	CBS 122068	**100/99**
BMA7_1	**55578**	*D. longicollis*	MH863172	CBS 122068	**100/99**
BMA11_2	**55579**	*D. longicollis*	MH863172	CBS 122068	**100/99**
BMD8_1	**55575**	*D. longicollis*	MH863172	CBS 122068	**100/99**
BMA11_6	**55576**	*D. longicollis*	MH863172	CBS 122068	**100/99**

Table. 2 (Continued)

BLASTn results of the fungal isolates obtained from branches of *Berchemia discolor* used for DNA sequencing

Isolate no	CMW no.	Closest NCBI match	GenBank accession no.	Reference code no.	Query cover/ ID %
BMA10_5	**57852**	*D. longicollis*	MH863172	CBS 122068	**100/99**
BMA8_4	**55583**	*D. mangifericola*	KC898221	IRAN1584C	**99/99**
BMA9_1	**55582**	*D. mangifericola*	KC898221	IRAN1584C	**100/100**
BMA13_3	**55587**	*D. oblonga*	MK959599	BT-RVCE02	**100/99**
BMA5_2	**55612**	*Epicoccum* sp.	MK809036	DS953	**100/100**
BMD1_3	**55609**	*Eutypella microtheca*	MF359643	STEU_8199	**100/100**
BMD7_2	**55608**	*Fusarium* sp.	MH582472	MRC 35	**99/100**
BMA13_1	**55607**	*Fusarium* sp.	MN498032	Anna6	**100/100**
BMA2_1	**55639**	*Hymenopleella austroafricana*	MH554119	CBS 144027	**99/97**
BMA12_2	**55601**	*Lasiodiplodia theobromae*	MN443736	MCR-1	**100/100**
BMA8_5	**55600**	*L. theobromae*	MN443737	MCR-2	**100/99**
BMA11_5	**55629**	*L. margaritacea*	MH790209	CR-12	**100/100**
BMA10_1	**55572**	*L. crassispora*	KJ450853	CMM 0283	**99/99**
BMA3_4	**55573**	*L. crassispora*	KJ450853	CMM 0283	**100/100**
BMA10_9	**55604**	*L. crassispora*	KJ450853	CMM 0283	**100/100**
BMA5_3		*Neopestalotiopsis foedans*	MN421910	63	**99/100**
BMA5_1	**55611**	*N. foedans*	MN421910	63	**100/100**

Table. 2 (Continued)

BLASTn results of the fungal isolates obtained from branches of *Berchemia discolor* used for DNA sequencing

Isolate no	CMW no.	Closest NCBI match	GenBank accession no.	Reference code no.	Query cover/ ID %
BMA12_1		*N. foedans*	MN421910	63	**100/100**
BMA5_4	**55640**	*Paracamarosporium fagi*	KY929154	CPC 31037	**100/98**
BMA2_6	**55641**	*Pa. fagi*	KY929154	CPC 31037	**100/97**
BMD2_1	**55606**	*Diaporthe* sp.	JN153072	CML 1930	**100/98**
BMD2_2	**55638**	*Diaporthe* sp.	JN153072	CML 1930	**99/98**
BMD10_2	**55637**	*Diaporthe* sp.	JN153072	CML 1930	**100/97**
BMD3_3	**55610**	*Pestalotiopsis bitilica*	MH855953	CBS 236.38	**100/100**

Table 3. Taxonomic relationships and placement of the fungi obtained in this study.

Order	Family	Taxa	Isolates	Branch type
Botryosphaeriales	***Botryosphaeriaceae***	*Dothiorella* spp.	**BMA8_4; BMA9_1; BMA11_9; BMA7_1; BMA11_2; BMA11_6; BMA13_3**	Asymptomatic branches
			BMD10_5; BMD8_1	Branches with dieback
		Lasiodiplodia spp.	**BMA3_4; BMA10_1; BMA10_9; BMA11_5; BMA12_2; BMA8_5**	Asymptomatic branches
		Alanphillipsia spp.	**BMA5_5, BMA5_8, and BMA5_9**	Asymptomatic branches
		Botryosphaeria sp.	**BMD13_3**	Branches with dieback
		Oblongocollomyces sp.	**BMD11_3**	Branches with dieback
Capnodialeales	***Cladosporiaceae***	*Cladosporium* spp.	**BMD3_4 and BMA5_6**	Branches with dieback and asymptomatic branch
Diaporthales	***Valsaceae***	*Cytospora* spp.	**BMD4_2 and BMD8_3**	Branches with dieback
	Diaporthaceae	*Diaporthe* spp.	**BMD10_2; BMD2_2; BMD2_1**	Branches with dieback
Hypocreales	***Nectriaceae***	*Fusarium* spp.	**BMD13_1; BMD7_2**	Branches with dieback
Pleosporales	***Pleosporaceae***	*Alternaria* spp.	**BMA7_2; BMA3_2**	Asymptomatic branches
			BMD9_2	Branches with dieback
	Didymellaceae	*Didymella* spp.	**BMD5_1; BMD1_1**	Branches with dieback
	Didymosphaeriaceae	*Paracamarosporium* spp.	**BMA2_6 and BMA5_4**	Asymptomatic branches
Xylariales	***Sporocadaceae***	*Pestalotiopsis* sp.	**BMD3_3**	Branches with dieback
		Neopestalotiopsis spp.	**BMA5_3;BMA5_1; BMA12_1**	Asymptomatic branches

Table 3. (Continued)

Taxonomic relationships and placement of the fungi obtained in this study.

Order	Family	Taxa	Isolates	Branch type
Xylariales	***Sporocadaceae***	*Hymenopleella* sp.	**BMA2_1**	Asymptomatic branches
	Diatrypaceae	*Eutypella* sp.	**BMD1_3**	Branches with dieback

8. Figures

Figure 1. *Berchemia discolor* samples collected in Limpopo Province. **A**: Cut pieces of the healthy-looking branch. **B**: Debarked branch showing the lesion between the dead part and alive part of the branch. **C**: *B. discolor* tree showing severe dieback on branches.

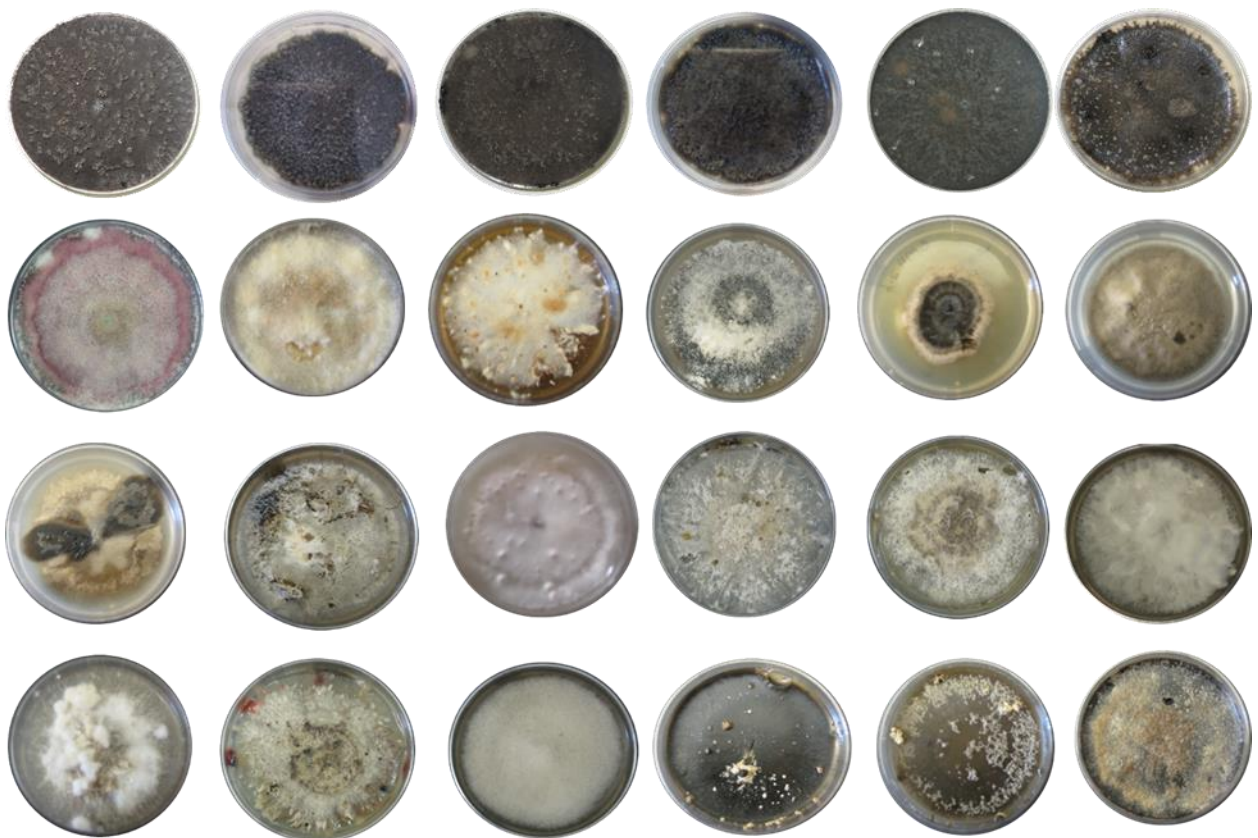


Figure 2. Photo plate illustrating the variation in culture morphology among isolates obtained in this study.

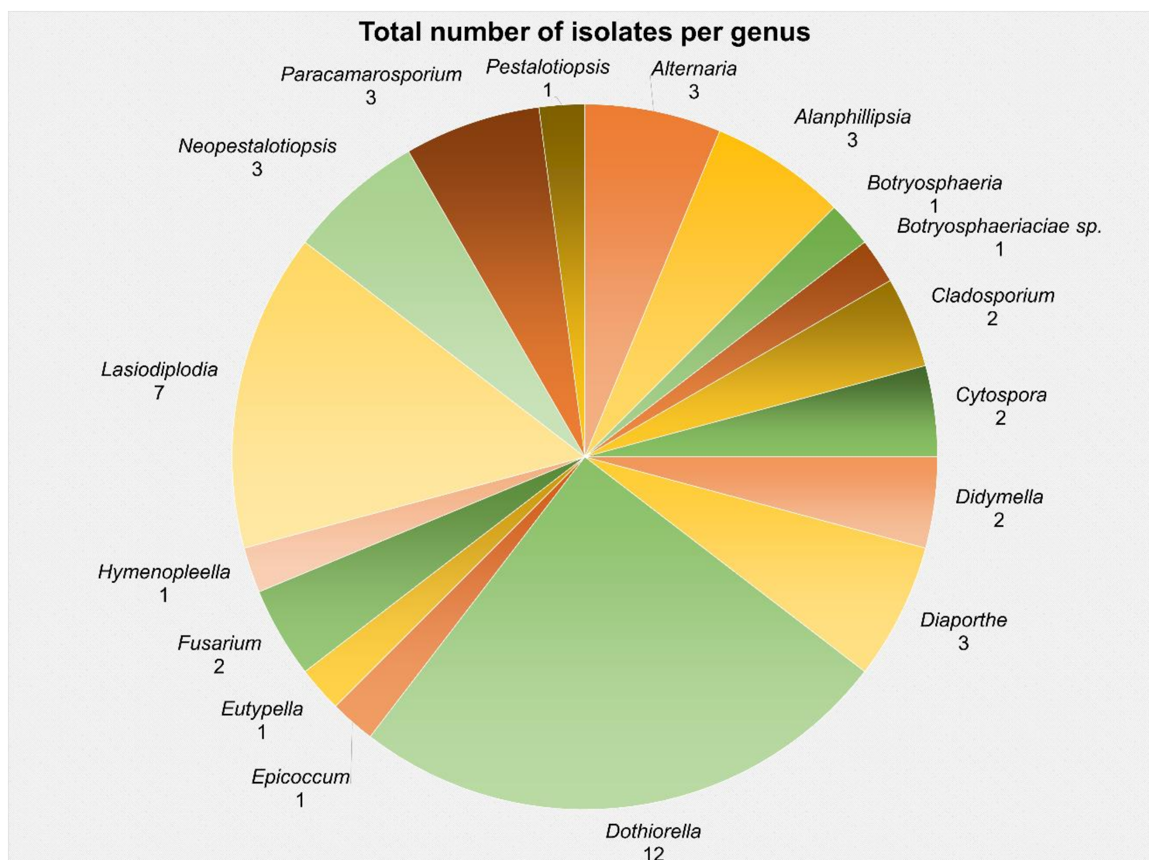
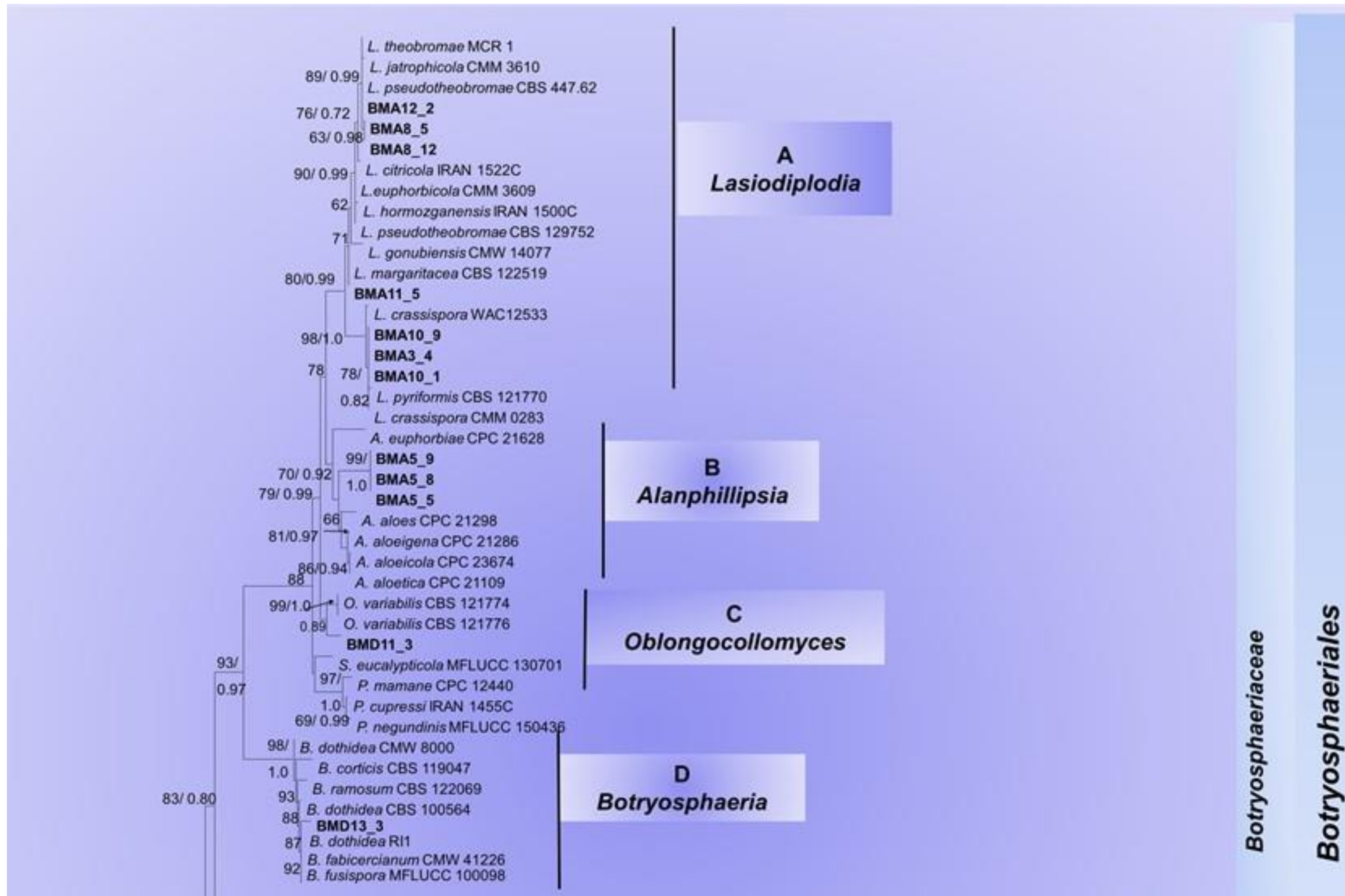
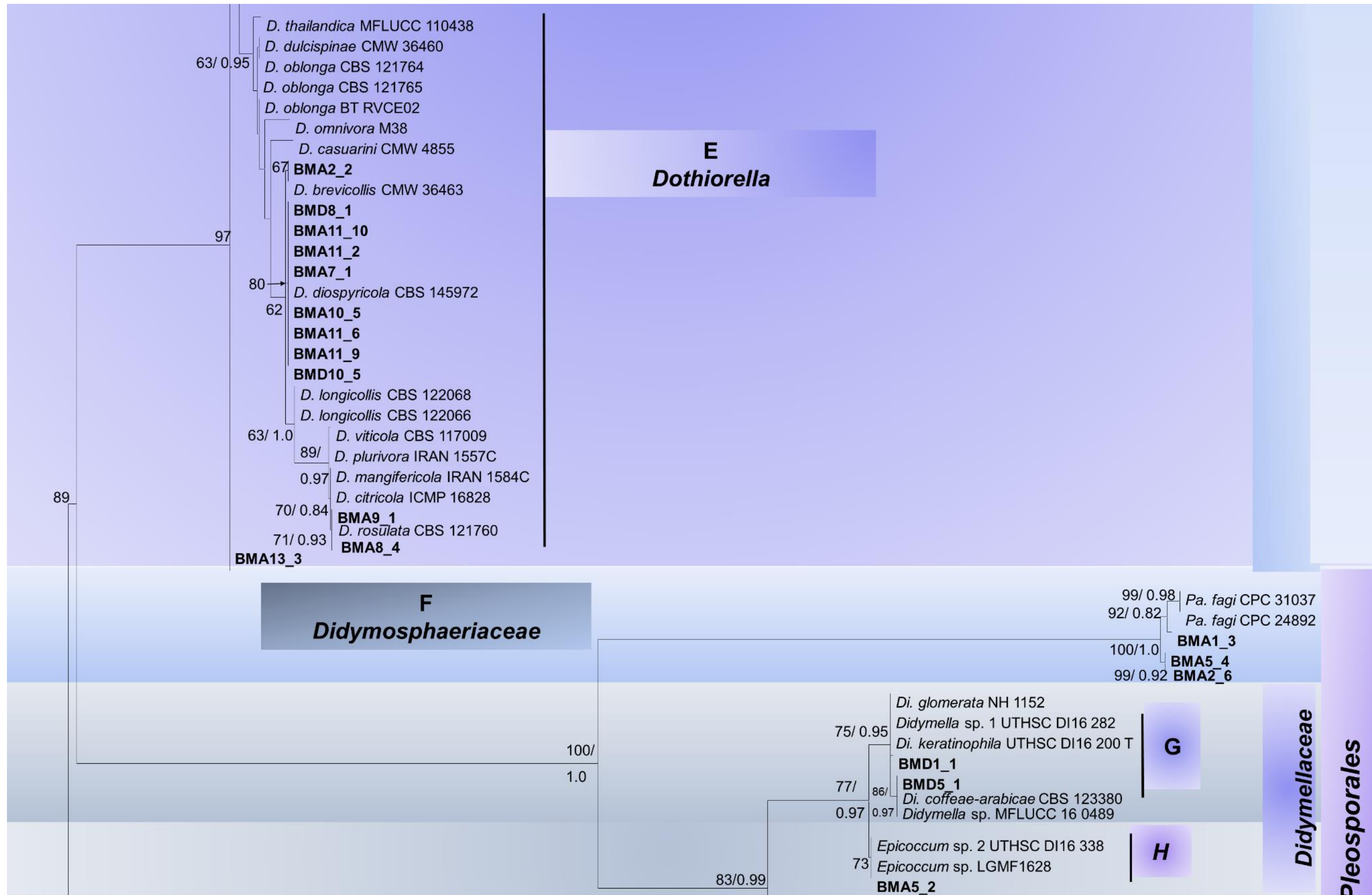


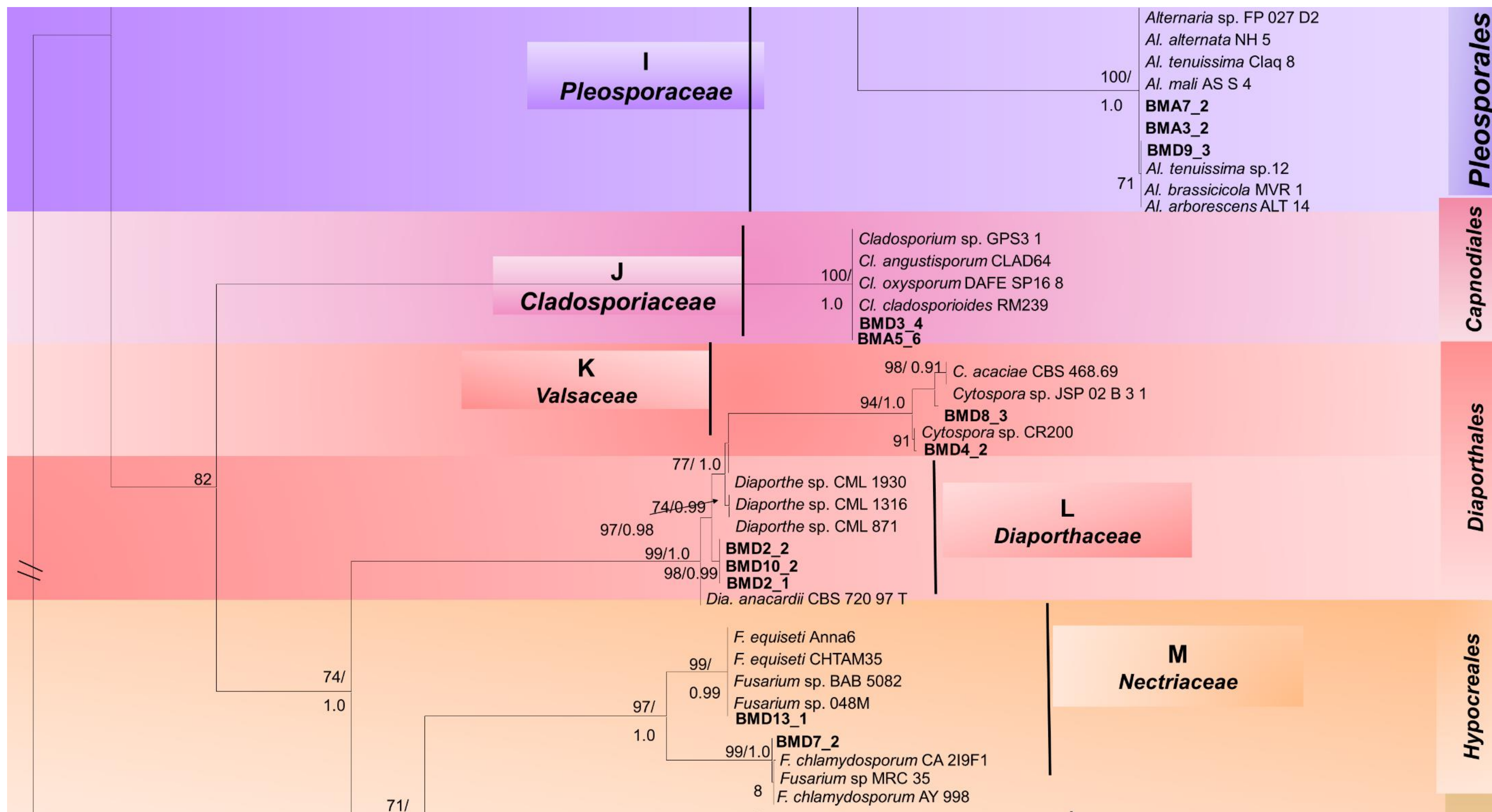
Figure 3. Abundance of fungal isolates per genus obtained from *B. discolor* branches.



L. theobromae MCR 1
L. jatrophicola CMM 3610
89/ 0.99
L. pseudotheobromae CBS 447.62
76/ 0.72
BMA12_2
BMA8_5
63/ 0.98
BMA8_12
90/ 0.99
L. citricola IRAN 1522C
L.euphorbicola CMM 3609
62
L. hormozganensis IRAN 1500C
L. pseudotheobromae CBS 129752
71
L. gonubiensis CMW 14077
80/0.99
L. margaritacea CBS 122519
BMA11_5
L. crassispora WAC12533
98/1.0
BMA10_9
BMA3_4
78
78/
BMA10_1
0.82
L. pyriformis CBS 121770
L. crassispora CMM 0283
A. euphorbiae CPC 21628
99/
BMA5_9
70/ 0.92
BMA5_8
1.0
79/ 0.99
BMA5_5
66
A. aloes CPC 21298
81/0.97
A. aloeigena CPC 21286
86/0.94
A. aloeicola CPC 23674
88
A. aloetica CPC 21109
99/1.0
O. variabilis CBS 121774
0.89
O. variabilis CBS 121776
BMD11_3
93/
S. eucalypticola MFLUCC 130701
0.97
97/
P. mamane CPC 12440
1.0
P. cupressi IRAN 1455C
69/ 0.99
P. negundinis MFLUCC 150436
98/
B. dothidea CMW 8000
1.0
B. corticis CBS 119047
93
B. ramosum CBS 122069
83/ 0.80
88
B. dothidea CBS 100564
BMD13_3
87
B. dothidea RI1
B. fabicercianum CMW 41226
92
B. fusispora MFLUCC 100098
A
Lasiodiplodia
B
Alanphillipsia
C
Oblongocollomyces
D
Botryosphaeria
Botryosphaeriaceae
Botryosphaeriales



D. thailandica MFLUCC 110438
D. dulcispinae CMW 36460
63/ 0.95
D. oblonga CBS 121764
D. oblonga CBS 121765
D. oblonga BT RVCE02
D. omnivora M38
D. casuarini CMW 4855
67
BMA2_2
D. brevicollis CMW 36463
BMD8_1
BMA11_10
97
BMA11_2
BMA7_1
80
D. diospyricola CBS 145972
62
BMA10_5
BMA11_6
BMA11_9
BMD10_5
D. longicollis CBS 122068
D. longicollis CBS 122066
63/ 1.0
D. viticola CBS 117009
89/
D. plurivora IRAN 1557C
0.97
D. mangifericola IRAN 1584C
89
D. citricola ICMP 16828
70/ 0.84
BMA9_1
D. rosulata CBS 121760
71/ 0.93
BMA8_4
BMA13_3
E
Dothiorella
F
Didymosphaeriaceae
99/ 0.98
Pa. fagi CPC 31037
92/ 0.82
Pa. fagi CPC 24892
BMA1_3
100/1.0
BMA5_4
99/ 0.92
BMA2_6
Di. glomerata NH 1152
Didymella sp. 1 UTHSC DI16 282
75/ 0.95
Di. keratinophila UTHSC DI16 200 T
G
100/
BMD1_1
1.0
BMD5_1
77/
86/
Di. coffeae-arabicae CBS 123380
0.97
0.97
Didymella sp. MFLUCC 16 0489
Epicoccum sp. 2 UTHSC DI16 338
H
73
Epicoccum sp. LGMF1628
83/0.99
BMA5_2
Didymellaceae
Pleosporales


I
Pleosporaceae
Pleosporales
Alternaria sp. FP 027 D2
Al. alternata NH 5
Al. tenuissima Claq 8
Al. mali AS S 4
BMA7_2
BMA3_2
BMD9_3
Al. tenuissima sp.12
Al. brassicicola MVR 1
Al. arborescens ALT 14
100/
1.0
71
J
Cladosporiaceae
Capnodiales
Cladosporium sp. GPS3 1
Cl. angustisporum CLAD64
Cl. oxysporum DAFE SP16 8
Cl. cladosporioides RM239
BMD3_4
BMA5_6
100/
1.0
K
Valsaceae
Diaporthales
98/ 0.91
C. acaciae CBS 468.69
Cytospora sp. JSP 02 B 3 1
94/1.0
BMD8_3
91
Cytospora sp. CR200
BMD4_2
77/ 1.0
82
Diaporthe sp. CML 1930
74/0.99
Diaporthe sp. CML 1316
97/0.98
Diaporthe sp. CML 871
99/1.0
BMD2_2
BMD10_2
98/0.99
BMD2_1
Dia. anacardii CBS 720 97 T
L
Diaporthaceae
M
Nectriaceae
Hypocreales
F. equiseti Anna6
F. equiseti CHTAM35
99/
0.99
Fusarium sp. BAB 5082
Fusarium sp. 048M
BMD13_1
74/
1.0
97/
1.0
BMD7_2
99/1.0
F. chlamydosporum CA 2I9F1
Fusarium sp MRC 35
8
F. chlamydosporum AY 998
71/

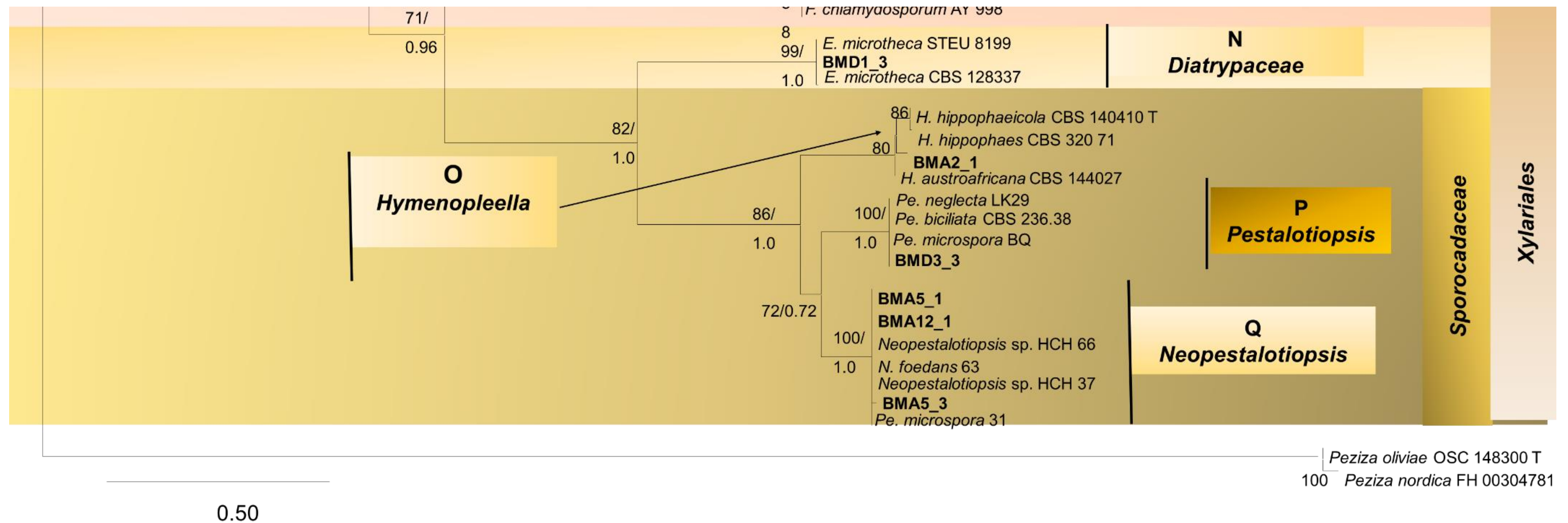


Figure 4. Phylogenetic tree based on Maximum Likelihood analysis of the ITS sequence dataset. Bootstrap values >60% and Posterior probability values > 0.9 respectively for ML and Bayesian inference are shown on the nodes. Isolates obtained in this study are shown in bold and the tree was rooted to *Peziza oliviae* (OSC 148300) and *Peziza Nordica* (FH 00304781).

CHAPTER 3

Diversity of *Botryosphaeriaceae* and their overlap on asymptomatic and symptomatic branches of *Berchemia discolor* in agricultural and natural ecosystems in the Limpopo Province

ABSTRACT

Botryosphaeriaceae includes important latent pathogens that cause disease on various indigenous and exotic trees, usually when the trees are subjected to stress. Nothing is known regarding the diversity and occurrence of *Botryosphaeriaceae* on indigenous *Berchemia discolor* trees worldwide. Like any other tree species, these plants are subjected to fungal diseases that affect their overall productivity and the quality of their by-products. This study aimed to explore the identity of endophytic species belonging to mainly the *Botryosphaeriaceae* on symptomatic and asymptomatic branches of *B. discolor* in agricultural and natural ecosystems in some areas of Limpopo Province. Thirteen species in the *Botryosphaeriaceae* and one species in the *Pseudofusicoccumaceae*, a family that was previously part of *Botryosphaeriaceae* were identified based on analyses of DNA sequence data of the ITS rDNA region and portions of the *β-tubulin*, *TEF-1α* and *rpb2* genes. The species identified included three potentially new species, designated as *Alanphillipsia* sp. Group B nov., *Dothiorella* sp. Group F nov. and *Oblongocollomyces* sp. Group A nov., which await morphological descriptions and formal naming. In addition, isolates of *Dothiorella diospyricola, D. brevicollis*, *Lasiodiplodia crassispora, L. mahajangana, L. pseudotheobromae*, *L. margaritacea*, *Pseudofusicoccum stromaticum,* and various isolates with uncertain identity, belonging to *Botryosphaeria* and *Dothiorella* were identified. Species in the genera *Dothiorella*, *Lasiodiplodia* and *Oblongocollomyces* were collected most frequently. Some species occurred on both symptomatic and asymptomatic branches of *B. discolor* trees in natural and agricultural ecosystems in the Limpopo Province. These results showed *B. discolor* trees as one of the many rich reservoirs for *Botryosphaeriaceae* in the Limpopo Province.

Keywords: *Berchemia discolor*, *Botryosphaeriaceae*, endophytes, gene regions, pathogens, phylogenetic analyses.

1. Introduction

Berchemia discolor (*Rhamnaceae*) is a tree species native to Africa, occurring from Ethiopia through Angola to South Africa (Orwa *et al.*, 2009; Cheikhyoussef and Embashu, 2013). It is extensively valued and used by local communities and is well-known for its nutritional values, medicinal properties and its use in the production of liquor (Green *et al.*, 2010; Debela *et al.*, 2012; Cheikhyoussef and Embashu, 2013). Information regarding fungi associated with *B. discolor* is not available. However, in the phylogenetic analyses using the ITS data set of isolates obtained from *B. discolor* branches (Chapter 2 of this dissertation), a large number of isolates were found belonging to *Botryosphaeriaceae* (*Botryosphaeriales*: *Ascomycota*). Research in the present chapter therefore focuses on the identity of the species and those from an agricultural ecosystem.

Schoch *et al.* (2006) introduced the *Botryosphaeriaceae* as a family in the order *Botryosphaeriales*. Based on recent molecular phylogenetic studies, a number of genera were excluded from *Botryosphaeriaceae* and assigned to families within the same order; these include *Aplosporellaceae* (*Aplosporella* and *Bagnisiella*), *Saccharataceae* (*Saccharata*) and *Melanopsaceae* (*Melanops*) (Slippers *et al.*, 2013), *Planistromellaceae* (*Kellermania*) (Minnis *et al.*, 2012), and most recently *Endomelanconiopsisaceae* (*Endomelanconiopsis*) and *Pseudofusicoccumaceae* (*Pseudofusicoccum*) (Yang *et al.*, 2017). Zhang *et al.* (2021) recently evaluated the *Botryosphaeriales* in the culture collection (CBS) of the Westerdijk Institute, the authors synonymised 58 species and identified eight novel species, of which six belonged to *Botryosphaeriaceae*. The *Botryosphaeriaceae* is the predominant family within *Botryosphaeriales*, and is represented by 23 genera, most of which have a cosmopolitan distribution (Slippers *et al.*, 2017).

Species of *Botryosphaeriaceae* are among the most commonly found fungi associated with native and non-native woody plants worldwide (Slippers and Wingfield, 2007). The association between these fungi and their plant hosts can be saprophytic, endophytic or pathogenic (Slippers and Wingfield, 2007). They typically manifest as pathogens on trees experiencing stresses such as drought (Paoletti *et al.*, 2001; Mehl *et al.*, 2011) and extreme cold or heat (Rayachhetry *et al.*, 1996). The symptoms that follow include tip dieback, stem and branch cankers, fruit rots, leaf spots and under

severe infections it can result in tree mortality (Slippers and Wingfield, 2007). Because of the fungal patterns of association with host stress, it has been predicted that members of the *Botryosphaeriaceae* may expand their host range and location as the global climate changes (Slippers and Wingfield, 2007; Batista *et al.*, 2021). This would result in an increased prevalence of disease in areas where it was previously not observed (Slippers and Wingfield, 2007).

In South Africa, *Botryosphaeriaceae* is relatively well studied on various plant hosts, particularly those of agricultural or of forestry importance. For example, various species in the family have been isolated from tree species in the genera *Mangifera* (*Anacardaceae*) (Mehl *et al.*, 2017), *Prunus* (*Rosaceae*) and *Vitis* (*Vitaceae*) (Damm *et al.*, 2007), *Eucalyptus* (*Myrtaceae*) (Pillay *et al.*, 2013; Slippers *et al.*, 2013), *Protea* (*Proteaceae*) (Denman *et al.*, 2003), *Vachellia* (*Fabaceae*) (Jami *et al.*, 2012; 2014; 2015), *Terminalia* (*Combretaceae*) (Begoude *et al.*, 2010) and *Pinus* (*Pinaceae*) (Bihon *et al.*, 2012). These fungi reduce the product quality and quantity of fruits, timber products, and the overall productivity of the trees; thus, having significant socio-economic impacts (Slippers and Wingfield, 2007; Jami *et al.*, 2017; 2018; Zlatković *et al.*, 2018).

Fungi in *Botryosphaeriaceae* are also associated with native tree species. Recent publications reporting their association with indigenous trees have increased and diverse species of *Botryosphaeriaceae* in different ecosystems were discovered. In 2017, Jami *et al.* documented the numbers of published species associated with various native and non-native trees in South Africa and Namibia. That study showed that 62 species are known from 66 different hosts, of which 37 species are known only from native trees (Jami *et al.*, 2017). These numbers are, however, bound to change with the increase of studies on new tree hosts.

Species from *Botryosphaeriaceae* were found on both asymptomatic and symptomatic indigenous trees in earlier studies (Mehl *et al.*, 2011; Jami *et al.*, 2012; Jami *et al.*, 2013, 2014; Jami *et al.*, 2017; Osorio *et al.*, 2017; Jami *et al.*, 2018). For example, healthy mangrove trees were host to *Botryosphaeria* sp., *Diplodia estuarine*, *D. sapinae*, *Lasiodiplodia avicenniae L. bruguierae, L. gonubiensis, L. theobromae, Neofusicoccum cryptoaustrale, N. kwambonambiense, N. lumnitzerae, N. luteum, N. mangroviorum, N. parvum* and *N. umdonicola* (Osorio *et al.*, 2017). Some of

Botryosphaeriaceae are, however, mostly associated with diseased plant parts and include *L. crassispora* isolated from *Pterocarpus angolensis* (Mehl *et al.*, 2011), *L. gonubiensis* from *Syzygium cordatum* (Pavlic *et al.*, 2004; 2007), *L. mahajangana* and *L. theobromae* from *Euphorbia ingens* (Van der Linde *et al.*, 2011), *L. pseudotheobromae* from *Terminalia catappa* (Begoude *et al.*, 2010), *N. parvum* and *N. vitifusiforme* from *Schizolobium parahyba* (Mehl *et al.*, 2014), *N. variabile* and *Pseudofusicoccum africanum* from *Mimusops caffra* (Jami *et al.*, 2018). The genus in which the latter species resides is now placed in the *Pseudofusicoccumaceae* (Yang *et al.*, 2017). Based on these studies, *Lasiodiplodia* and *Neofusicoccum* species were most frequently isolated from native trees. This was not surprising as these genera constitute the largest number of described species (Dissanayake *et al.*, 2016b), suggesting that native trees in South Africa represent significant reservoirs for new species in *Botryosphaeriaceae*.

Many of the species in the *Botryosphaeriaceae* are best described as latent pathogens that can cause significant damage to trees, particularly when the trees are under stress (Slippers and Wingfield, 2007). Some studies observed such species to simultaneously occur in asymptomatic and diseased parts of the plants (Sakalidis *et al.*, 2011a; Bihon *et al.*, 2012; Jami *et al.*, 2012; 2014; 2015; Cruywagen *et al.*, 2017; Mehl *et al.*, 2017). However, it is difficult to understand the host-tissue association and epidemiology of diseases caused by these fungi, as the occurrence of the isolates from either tissue showing dieback or from the tissue of trees showing no symptoms were not clearly documented. This information is important as it indicates and provides clues to the biology of species in the *Botryosphaeriaceae*.

The aim of this study was to identify isolates belonging to *Botryosphaeriaceae* isolated from symptomatic and asymptomatic branches of *B. discolor* in agricultural and natural ecosystems by making use of a phylogenetic species concept, with gene Genealogical concordance (GCPSR) as the recognition criterion. Based on this concept, isolates were identified to species if they grouped with strong statistical support with sequences of known species *from* different gene trees. Isolates that did not group with any sequences and that formed exclusive monophylogenetic groups in all the gene trees were considered species new to science. Phylogenetic trees were constructed from DNA sequence information obtained from the Internal Transcribed Spacer region (ITS)

of the ribosomal RNA, as well as portions of the gene encoding β-tubulin, translation elongation factor 1α (*TEF-1α*), and in some cases the second largest subunit of the RNA polymerase (*rpb2*) were used.

2. Materials and methods

2.1. Fungal isolates

A set of 247 isolates originating from branches of *B. discolor* were used in this study. Of these, 170 (68.8%) isolates were from asymptomatic branch pieces, whereas 77 (31.2%) isolates were from branch pieces having dieback symptoms. These isolates had typical cultural morphological characteristics that resembled the *Botryosphaeriaceae* (white and grey mycelium with aerial hyphae and rapid growth) (Figure 1). Some of the isolates originated from previous work and were collected in Mapungubwe National Park (see Chapter 2 of this dissertation) and an expansion of the survey was done in Dambale and Tshipise. The sampling and isolation strategies employed were the same as in Chapter 2 of this dissertation. Samples were collected under the SANParks project permit number KUNE 1442, under the supervision of Professor E.C. Kunjeku and co-worker Prof M.P.A. Coetzee. Pure isolates are housed in the Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria (CMW).

2.2. DNA extractions, PCR amplification and Sequencing

DNA was extracted from fungal colonies using a modified CTAB (Cetyltrimethylammonium bromide) method described by Brunner *et al*. (2001). The quality and quantity of the DNA was determined with a Nanodrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and then diluted to 50ng/µl with sterile water for use in PCR reactions. Sequence data were obtained for the ITS region after PCR using primers ITS-1 and ITS-4 (White *et al*., 1990) as an initial screen. In total, 247 isolates were sequenced in the forward direction using ITS primer, after which 63 isolates were selected from a phylogenetic tree (see below) and sequenced in the reverse direction using the ITS-4 primer. Subsequently, primer sets used to amplify other gene regions for the 63 representative samples included Bt2a

and Bt2b for the *β-tubulin* gene (Glass and Donaldson, 1995), Ef1-F and Ef2-R for *TEF-1α* (Jacobs *et al.*, 2004). Primers *rpb2* F-Las and *rpb2*-R-Las (Cruywagen *et al.*, 2017) for the second largest subunit of the RNA polymerase (*rpb2*) were only used for the *Lasiodiplodia* isolates. Amplifications of the different loci were performed under the same cycling conditions except for the annealing temperatures that varied per locus (ITS, *B-tub, rpb2* = 54°C and *TEF1-α* = 56°C). The amplification reactions, the PCR sequencing master mixes and conditions were the same as described before (see Chapter 2 of this dissertation).

2.3. DNA sequence and phylogenetic analyses

Sequences of the isolates examined in this study were assembled using CLC BioWorkbench v.8 (QIAGEN, Aarhus, Denmark) (Sequencing, 2011) and the nucleotide sequences were manually checked using BioEdit v7.0.9.0 software (www.mbio.ncsu.edu/BioEdit?BioEdit.html) (Hall *et al.*, 2011). Base calling errors were corrected in CLC BioWorkbench. The sequences were then compared to the nucleotide sequences included in the NCBI database (http:// blast.ncbi.nih.gov/blast.cgi) using BLASTn (Altschul *et al.*, 1990). To confirm species identities, additional datasets (as per gene regions) were created, which included additional isolates for each unidentified taxon, as well as extra isolates of the *Botryosphaeriaceae* and *Pseudofusicoccumaceae* representing closely related known species and the type species retrieved from GenBank (http://www.ncbi.nlm.gov) (Table 2).

Neighbor-Joining (NJ) analyses was done using 247 ITS sequences obtained in this study. The NJ tree was constructed with MEGA v7.2, using a GTR+G model. From the 247, 63 isolates were selected for further phylogenetic analyses. Selections were done from each clade on the phylogeny (results not shown). Two criteria were used to select isolates, firstly, each isolate chosen from the clades was representative of the branch-type it was obtained from (i.e., asymptomatic branch or dieback branch), secondly, the type of ecosystem the isolates were obtained from (i.e., agricultural or natural ecosystem) was considered (Table 1).

Phylogenetic analyses for ITS, *TEF-1α* and *β-tub* were done separately after which an analysis based on the combined datasets was done. These analyses were conducted

to infer the relationships and included the 63 isolates (Table 1) and the relevant *Botryosphaeriaceae* and *Pseudofusicoccumaceae* sequences obtained from GenBank (Table 2). Datasets for each gene at the genus level were also created for *Alanphillipsia, Botryosphaeria*, *Dothiorella*, *Lasiodiplodia*, *Oblongocollomyces* and *Pseudofussicoccum*. *Phyllosticta citricarpa* CBS102374 was the outgroup for *Alanphillipsia, Botryosphaeria*, *Oblongocollomyces* and *Pseudofusicoccum*. The outgroup for *Dothiorella* was *Lasiodiplodia mahajangana* CMW27820 and *Dothiorella longicollis* CBS122068 was the outgroup for *Lasiodiplodia*. The combined datasets for each genus were as follows: *Alanphillipsia, Oblongocollomyces* and *Pseudofusicoccum* consisted of ITS+*TEF-1α* sequences, while *Dothiorella* consisted of ITS+*TEF-1α*+*β-tub* sequences and *Lasiodiplodia* consisted of ITS+*TEF-1α*+*β-tub*+*rpb2* sequences. The sequence alignments were done online using MAFFT (https://mafft.cbrc.jp/alignment/server/) version 7 (Katoh and Standley, 2013) and assessed manually for alignment errors using BioEdit v7.0.9.0 software (www.mbio.ncsu.edu/BioEdit?BioEdit.html).

Bayesian inference of phylogenies were conducted only for the large ITS data matrix that included all genera from the representative isolates in this study using Mr. Bayes v3.2 (Huelsenbeck and Ronquist, 2001). The best nucleotide substitution model used was TIMef+I+G model as determined with J-ModelTest (Posada, 2008). An MCMC analysis was run for six million generations, with four runs consisting of four chains heated at the default temperature. Tree sampling frequency was 100 generations. Effective sampling size (ESS) were assessed using Tracer (Lanfear *et al.*, 2016) after the MCMC analysis was completed. Thereafter, a 25% burn-in was used to summarise a consensus from 45, 000 trees. The ITS trees obtained from Bayesian analysis were viewed using Figtree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Tree annotations for the ITS tree that included all the genera were done online using the iTOL web-interface (https:itol.embl.de/).

Phylogenetic trees based on maximum likelihood (ML) were constructed for all the remaining data sets. Searches for the best-scoring tree were conducted with RaxMLHPC v.8 (Stamatakis, 2014) using the General Time Reversal (GTR) model with a gamma distribution. Support for the nodes were obtained using 1000 bootstrap

replications in RaxML. The phylogenetic trees obtained from the ML analyses were visualised and edited in MEGA 7 (Kumar *et al.*, 2016).

Genealogical concordance phylogenetic species recognition (GCPSR) was used to delineate species. In application of the species recognition, single gene (ITS, *TEF-1α, β-tub* and *rpb2*) phylogenetic trees were constructed and the grouping of isolates compared. Isolates were identified to species when they grouped consistently or within the majority of gene trees with sequences of known species. Isolates were recognised as being a newly discovered species if they clustered into exclusive monophyletic groups in the majority of gene trees. To consider the congruency of the isolate clades, a statistical support of 70%- 100% was accepted. Concatenated datasets were used to infer the phylogenetic relationships of the recognised species in relation to other species.

3. Results

3.1. DNA sequencing and phylogenetic analyses

Preliminary identities of the isolates were determined by comparing the ITS sequences of the isolates against those in GenBank. The results revealed isolates belonged to six genera (Table 1). Five genera (*Alanphillipsia, Botryosphaeria, Dothiorella, Lasiodiplodia* and *Oblongocollomyces*) belonged to the family *Botryosphaeriaceae.* The remaining genus was *Pseudofusicoccum* that resides in *Pseudofusicoccumaceae*.

The datasets for ITS, *TEF-1α, β-tub* and *rpb2* were analysed individually and in combinations. ITS sequences were obtained for 247 isolates. Sequences for *TEF-1α* and *β-tub* regions were obtained for 63 isolates and sequences for *rpb2* were obtained for 15 isolates. Sequences matrices of approximately 550 base pairs (bp) for ITS, 430 bp for *β-tub*, 280 bp for *TEF-1α* and 620 bp for *rpb2* were obtained after alignment.

A ML and Bayesian analyses of the ITS dataset grouped the isolates into six clades corresponding to different genera of the *Botryosphaeriaceae* and *Pseudofusicoccum* (Group O) within *Pseudofusicoccumaceae* (Figure 2). The genera identified included

Alanphillipsia (Group B), *Botryosphaeria* (Group C), *Dothiorella* (Group D- J), *Lasiodiplodia* (Group K- N), *Oblongocollomyces* (Group A) from the *Botryosphaeriaceae*. The grouping of isolates in the different genera was supported with high bootstrap and/or posterior probability values.

3.2.1. Species of *Oblongocollomyces*

Phylogenetic analyses grouped 13 isolates in a single cluster referred to as Group A with strong support which is related to *Oblongocollomyces* species (Figure 3 A-C, Table 1). The isolates in this group comprised representatives from tree branches with dieback and branches that were asymptomatic. They formed a sister group to *Oblongocollomyces variabilis* and an unidentified *Oblongocollomyces* sp. (CMW2996 2) (Van der Linder, unpublished data; *Botryosphaeriaceae* sp. JAL-2011a strain CMW 29962, GeneBank Number: HM176528.1) in the phylogenetic tree generated from *TEF-1α* sequences and from the combined ITS and *TEF-1α* dataset. At the time of this study there were no *β-tub* reference sequences in GenBank for this genus, therefore this gene was not included here. Based on the distinct grouping of the isolates, they are treated as *Oblongocollomyces* sp. Group A nov., because it possibly represents a new species in this genus.

3.2.2. Species of *Alanphillipsia*

Two isolates (BMA5.5 and BMA5.9) grouped together on the phylogenetic tree based on *TEF-1α* sequences. In the ITS tree and combined tree, it formed a polytomy with an ancestral node shared by all the other species included in the dataset. Similarly, the analysis showed that their ITS sequences are more similar to each other than with any other isolates in this study or sequences on GenBank (Figure 4, Table 2). The isolates were only obtained from asymptomatic branches. They were most closely related to *A. aloeicola* and *A. aloetica* based on *TEF*-1α sequences (51% bootstrap) (Figure 4 B) but did not cluster with *Alanphillipsia* spp. for which ITS sequence data is available. The isolates are treated as a new undescribed species in *Alanphillipsia (Alanphilipsia* sp. Group B nov.).

3.2.3. Species of *Botryosphaeria*

One isolate (BMD13.3) from symptomatic branch was placed in a cluster of sequences from species belonging to *Botryosphaeria* (Figures 2 and 5). The isolate did not group with any of the known *Botryosphaeria* species with statistical support (Figures 5 A-D) for which it had sequence similarity on GenBank. Based on these results the isolate represents a unique taxon and was treated as *Botryosphaeria* sp. Group C.

3.2.4. Species of *Dothiorella*

The isolates identified as *Dothiorella* spp. grouped into seven broad phylogenetic clades based on the ITS dataset, but not all the groups were supported by bootstrap analyses (Figure 6 A). They grouped into six clades using both *TEF-1α* (Figure 6 B) and *β-tub* datasets (Figure 6 C). The phylogenetic groupings of the isolates into the different clades (Groups D-J) and those switching clades are observed on the phylogenies (Figures 6 A-D). The reference strains of species used in this study that were recently reduced to synonymy, are shown in Table 3 (Zhang *et al.*, 2021). Many of the clades included multiple species, therefore the identities of the isolates were not conclusive.

Phylogenetic analyses of the ITS and *β-tub* datasets resulted in the isolates from this study being placed in a single clade with *D. longicollis*, *D. brevicollis* and *D. diospyricola* (Figures 6 A, C). Phylogenetic trees based on *TEF-1α* and the combined datasets separated the isolates into two groups, Group D and E (Figures 6 B, D). Phylogenetic analyses of the *TEF-1α* dataset resulted in Group D isolates clustering with *D. diospyricola* (82% bootstrap), and Group E isolates grouping with *D. brevicollis* with low bootstrap support (Figure 6 B). Similarly, the phylogenetic tree from the combined dataset clustered Group D isolates with *D. diospyricola* (84% bootstrap), and Group E isolates formed a polytomy with *D. brevicollis* (Figure 6 D). Thus, the identities of Groups D and E were regarded as *D. diospyricola* and *D. brevicollis,* respectively.

Two isolates (BTD13.1 and BDA6.3) grouped together in all phylogenetic trees (Figures 6 A-D) and were referred to as Group F. The group was placed in different positions relative to other species and groups in the phylogenetic trees. They also

received low nodal support when grouping with other species in the phylogenetic trees. The isolates in this group are treated as a new undescribed species in *Dothiorella* (*Dothiorella* sp. F nov.).

Six isolates (BMA9.1, BMA8.4, BTA18.3, BDT8.2, BDA22.3 and BTA20.4) clustered together in three phylogenetic trees (*TEF-1α, β-tub* and the combined dataset) (Figures 6 B-D) with strong bootstrap support, however, within the ITS phylogenetic tree, two isolates, BMA8.4 and BMA9.1, formed a separate group. The isolates in this group are referred to as Group G, while the remainder of the isolates belong to Group H. This separation was, however, not supported in the other phylogenetic trees. In all the phylogenetic trees, the isolates from these two groups clustered within clades with *Dothiorella* sp., *D. viticola*, *D. plurivora*, *D. citricola* and *D. mangifericola* and were closest to *D. mangifericola.* However, they grouped with strong bootstrap support in trees generated from *TEF-1α* (73% bootstrap) and combined datasets (91% bootstrap) (Figures 6 B, D). The identities of Groups G and H isolates are therefore uncertain, and are regarded as *Dothiorella* sp. Group G and H.

In all the individual analyses of ITS, *β-tub* and *TEF-1α* datasets, isolate BMA13.3 was distinct from all other isolates included in this study, and is referred to as Group I. This isolate grouped in a clade with known species, namely, *D. dulcispinae* (= *D. oblonga*) and *D. thailandica* (ITS = 86%, *β-tub* and *TEF-1α* = 100%) (Figures 6 A-D). The isolate was most closely related to *D. thailandica* based on ITS (no support) and *β-tub* dataset (72% bootstrap) and sister to *D. dulcispinae* with bootstrap support of 86% and 100%, respectively (Figures 6 A, C). In the *Tef1-α* dataset analysis, the isolate grouped distinctly (88%) in a large group together with the same species with a 100% bootstrap support (Figure 6 B), which was also confirmed by the combined ITS+*TEF-1α*+*β-tub* dataset analyses (82% bootstrap) (Figure 6 D). The Group I isolate is considered representing a unique species.

Three isolates (BDA47.3, BDA43.6 and BTD38.1) clustered in all phylogenetic trees within a group that included different *Dothiorella* species (Figures 6 A-D). These isolates are referred to as Group J. The identity of these isolates could not be established due to taxonomic uncertainty based on the phylogenetic results.

3.2.5. Species of *Lasiodiplodia*

Isolates residing within *Lasiodiplodia* were recovered from asymptomatic and branches with dieback. Their phylogenetic placement with relation to other *Lasiodiplodia* spp. were determined using four gene regions including ITS, *β-tub*, *TEF-1α* and *rpb2* for the individual gene trees and an analysis based on concatenation of the sequence matrices (ITS+*TEF-1α*+*β-tub*+*rpb2*) (Figure 7 A-E). The isolates grouped into four clades throughout all four loci examined. Some of the *Lasiodiplodia* reference strains used in the current study were reduced to synonymy by Rodríguez-Gálvez *et al.* (2017) and Zhang *et al.* (2021), and are shown in Table 3.

The ITS and *β-tub* analyses could not resolve the identities of the isolates in Group K and grouped them closest to four (65% bootstrap) and 11 (no support) reference species, respectively. *TEF-1α*, *rpb2* and the combined ITS+*TEF1-α*+*β-tub*+*rpb2* datasets grouped the isolates most closely to *L. pseudotheobromae* with strong BS support of 91%, 84% and 99%, respectively (Figures 7 B, D, E). Thus, Group K isolates were treated as *L. pseudotheobromae.*

Four isolates (BTA43.1, BDA21.1, BDD30.1, AND BTA36.3) clustered together and are referred to as Group L. These isolates grouped with different *Lasiodiplodia* species in the phylogenetic tree generated from ITS, *TEF-1α* and *β-tub* (Figures 7 A, B, C). They formed a supported monophyletic group with *L. mahajangana* in the tree generated from *rpb2* (94% bootstrap) and combined datasets (99% bootstrap) (Figures 7 D and E). Isolates in group L are therefore considered being *L. mahajangana*.

Isolate BMA11.5 represented Group M and grouped with *L. margaritacea* in all phylogenetic trees generated and with strong bootstrap support (*β-tub* = 71%, *TEF-1α* = 99%, *rpb2* = 99% and combined data = 100%) except for the ITS dataset (55% bootstrap) (Figure 7 A- D). Based on these results, the isolate is identified as *L. margaritacea*.

Several isolates clustered in a group referred to as Group N. In most phylogenetic trees, these isolates grouped with sequences of *L. crassispora* (= *L. pyriformis*) with high bootstrap support (ITS = 99%, *TEF-1α* = 93%, *rpb2* = 100% and combined dataset = 98%) (Figure 7 A, B, D, E). The exception was the tree obtained from *β-tub*

dataset *that* grouped some of the isolates as a distinct group from the others with no support (Figure 7 C). Isolates in this group are treated as *L. crassispora*.

3.2.6. Species of *Pseudofusicoccum*

Two isolates (BTD15.1 and BTD15.3) clustered together in phylogenetic trees generated from the datasets that included sequences of *Pseudofusicoccum* spp. (Figure 8 A- C). These isolates represented Group O in this study and were closely related to *Ps. stromaticum* in all phylogenetic trees with high bootstrap support (ITS = 99%, *TEF-1α* = 90%, combined dataset = 84%) (Figures 8 A- C). These isolates were designated as *Ps. stromaticum.*

3.3. Species distribution in the agricultural and natural ecosystem and their overlap on branches of *B. discolor*

Four species were found to overlap in the agricultural and natural ecosystems. These were *D. diospyricola*, *Dothiorella* sp. Group GH, *Oblongocollomyces* sp. Group A nov. and *L. crassispora*. An equal number of five species were found distributed separately in the two ecosystems. In the agricultural ecosystem, the obtained species were, *D. brevicollis, Dothiorella* sp. Groups F and J, *L. mahajangana* and *Ps. stromaticum* (Figure 9 A), whereas *Alanphillipsia* sp. Group B nov., *Botryosphaeria* sp. Group C, *Dothiorella* sp. Group I, *L. pseudotheobromae* and *L. margaritacea* were found in the natural ecosystem.

The fungal isolates obtained from the branches of *B. discolor* in this study showed that a higher number of the species were found to overlap on both *B. discolor* branches collected (Figure 9 B). In total, eight species overlapped on asymptomatic and symptomatic branches. Four species (*Alanphillipsia* sp. Group B nov., *Dothiorella* sp. Group I, *L. margaritacea* and *L. pseudotheobromae*) were obtained only in asymptomatic branches, whereas two (*Botryosphaeria* sp. Group C and *Ps. stromaticum*) species were obtained only on branches with dieback (Figure 9 B).

4. Discussion

Before this study, no information was available about the diversity of endophytic and potentially pathogenic species in the *Botryosphaeriaceae* on *B. discolor* in South Africa. Fungal isolates from asymptomatic and symptomatic branches of *B. discolor* from three locations (Mapungubwe National Park, Tshipise, and Dambale) in the Limpopo Province were therefore collected during this study and their identities investigated. Results from phylogenetic analyses revealed at least fourteen species belonging to five genera of the *Botryosphaeriaceae* and a species in *Pseudofusicoccumaceae*. Of these, three species potentially represent novel species which we refer to as *Alanphilipsia* sp. Group A nov., *Dothiorella* sp. Group F nov. and *Oblongocollomyces* sp. Group B nov. Some of the isolates were identified as *D. diospyricola, D. brevicollis, L. pseudotheobromae*, *L. crassispora, L. mahajangana, L. margaritacea* and *Ps. stromaticum*. However, the taxonomic status of several isolates that were identified as members of *Dothiorella* and *Botryosphaeria* could not be resolved as they were placed within clades that included more than one known species.

The results of this study illustrated the richness in fungal biodiversity from unexplored indigenous trees of South Africa. In addition to three new species, four species had unresolved identities and could also represent novel species. Crous *et al.* (2006a) indicated that fungal diversity is poorly studied worldwide, and it was suggested that fungal species diversity can be as many as seven undescribed species per indigenous host trees and plant. Thus the results of this study suggest that the estimates done by Crous *et al.* (2006b) were conservative. It also suggests that native trees in South Africa are rich reservoirs for new species in *Botryosphaeriaceae* and other fungal families that were not considered in this study.

Species identification of the strains were primarily based on DNA sequence comparisons of four loci. The selection of the loci ITS, *β-tub*, *TEF-1α* and *rpb2* (*rpb2* being used only for *Lasiodiplodia*) were based on their use in previous studies that considered the taxonomy of species in *Botryosphaeriaceae* (Cruywagen *et al.*, 2017; Yang *et al.*, 2017; Batista *et al.*, 2021). These four loci allowed delineation of the species boundaries of most of the isolate groups obtained in this study.

In this study, several highly supported clades included several species, making the identification of isolates based on DNA sequence data complex. Species of these clades must be resolved in future studies. The misidentification of species in earlier studies was frequent within the *Botryosphaeriaceae*, and has improved over the years due to the substantial revisions of the taxonomic status of the species in the family based on DNA sequence data from multiple loci (Phillips *et al.*, 2013; Dissanayake *et al.*, 2016b; Cruywagen *et al.*, 2017; Zhang *et al.*, 2021). Accordingly, the species groups in which the isolates from this study reside could be reduced to synonymy or alternative loci that provide strong phylogenetic signal should be used in future to resolve this issue.

Dothiorella brevicollis and *D. diospyricola* were identified in this study. *Dothiorella diospyricola* is a recently described species that is closely related to *D. longicollis* and *D. brevicollis*, and it was first isolated from *Diospyros mespiliformis* in the Kruger National Park (Zhang *et al.*, 2021). In this study, *D. diospyricola* was abundant from both tissue types. Previous studies identified *D. brevicollis*, *D. mangifericola* (= *D. rosulata*), *D. capri-amissi*, *D. dulcispinae* (=*D. oblonga*), *D. viticola* and *D. pretoriensis* species as endophytes and pathogens of various indigenous trees in South Africa (Jami *et al.*, 2012; 2013; Slippers *et al.*, 2013; 2014). In Kenya, an unidentified *Dothiorella* sp. grouping closely to *D. brevicollis* and *D. longicollis* (possibly *D. diospyricola*) was also recently isolated from *B. discolor* trees showing cankers and dieback symptoms in Kenya (Karani *et al.*, 2022).

Other species identified were *L. pseudotheobromae, L. margaritacea*, *L. mahajangana* and *L. crassispora*. Of these, *L. pseudotheobromae* has a wide host range particularly on indigenous trees, causing symptomless infections and diseases on *V. karroo*, *Sclerocarya birrea*, and *Adansonia digitata* in the Limpopo Province (Jami *et al.*, 2015; Cruywagen *et al.*, 2017; Mehl *et al.*, 2017), *Pterocarpus angolesis* in Mpumalanga (Mehl *et al.*, 2011), and *Syzygium cordatum* in KwaZulu-Natal (Pillay *et al.*, 2013). In this study however, *L. pseudotheobromae* was only obtained from asymptomatic branches on a single tree. Also, *L. mahajangana* was reported on diseased *Euphorbia ingens* (Van der Linde *et al.*, 2011), *Sclerocarya birrea* (symptoms not reported) (Mehl *et al.*, 2017) and on healthy and diseased *A. digitata* (Cruywagen *et al.*, 2017) in the Limpopo Province. This species was also isolated from asymptomatic and

symptomatic branches of *B. discolor* in the current study. Furthermore, *L. margaritacea* was only found in asymptomatic branches whereas, *L. crassispora* was found in asymptomatic and branches with dieback. The fungi obtained in this study are reported here for the first time from *B. discolor*. Therefore, these *Lasiodiplodia* species, including the other species obtained in this study, should be considered in future pathogenicity studies to further understand their association with *B. discolor* trees.

Two isolates resided within *Pseudofusicoccum* and were identified as *Ps. stromaticum*. This genus was recently revised and moved by Yang *et al.* (2017) from *Botryosphaeriaceae* to *Pseudofusicoccumaceae.* Currently, the family includes only the genus *Pseudofusicoccum* with nine accepted species (Zhang *et al.*, 2021). *Pseudofusicoccum* species are mostly isolated as endophytes of woody hosts and are more prevalent in the tropics and/or subtropics (Jami *et al.*, 2017; Slippers *et al.*, 2017; Zhang *et al.*, 2021). In this study, however, *Ps. stromaticum* isolates were isolated only from diseased branches.

Three taxa obtained in this study are potentially new to science and were not given species names but regarded as *Alanphillipsia* sp. Group B nov., *Dothiorella* sp. Group F nov. and *Oblongocollomyces* sp. Group A nov. These species discovered are relatively closely related to multiple known species belonging to *Botryosphaeriaceae*. From the loci used in this study (*ITS, β-tub*, *TEF-1α* and *rpb2*), only five ITS and two *TEF-1α* sequences were available for the genus *Alanphillipsia*, and the isolates in this study grouped closely to *Alanphillipsia* species. Five *Alanphilllipsia* species are currently known and four of them have been reported from the Western Cape and one from the Northern Cape Provinces of South Africa associated with *Aloe dichotoma*, *Aloe melanacantha* and *Euphorbia* sp. (Crous *et al.*, 2013; 2014). Our species is a new record for a host association and distribution of *Alanphillipsia*. *Alanphillipsia* sp. Group B nov. in this study was found only in asymptomatic branches in the natural ecosystem.

Two isolates assigned to *Dothiorella* sp. Group F nov. are phylogenetically closely related to sister species, *D. striata* and *D. uruguayensis. Dothiorella striata* was first isolated from twigs of *Citrus sinensis* in New Zealand (Abdollahzadeh *et al.*, 2014) while *D. uruguayensis* was found as an endophyte from *Hexachlamis edulis* in

Uruguay (Pérez *et al.*, 2010). Since 2014, there aren't any reports of new host-associations and according to Dissanayake *et al.* (2016a), both species are reported to be limited to single host species. Isolates obtained in this study were from asymptomatic and diseased branches of *B. discolor*.

Oblongocollomyces, previously known as *Sphaeropsis*, is a newly erected genus within *Botryosphaeriaceae* (Yang *et al.*, 2017). *Oblongocollomyces* is monotypic and includes only *Oblongocollomyces variabilis* (Yang *et al.*, 2017). In this study, isolates from *B. discolor* and belonging to this genus formed a monophyletic group sister to *O. variabilis*. *Oblongocollomyces variabilis* was discovered from indigenous tree species *Vachellia hebeclada*, *Senegalia mellifera* (Slippers *et al.*, 2013) and *V. karroo* (Jami *et al.*, 2015) causing symptomless infections. However, the isolates in this study were obtained from both diseased and asymptomatic branches. Future studies must include formal descriptions of this group.

Species with uncertain identities were only identified to genus level. Six isolates assigned to *Dothiorella* sp. Group GH are closely related to *D. mangifericola*, *D. citricola*, *D. plurivora* and *D. viticola*, while *Dothiorella* sp. Group I grouped with *D. dulcispinae* and *D. thailandica* and lastly, *Dothiorella* sp. Group J was closely related to *D. casuarina* and *D. capriamissi*. *Dothiorella* sp. Groups GH and J were isolated from both asymptomatic and symptomatic branches, while Group I was only found in asymptomatic branches. Currently, there are 431 records of *Dothiorella* species names listed in MycoBank (accessed October 2021). Many of these species do not have cultures linked to them. It is therefore possible, as with any other study, that some of the isolates may represent previously described species for which DNA sequences are not available.

Cryptic species are defined as species that have undergone genetic divergence, but cannot be separated by ecological or morphological characteristics, and the statistical support values separating them from other species are sometimes poor (Sakalidis *et al.*, 2011b). In this study, this was observed for isolates belonging to *Botryosphaeria* (Group C), *Dothiorella* (Groups GH and I) and *Lasiodiplodia* (Group L). The isolates in these groups formed monophyletic clades clades that included several species, moreover, the bootstrap support values were very low. This raises questions as to whether the species are cryptic undescribed species or whether they represent some

of the genetic variations observed in *Botryosphaeria*, *Dothiorella* and *Lasiodiplodia* species. Phillips *et al.* (2013) recommended the use of at least two loci (ITS and *Tef-1α*) for separating species within *Botryosphaeriaceae*. Four gene regions (ITS, *Tef-1α*, *β-tub* for all genera and *rpb2* as additional gene region for *Lasiodiplodia*) were used in this study, and the individual trees showed inconsistent groupings between gene regions of the isolates belonging to *Botryosphaeria*, *Dothiorella* and *Lasiodiplodia* to the known species. According to Yang *et al.* (2017), variation within a species becomes more apparent when more isolates are added to the dataset, which could be the case in this study. Based on the definition of cryptic species by Sakalidis *et al.* (2011b), the species mentioned above are considered being cryptic. Additional analyses will be required to resolve these groups, and future studies must employ additional gene regions in order to resolve the uncertainties in the clades either by including more gene regions or using specific microsatellite markers to consider the problem at a population level.

The most dominant fungi identified from branches of *B. discolor* were *D. diospyricola* (46 of the total number of isolates), *Oblongocollomyces* sp. Group A nov. (30 of the total isolates) and *L. crassispora* (18 of the total number of isolates). All these taxa were found on both asymptomatic and symptomatic branches and also occurred in both ecosystems. Other overlapping species included *D. brevicollis, Dothiorella* sp. Groups F nov., GH and J, and *Lasiodiplodia mahajangana*. Pathogenicity studies must be considered to confirm whether they can play a role in dieback of the *B. discolor* trees. Four of the *Botryosphaeriaceae* species were obtained only from asymptomatic tissue whilst two were obtained only on branches with dieback. These species included, *Dothiorella* sp. Group I, *L. margaritacea*, *L. pseudotheobromae* and *Alanphillipsia* sp. Group B nov. on asymptomatic whereas *Botryosphaeria* sp. Group C and *Ps. stromaticum* on branches with dieback. These species were generally present in very low numbers, ranging from one isolate to four isolates per fungal species. The other eight species that overlapped on both the branches, however, ranged from five isolates to 46 isolates per fungal species, of which asymptomatic isolates dominated. This high number of isolates found in asymptomatic branches is not surprising and agrees with reports on other indigenous trees (Sakalidis *et al.*, 2011a; Jami *et al.*, 2013; Osorio *et al.*, 2017). These studies illustrate that most *Botryosphaeriaceae* occur as endophytes and/ or are latent opportunistic pathogens,

persisting asymptomatically in the host until the host is under stress (Slippers and Wingfield, 2007). In certain cases, they dominate the endophytic communities (Smith *et al.*, 1996). The results of this study further add credibility to the notion that *Botryosphaeriaceae* are endophytes. However, because of the non-host specificity, wide geographic distribution and the disease associations of *Botryosphaeriaceae* with various indigenous host trees, their ecological roles as endophytes in nature need to be attended to more explicitly.

In this study, there seems to be no difference in the diversity of *Botryosphaeriaceae* between the natural and agricultural ecosystems. The species diversity in the natural ecosystem may have been highly influenced by the low number of trees examined as compared to the agricultural ecosystem, which was due to difficulties in reaching branches. A noteworthy difference was observed in the frequent isolations between the two ecosystems. Various studies illustrated that high levels of species diversity may be observed in the natural ecosystem (Burgess and Wingfield, 2002; Sieber, 2007; Hardoim *et al.*, 2015; Wood, 2017), in this study, it was quite the opposite which may have been influenced by the low number of samples. More samples were collected in the agricultural ecosystems than in the natural ecosystem because of the availability of the host tree in the agricultural ecosystem. Even in this case, an equal number of species, in total five potential species, were found separately in each ecosystem. The remainder of the species were found overlapping in both ecosystems in asymptomatic and symptomatic branches. To fully understand and uncover the diversity, distribution and host interaction of these fungi, further studies are required that involve more intensive sampling in both ecosystems, and in cases where the host is not available, surrounding vegetation should also be considered for sampling.

5. Conclusions

There seem to be a great diversity of unexplored *Botryosphaeriaceae* species associated with *B. discolor* trees. Studies like the one presented here emphasise the extensive diversity of the *Botryosphaeriaceae* species that can exist on an individual tree species in a limited area. Due to their associations with various hosts and the apparent ease with which they shift and switch host species (Slippers and Wingfield,

2007), these fungi pose threats as potential pathogens on indigenous trees. More indigenous trees species in the Limpopo Province must be sampled, as there seems to be a great diversity of new species, particularly the trees utilised by the rural communities. Our understanding of *Botryosphaeriaceae* on these trees will contribute to a better understanding of the ecology, biology and distributions of these fungi in South Africa. This study has contributed to our understanding of *Botryosphaeriaceae* on indigenous *B. discolor* trees in the Limpopo Province, much remains to be learnt regarding the ecological relevance of these species on *B. discolor* trees and other indigenous trees in the surrounding areas.

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7. Tables

Table 1. Species identity and geographic origin of the representative isolates obtained from *Berchemia discolor* branches in agricultural and natural ecosystems used for the phylogenetic analyses.

Agricultural ecosystem				Natural ecosystem			
Isolate	**CMW No.**	**Identity**	**Location**	**Isolate**	**CMW No.**	**Identity**	**Location**
BDA27_1	**55596**	*Oblongocollomyces* sp. Group A nov.	Dambale	**BMD11_3**	**55597**	*Oblongocollomyces* sp. Group A nov.	
BDA33_4	**55591**		Dambale	**BMA5_5**	**55624**	*Alanphillipsia* sp. Group B nov.	
BTA44_2	**55594**		Tshipise	**BMA5_9**	**55589**		
BDA33_6	**55592**		Dambale	**BMD13_3**	**55916**	*Botryosphaeria* sp. Group C	
BDA20_2	**57854**		Dambale	**BMA7_1**	**55578**		
BDA9_2	**55598**		Dambale	**BMA11_9**	**55580**		Mapungubwe National Park
BDD26_2	**55590**		Dambale	**BMD10_5**	**55616**	*D. diospyricola*	
BTD7_1	**55593**		Tshipise	**BMD8_1**	**55575**		
BTA29_3	**55595**		Tshipise	**BMA11_6**	**55576**		
BTD47_1	**55625**		Tshipise	**BMA11_2**	**55579**		
BTA12_4	**57855**		Tshipise	**BMA13_3**	**55587**	*Dothiorella* sp. Group I	
BTD15_1	**55644**	*Ps. stromaticum*	Tshipise	**BMA8_4**	**55583**	*Dothiorella* sp. Group GH	
BTD15_3			Tshipise	**BMA9_1**	**55582**		

Table 1. (Continued) Species identity and geographic origin of the representative isolates obtained from *Berchemia discolor* branches in agricultural and natural ecosystems used for the phylogenetic analyses.

Agricultural ecosystem				**Natural ecosystem**			
Isolate no.	**CMW No.**	**Identity**	**Location**	**Isolate no**	**CMW No.**	**Identity**	**Location**
BDA34_2	**57853**		Dambale	**BMA8_5**	**55600**	*L. pseudotheobromae*	
BTD4_3	**55615**	*Dothiorella diospyricola*	Tshipise	**BMA11_5**	**55629**	*L. margaritacea*	Mapungubwe
BDA27_1	**55596**		Tshipise	**BMA12_2**	**55601**	*L. pseudotheobromae*	National Park
BDA12_2	**55618**		Dambale	**BMA3_4**	**55573**	*L. crassispora*	
BDD8_4	**55577**		Dambale	**BMA10_1**	**55572**	*L. crassispora*	
BDD8_2	**55574**		Dambale	**BMA10_9**	**55604**	*L. crassispora*	
BDD6_1	**55614**	*D. brevicollis*	Dambale	**BMA10_5**	**57852**	*D. diospyricola*	
BDA10_1	**55617**		Dambale				
BTD47_3	**57856**		Tshipise				
BTA18_3	**55584**	*Dothiorella* sp. Group GH	Tshipise				
BTA20_4	**55620**		Tshipise				
BTD8_2	**55585**		Tshipise				
BDA22_3	**55619**		Dambale				
BDA6_3	**55586**	*Dothiorella* sp. Group F	Dambale				
BTD13_1	**55621**		Tshipise				
BTD38_1	**55623**	*Dothiorella* sp. Group J	Tshipise				

Table 1. (Continued) Species identity and geographic origin of the representative isolates obtained from *Berchemia discolor* branches in agricultural and natural ecosystems used for the phylogenetic analyses.

Agricultural ecosystem				Natural ecosystem			
Isolate No.	**CMW No.**	**Identity**	**Location**	**Isolate No.**	**CMW No.**	**Identity**	**Location**
BDA47_3	**55588**	*Dothiorella* sp. Group J	Dambale				
BDA19_2	**55632**	*Lasiodiplodia crassispora*	Dambale				
BDA43_6	**55622**		Dambale				
BTA36_3	**55628**	*L. mahajangana*	Tshipise				
BDA21_1	**55627**		Dambale				
BTA43_1	**55626**		Tshipise				
BDD30_1	**55599**		Dambale				
BDA11_1	**55603**	*L. crassispora*	Dambale				
BDA30_1	**55602**		Dambale				
BTD31_3	**55631**		Tshipise				

Table 2. Isolates for which sequences were obtained from GenBank and representing species in the *Botryosphaeriaceae.*

Identity	Strain number	Host	Location	GenBank accession number			
				ITS	*β-tub*	*TEF-1α*	*rpb2*
Alanphillipsia aloes	CPC 21298	*Aloe dichotoma*	Western Cape	NR_137122	-	-	-
A. aloeicola	CPC 23674	*Aloe* sp.	Western Cape	NR_137926	-	MT592027	-
A. aloeigena	CPC 21286	*A. melanacantha*	Namakwaland	NR_137121	-	-	-
A. aloetica	CPC 21109	*Aloe* sp.	Eastern Cape	NR_137123	-	-	-
A. euphorbiae	CPC 21628	*Euphorbia* sp.	Western Cape	NR_137124	-	MT592029	-
Botryosphaeria agaves	MFLUCC 11-0125	*Agave* sp.	Chiang Rai	NR_111792	JX646841	JX646856	-
B. corticis	CBS 119047	*Va. corymbosum*	USA	DQ299245	-	EU017539	-
B. dothidea	CMW 8000	*Prunus* sp.	Switzerland	AY236949	AY236927	AY236898	-
B. dothidea	CBS 100564	*Paeonia* sp.	Netherlands	KX464085	KX464781	KX464555	-
B. fabicercianum	CMW 41226	*Avicennia marina*	South Africa	KP860875	KP860795	KP860718	-
B. fusispora	MFLUCC 10-0098	*Entada* sp.	Chiang Rai	NR_121552	JX646839	JX646854	-

Table 2. (Continued) Isolates for which sequences were obtained from GenBank and representing species in the *Botryosphaeriaceae*.

Identity	Strain number	Host	Location	GenBank accession number			
				ITS	*β-tub*	*TEF-1α*	*rpb2*
B. ramosum	CBS 122069	*E. camaldulensis*	Perth	EU144055	-	EU144070	-
Dothiorella acaciacola	CBS 141295	*Acacia mearnsii*	France	KX228269	-	KX228376	-
D. brevicollis	CMW 36463	*V. karroo*	Gauteng	NR_111703	JQ239371	JQ239390	-
D. capriamissi	CBS 121763	*V. erioloba*	Gauteng	EU101323	KX464850	EU101368	-
D. casuarini	CMW 4855	*Casuarini* sp.	Australia	DQ846773	DQ875340	DQ875331	-
D. citricola	ICMP 16828	-	New Zealand	EU673323	EU673145	EU673290	-
D. dulcispinae	CMW 36460	*V. karroo*	South Africa	NR_111702	JQ239373	JQ239387	-
D. diospyricola	CBS 145972	*Di. mespiliformis*	South Africa	MT587398	MT592581	MT592110	-
D. iberica	CBS 115041	*Quercus ilex*	Spain	NR_111165	EU673096	AY573222	-
D. longicollis	CBS 122068	*L. cunninghamii*	South Africa	NR_136999	KF766130	EU144069	KX463972
D. longicollis	CBS 122066	*Terminalia* sp.	Australia	EU144052	KX464857	EU144067	-
D. mangifericola	IRAN 1584C	-	Iran	KC898221	-	KC898204	-
D. oblonga	CBS 121764	*Senegalia mellifera*	Namibia	EU101299	KX464854	EU101344	-

Table 2. (Continued) Isolates for which sequences were obtained from GenBank and representing species in the *Botryosphaeriaceae.*

Identity	Strain number	Host	Location	GenBank accession number			
				ITS	*β-tub*	*TEF-1α*	*rpb2*
D. oblonga	CBS 121765	*S. mellifera*	South Africa	NR_137689	KX464862	EU101345	-
D. rosulata	CBS 121760	*V. karroo*	Namibia	NR_136991	KX464877	EU101335	-
D. omnivora	M 38	*Vitis vinifera-*	Hungary	KY672851	-	KY681038	
D. plurivora	IRAN 1557C		Iran	KC898225	-	KC898208	
D. nigra	CBS 121783	*A. mearnsii*	South Africa	EU101333	KX464859	EU101378	-
D. striata	ICMP 16819	*C. sinensis*	New Zealand	EU673320	EU673142	EU673287	-
D. thailandica	MFLUCC 11-0438	*Bambusa* sp.	Thailandica	NR_111794	JX646844	JX646861	-
D. tectonae	MFLUCC12-0382	*Tectona grandis*	Thailand	KM396899	KM510357	KM409637	-
D. uruguayensis	UY 672	*Eucalyptus* sp.	USA	EU080923	KX464886	EU863180	-
D. viticola	CBS 117009	*V. vinifera cv. G. Negra*	Spain	NR_111186	EU673104	AY905559	-
D. vinea-gemmae	B 116-3	*V. vinifera Chardonnay*	Australia	KJ573644	KJ577552	KJ573641	-

Table 2. (Continued) Isolates for which sequences were obtained from GenBank and representing species in the *Botryosphaeriaceae.*

Identity	Strain number	Host	Location	GenBank accession number			
				ITS	*β-tub*	*TEF-1α*	*rpb2*
D. westrale	WA10NO 01	*V. vinifera*	Australia	HM009376	HM800519	HM800511	-
Lasiodiplodia americana	CERC 1960	*Pistachio*	China	KP217058	KP217074	KP217066	MF410161
L. avicenniae	CMW 41467	*Avicennia marina*	South Africa	NR_147359	KP860758	KP860680	KU587878
L. bruguierae	CMW 42480	*B. gymnorrhiza*	South Africa	KP860832	KP860755	KP860677	KU587876
L. citricola	IRAN 1522C	*Citrus* sp.	Iran	GU945354	KP872405	GU945340	KP872455
L. crassispora	WAC 12533	*Santalum album*	Australia	NR_111194	-	DQ103557	KP872457
L. euphorbicola	CMM 3609	*Jatropha curcas*	Brazil	KF234543	KF254926	KF226689	KU887395
L. exigua	BL 104	Broom bush	Tunisia	KJ638317	KU887509	KJ638336	-
L. gonubiensis	CMW 14077	*Sy. cordatum*	South Africa	AY639595	-	DQ103566	KU587887
L. gravistriata	CMM 4564	*Anacardium humile*	Brazil	KT250949	-	KT250950	-
L. hormozganensis	IRAN 1500C	*Olea* sp.	IRAN	GU945355	KP872413	GU945343	KP872466
L. iraniensis	IRAN 921C	-	Iran	GU945346	-	GU945334	KU887388
L. jatrophicola	CMM 3610	*Jatropha curcas*	Brazil	KF234544	KF254927	KF226690	KU887417

Table 2. (Continued) Isolates for which sequences were obtained from GenBank and representing species in the *Botryosphaeriaceae.*

Identity	Strain number	Host	Location	GenBank accession number			
				ITS	*β-tub*	*TEF-1α*	*rpb2*
L. macrospora	CMM 3833	*Jatropha curcas*	Brazil	NR_147349	KF254941	KF226718	-
L. mahajangana	CMW 27820	*Terminalia catappa*	Madagascar	FJ900597	FJ900632	FJ900643	KP872472
L. margaritacea	CBS 122519	*Adansonia gibbosa*	Australia	EU144050	-	EU144065	KU696367
L. pontae	IBL 12	Tropical fruit trees	Brazil	KT151794	KT151797	KT151791	-
L. pseudotheobromae	CBS 129752	*A. mangium*	Venezuela	MH865368	JX545131	JX545111	KP872481
L. pseudotheobromae	CBS 447.62	*Citrus aurantium*	Suriname	EF622081	-	EF622060	MF410182
L. pseudotheobromae	CBS 116459	*Gmelina arborea*	Costa Rica	NR_111264	EU673111	-	KU696376
L. pyriformis	CBS 121770	*Senegalia mellifera*	Namibia	NR_136993	KU887527	EU101352	KU696378
L. thailandica	CPC 22795	*Mangifera indica*	Thailand	KJ193637	-	KJ193681	KY751297
L. theobromae	CBS 164.96	Fruit along coral reef coast	Papua New Guinea	NR_111174	KU887532	AY640258	KU696383
L. rubropurpurea	WAC 12535	*Eucalyptus grandis*	Australia	DQ103553	-	DQ103571	KP872485
L. subglobosa	CMM 3872	*Jatropha curcas*	Brazil	KF234558	KF254942	KF226721	-
L. venezuelensis	WAC 12539	*A. mangium*	Venezuela	DQ103547	-	DQ103568	KP872490

Table 2. (Continued) Isolates for which sequences were obtained from GenBank and representing species in the *Botryosphaeriaceae.*

Identity	Strain number	Host	Location	GenBank accession number			
				ITS	*β-tub*	*TEF-1α*	*rpb2*
Oblongocollomyces variabilis	CBS 121774	*V. karroo*	Namibia	NR_136994	-	EU101357	-
Ob. variabilis	CBS 121776	*Senegalia mellifera*	South Africa	EU101326	-	EU101371	-
Phaeobotryon cupressi	IRAN 1455C	-	Iran	FJ919672	-	FJ919661	-
Ph. mamane	CPC 12440	*S. chrysophylla*	USA: Hawaii	NR_111324	EU673121	EU673298	-
Ph. negundinis	MFLUCC 15-0436	-	Russia:Rostov region	NR_155669	KU853996	KU853997	-
Ph. rhois	CFCC 89662	*Rhus typhina L.*	China	KM030584	-	KM030598	-
Pseudofusicoccum adansoniae	WAC12689	*Mangifera indica*	Australia	EF585534	-	EF585567	-
Ps. kimberleyense	CBS 122061	*Ficus opposita*	Australia	EU144059	-	EU144074	-
Ps. stromaticum	CBS 117448	-	South Africa	KF766223	EU673094	-	-
Sphaeropsis citrigena	ICMP 16812	*Citrus sinensis*	New Zealand	NR_119697	EU673141	EU673294	-
S. eucalypticola	MFLUCC 13-0701	*Tectona grandis*	Thailand	KM396907	KM510365	KM409644	-

Table 2. (Continued) Isolates for which sequences were obtained from GenBank and representing species in the *Botryosphaeriaceae.*

Identity	Strain number	Host	Location	GenBank accession number			
				ITS	*β-tub*	*TEF-1α*	*rpb2*
S. porosa	STE-U 5046	Grapevines	South Africa	AY343378	-	AY343339	-
Sakireeta madreeya	CBS 532.76	Undetermined grass	India: Madras	KM108376	-	KM108427	-

Table 3. *Botryosphaeriaceae* species used as reference strains in this study that were recently reduced to synonymy.

Recent names	Previous names	References
Botryosphaeria fabicerciana	*B. fusispora*	Zhang *et al.* (2021)
Dothiorella dulcispinae	*D. oblonga*	Zhang *et al.* (2021)
D. mangifericola	*D. rosulata*	Zhang *et al.* (2021)
Lasiodiplodia iraniensis	*L. jatrophicola*	Zhang *et al.* (2021)
L. mahajangana	*L. exigua* *L. americana*	Rodríguez-Gálvez *et al.* (2017) and Zhang *et al.* (2021)
L. crassispora	*L. pyriformis*	Zhang *et al.* (2021)

*Synonymised names are highlighted in green on the phylogenies.

8. Figures

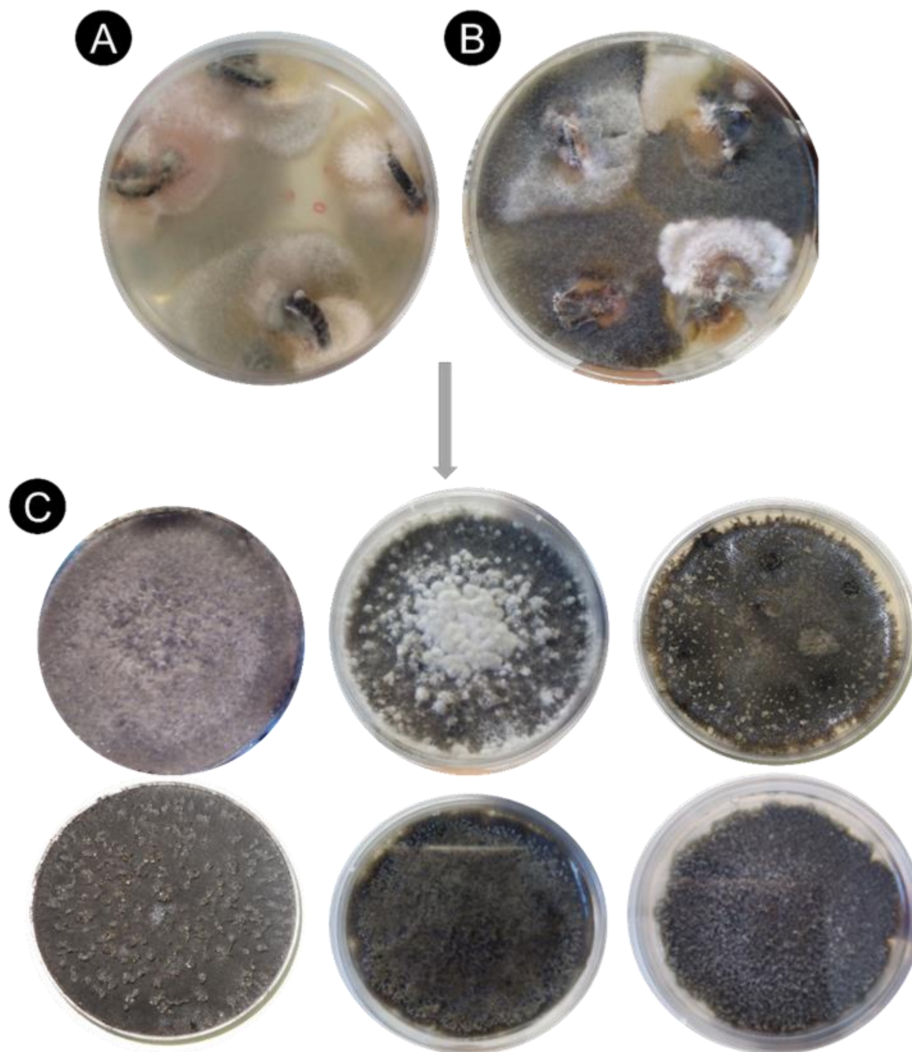


Figure 1 A-C. Isolations of fungi from branches of *B. discolor*. **A.** Primary culture from asymptomatic branches**. B.** Primary culture from branch with dieback branches. **C.** Pure fungal colonies obtained from *B. discolor* branches.

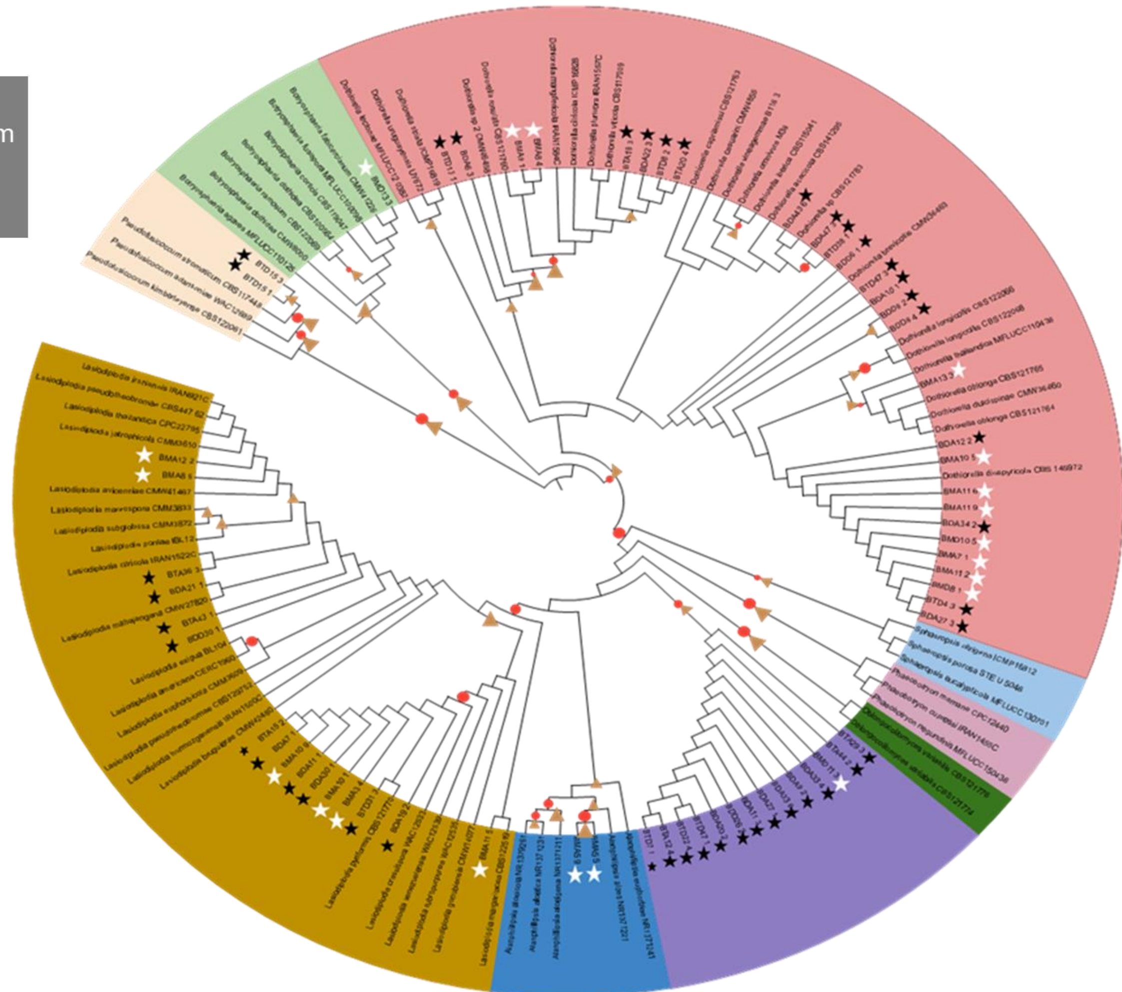


Figure 2. Maximum Likelihood (ML) tree of the *Botryosphaeriaceae* and *Pseudofusicoccumaceae* obtained from ITS sequence data, showing the different genera to which the isolates obtained from the branches of *Berchemia discolor* belong. Bootstrap values >80% and Posterior probability values >0.90 respectively are provided at the nodes and represented by bootstrap support = circles and posterior probability = triangles. Isolates in this study from the natural and agricultural ecosystems are represented by white and black star shapes.

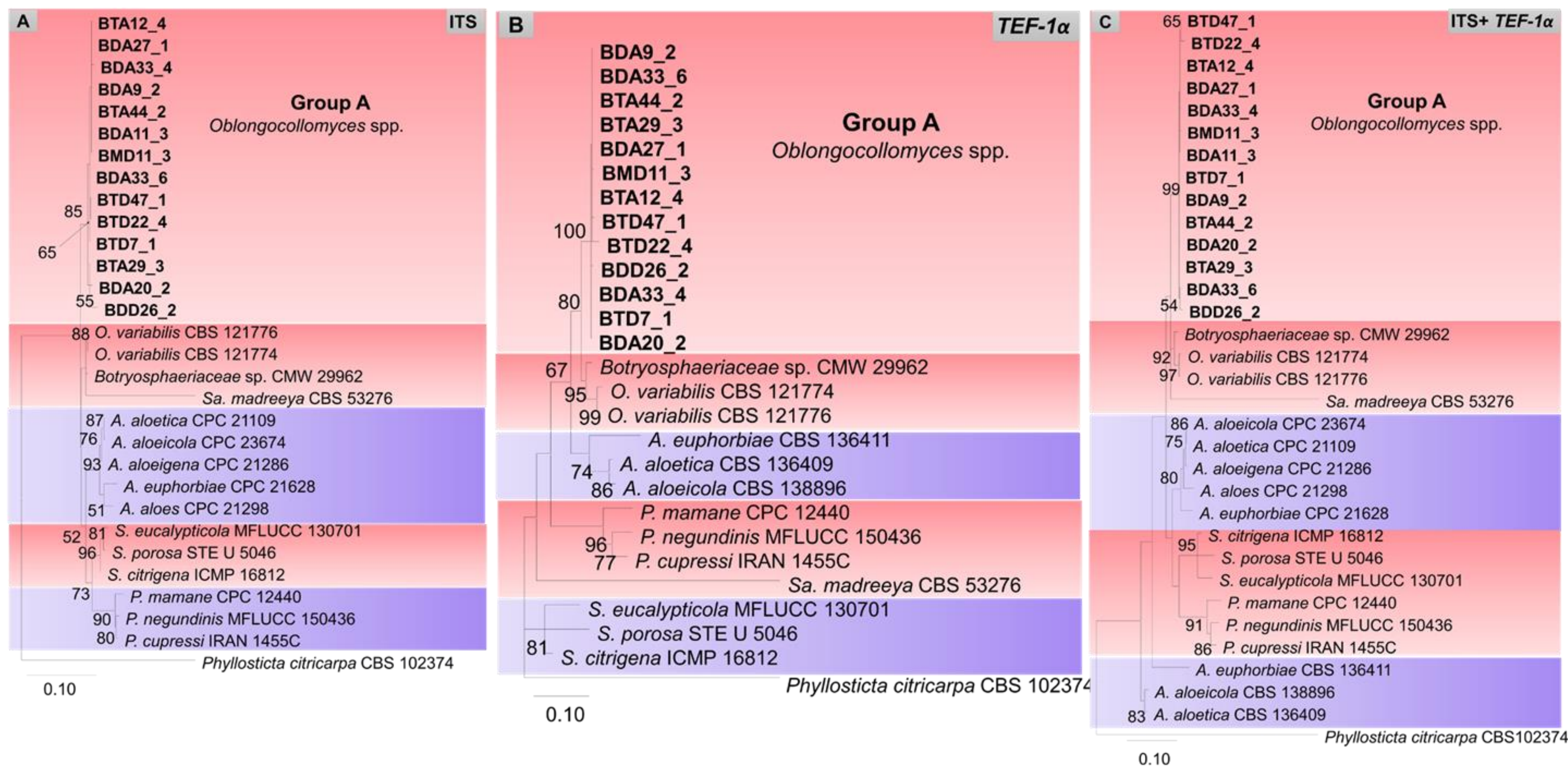


Figure 3 A-C. Maximum Likelihood (ML) trees of *Oblongocollomyces* species obtained from ITS, *TEF-1α*, and the combined sequence data ITS+ *TEF-1α*, showing the phylogenetic placement of isolates obtained from *B. discolor* branches. Bootstrap values above 60% are given at the nodes. Isolates in this study are shown in bold and the tree was rooted to *Phyllostica citricarpa* (CBS102374).

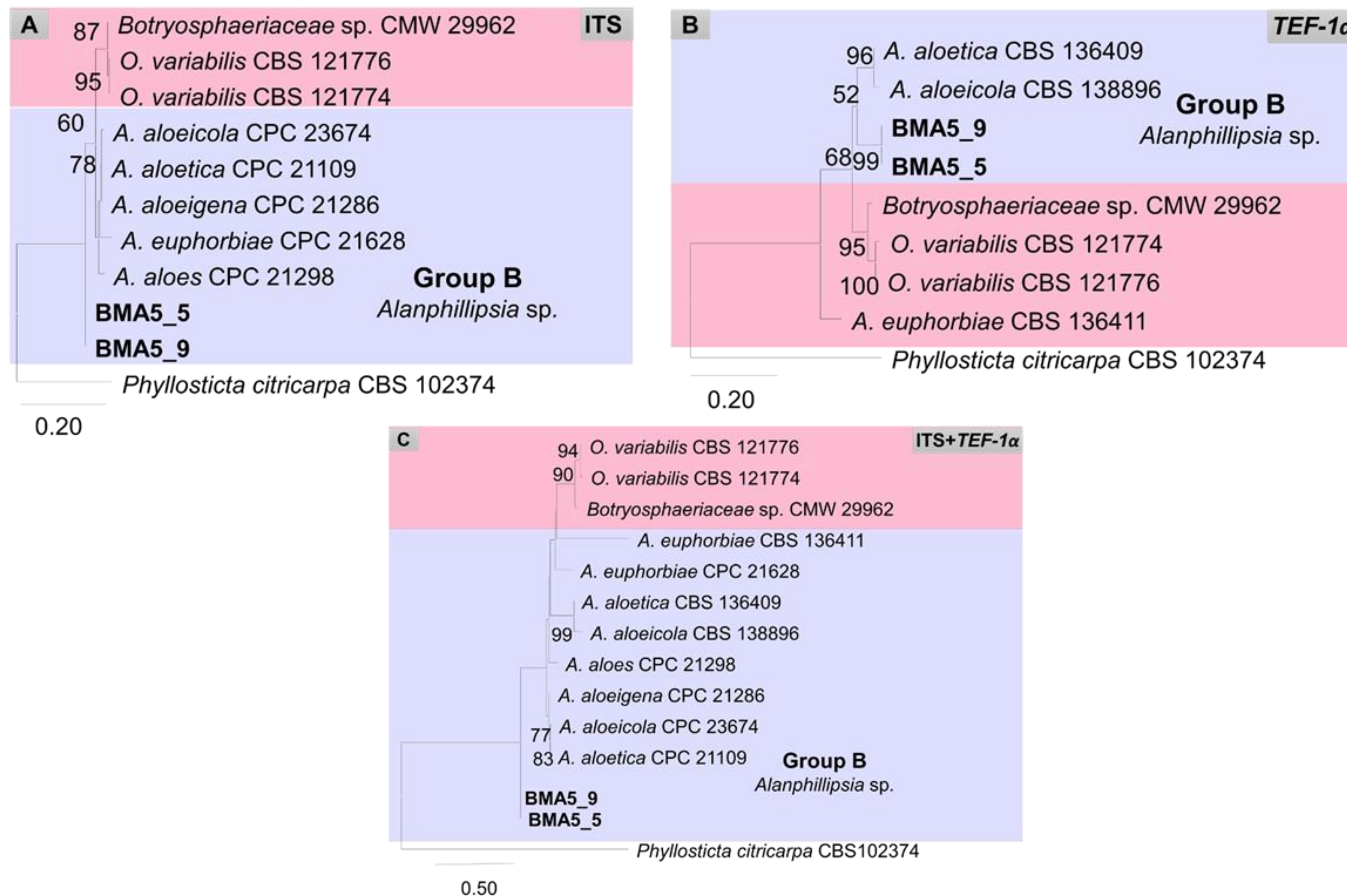


Figure 4 A-C. Maximum Likelihood (ML) tree of *Alanphillipsia* species obtained from ITS (A), *TEF-1α* (B) and combined ITS+*TEF-1α* (C) sequence data, showing the phylogenetic placement of isolates obtained from *B. discolor* branches. Bootstrap values above 60% are given at the nodes. Isolates in this study are shown in bold and the tree was rooted to *Phyllostica citricarpa* (CBS102374).

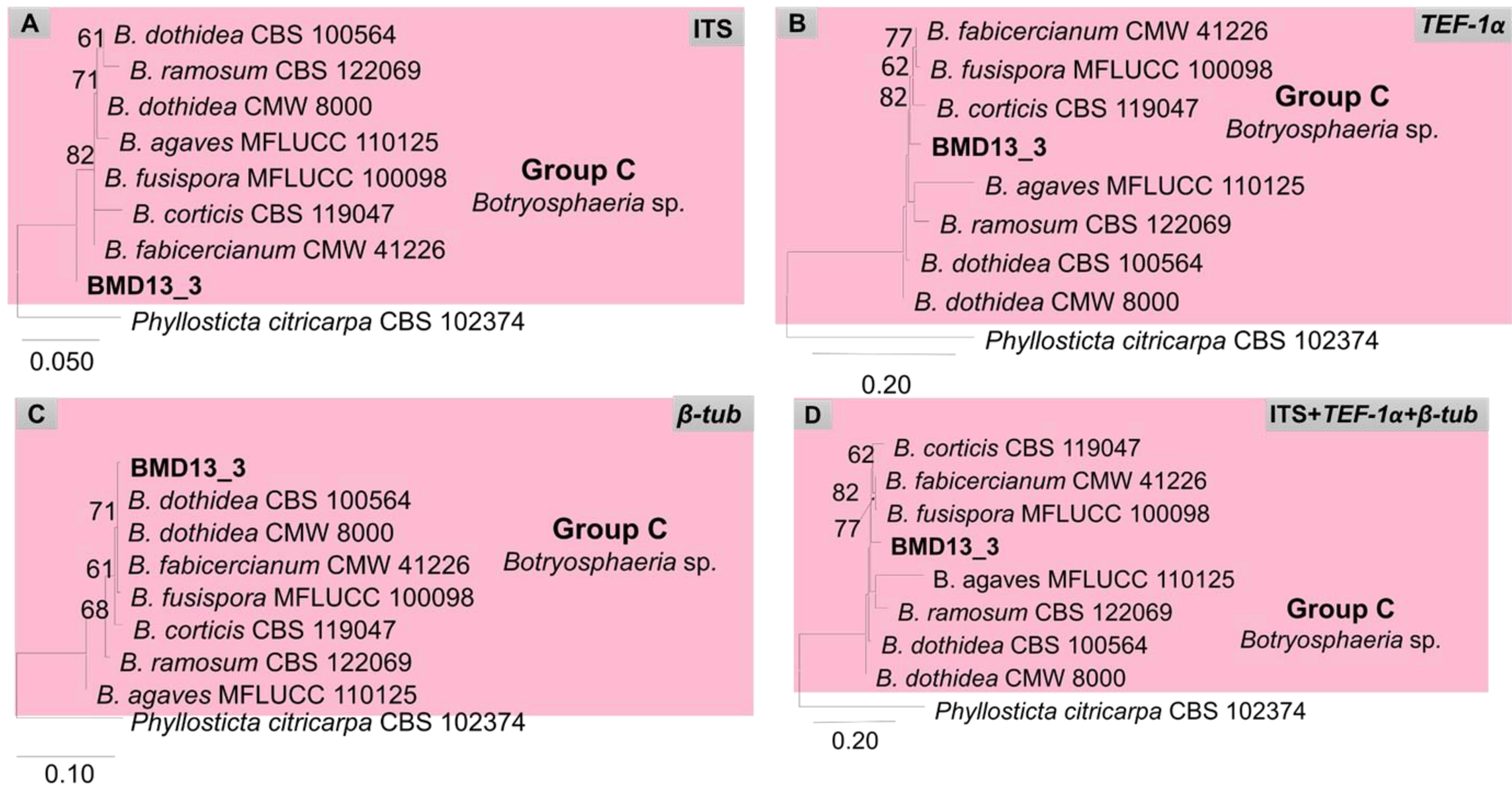


Figure 5 A-D. Maximum Likelihood (ML) trees of *Botryosphaeria* species obtained from ITS, *TEF-1α*, *β-tub* and the combined sequence data ITS+*TEF-1α*+*β-tub*, showing the phylogenetic placement of isolates obtained from *B. discolor* branches. Bootstrap values above 60% are given at the nodes. Isolates in this study are shown in bold and the tree was rooted to *Phyllostica citricarpa* (CBS102374).

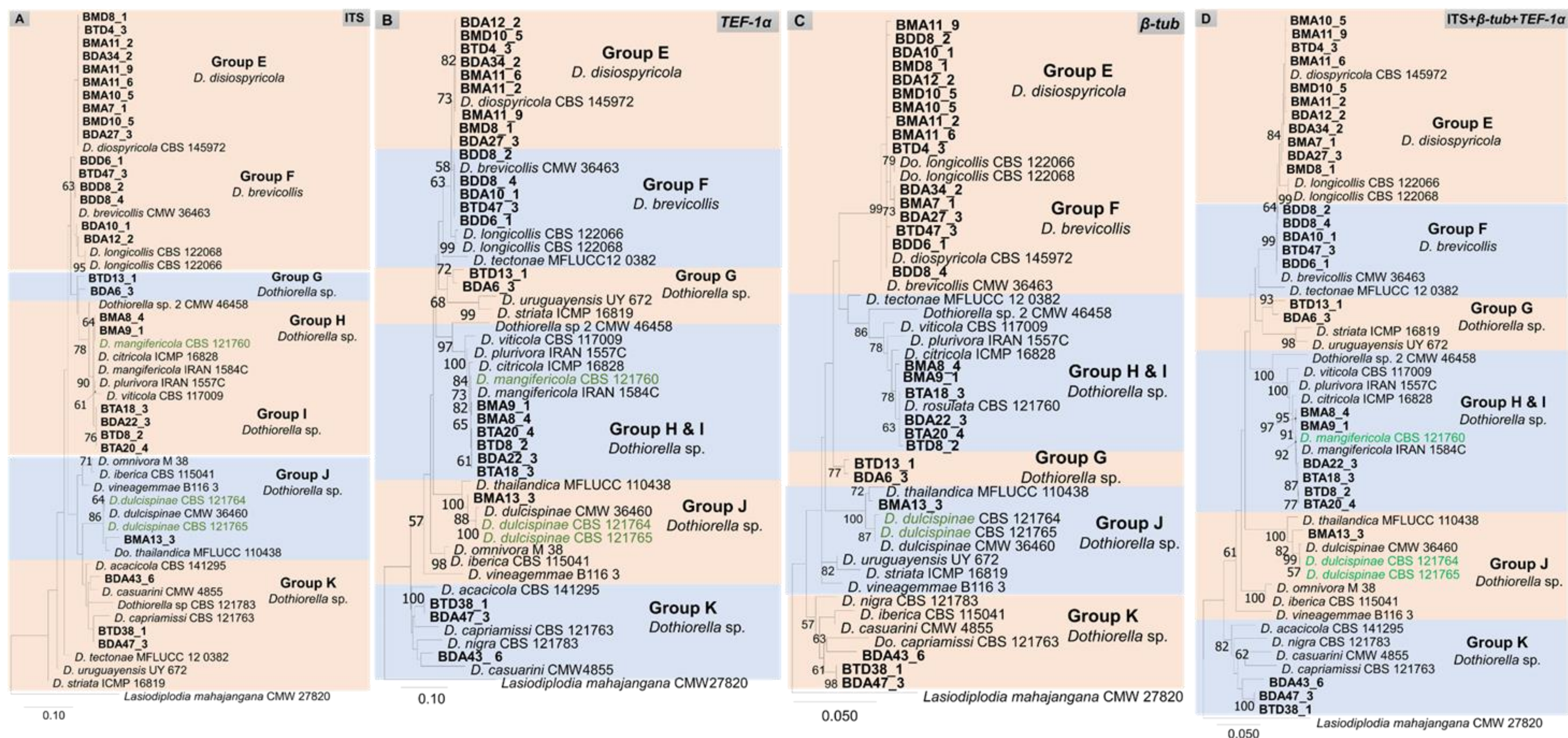
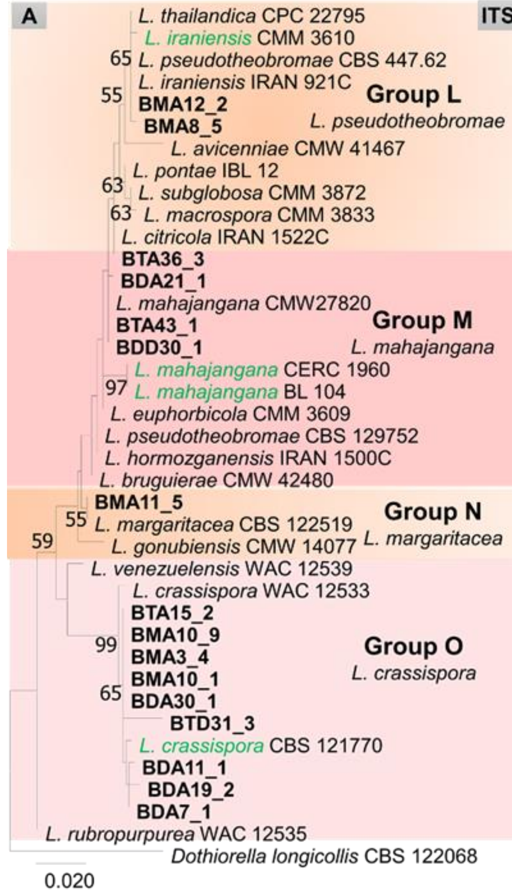
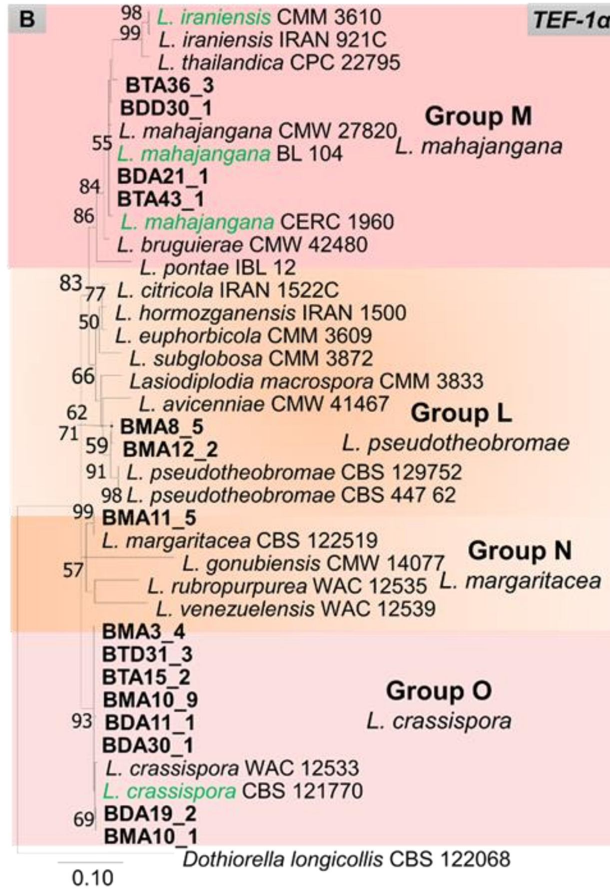
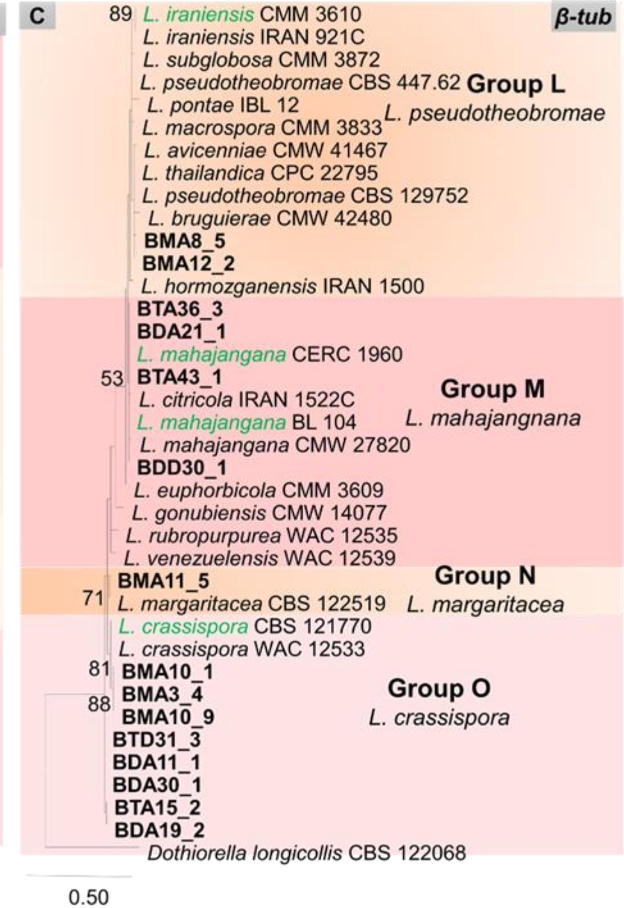


Figure 6 A-D. Maximum Likelihood tree of *Dothiorella* species obtained from ITS, *TEF-1α, β- tub* and ITS+*TEF-1α+β-tub* sequence data, showing the phylogenetic placement of isolates obtained from *B. discolor* branches. Bootstrap values above 60% are given at the nodes. Isolates in this study are shown in bold, the synonymised reference sequences are highlited in green and the tree was rooted to *Lasiodiplodia mahajangana* (CMW27820).


A
ITS
L. thailandica CPC 22795
L. iraniensis CMM 3610
65
L. pseudotheobromae CBS 447.62
L. iraniensis IRAN 921C
55
BMA12_2
BMA8_5
Group L
L. pseudotheobromae
L. avicenniae CMW 41467
L. pontae IBL 12
63
L. subglobosa CMM 3872
63
L. macrospora CMM 3833
L. citricola IRAN 1522C
BTA36_3
BDA21_1
L. mahajangana CMW27820
BTA43_1
BDD30_1
Group M
L. mahajangana
L. mahajangana CERC 1960
97
L. mahajangana BL 104
L. euphorbicola CMM 3609
L. pseudotheobromae CBS 129752
L. hormozganensis IRAN 1500C
L. bruguierae CMW 42480
BMA11_5
55
L. margaritacea CBS 122519
L. gonubiensis CMW 14077
Group N
L. margaritacea
59
L. venezuelensis WAC 12539
L. crassispora WAC 12533
BTA15_2
99
BMA10_9
BMA3_4
BMA10_1
65
BDA30_1
BTD31_3
L. crassispora CBS 121770
BDA11_1
BDA19_2
BDA7_1
Group O
L. crassispora
L. rubropurpurea WAC 12535
Dothiorella longicollis CBS 122068
0.020


B
TEF-1α
98
L. iraniensis CMM 3610
99
L. iraniensis IRAN 921C
L. thailandica CPC 22795
BTA36_3
BDD30_1
L. mahajangana CMW 27820
55
L. mahajangana BL 104
Group M
L. mahajangana
84
BDA21_1
BTA43_1
86
L. mahajangana CERC 1960
L. bruguierae CMW 42480
L. pontae IBL 12
83
77
L. citricola IRAN 1522C
L. hormozganensis IRAN 1500
50
L. euphorbicola CMM 3609
L. subglobosa CMM 3872
66
Lasiodiplodia macrospora CMM 3833
L. avicenniae CMW 41467
62
71
BMA8_5
59
BMA12_2
Group L
L. pseudotheobromae
91
L. pseudotheobromae CBS 129752
98
L. pseudotheobromae CBS 447 62
99
BMA11_5
L. margaritacea CBS 122519
57
L. gonubiensis CMW 14077
L. rubropurpurea WAC 12535
L. venezuelensis WAC 12539
Group N
L. margaritacea
BMA3_4
BTD31_3
BTA15_2
BMA10_9
93
BDA11_1
BDA30_1
L. crassispora WAC 12533
L. crassispora CBS 121770
69
BDA19_2
BMA10_1
Group O
L. crassispora
Dothiorella longicollis CBS 122068
0.10


C
β-tub
89
L. iraniensis CMM 3610
L. iraniensis IRAN 921C
L. subglobosa CMM 3872
L. pseudotheobromae CBS 447.62
L. pontae IBL 12
L. macrospora CMM 3833
L. avicenniae CMW 41467
L. thailandica CPC 22795
L. pseudotheobromae CBS 129752
L. bruguierae CMW 42480
BMA8_5
BMA12_2
L. hormozganensis IRAN 1500
Group L
L. pseudotheobromae
BTA36_3
BDA21_1
L. mahajangana CERC 1960
53
BTA43_1
L. citricola IRAN 1522C
L. mahajangana BL 104
L. mahajangana CMW 27820
BDD30_1
L. euphorbicola CMM 3609
L. gonubiensis CMW 14077
L. rubropurpurea WAC 12535
L. venezuelensis WAC 12539
Group M
L. mahajangnana
BMA11_5
71
L. margaritacea CBS 122519
Group N
L. margaritacea
L. crassispora CBS 121770
L. crassispora WAC 12533
81
BMA10_1
88
BMA3_4
BMA10_9
BTD31_3
BDA11_1
BDA30_1
BTA15_2
BDA19_2
Group O
L. crassispora
Dothiorella longicollis CBS 122068
0.50

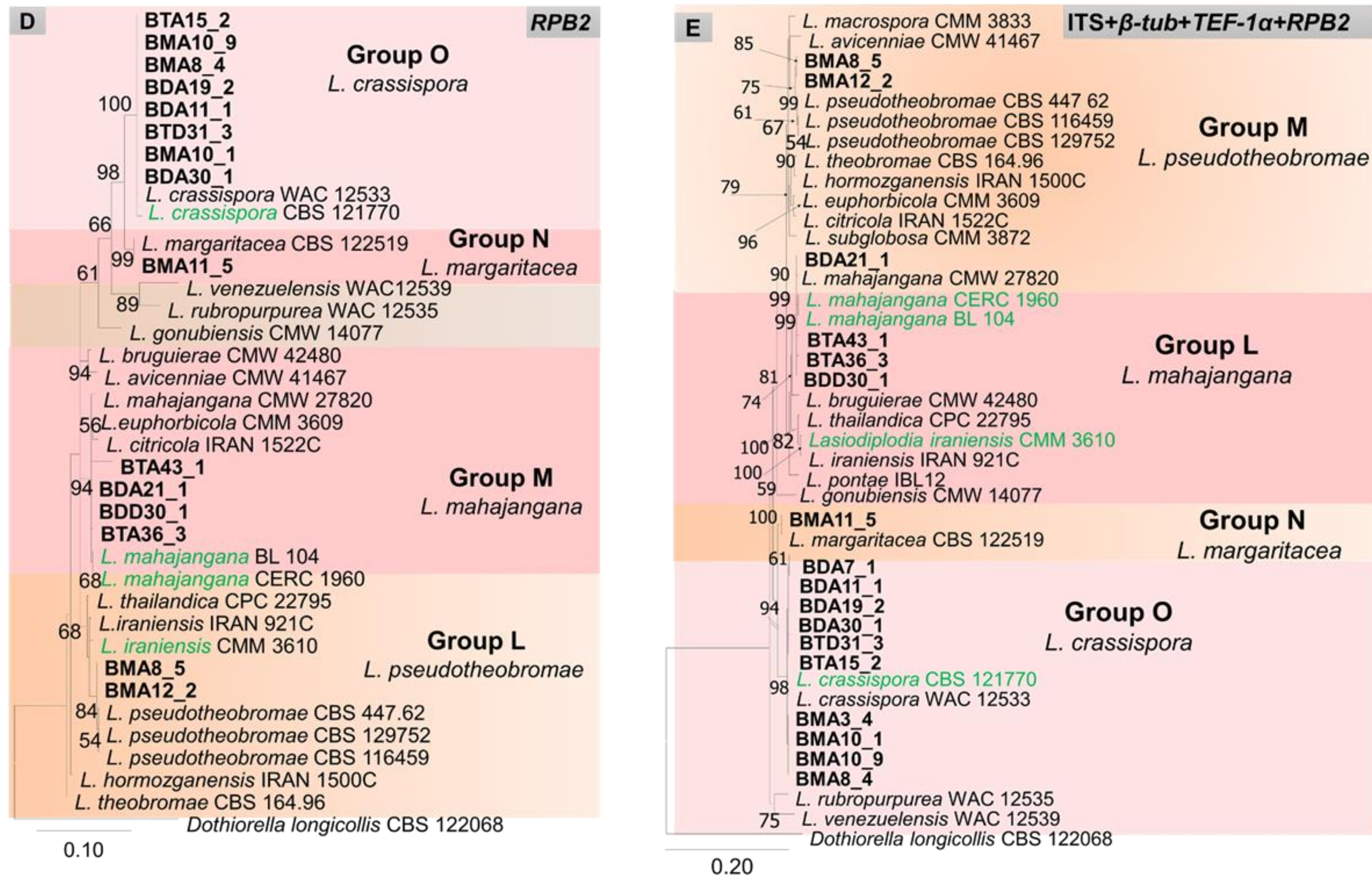


Figure 7 A-E. Maximum Likelihood (ML) tree of *Lasiodiplodia* species obtained from ITS, *TEF-1α, β-tub, rpb2* and ITS+*TEF-1α*+*β-tub*+*rpb2* sequence data, showing the phylogenetic placement of isolates obtained from *B .discolor* branches. Bootstrap values above 60% are given at the nodes. Isolates in this study are shown in bold, the synonymised reference sequences are highlited in green and the tree was rooted to *Dothiorella longicollis* (CBS122068).

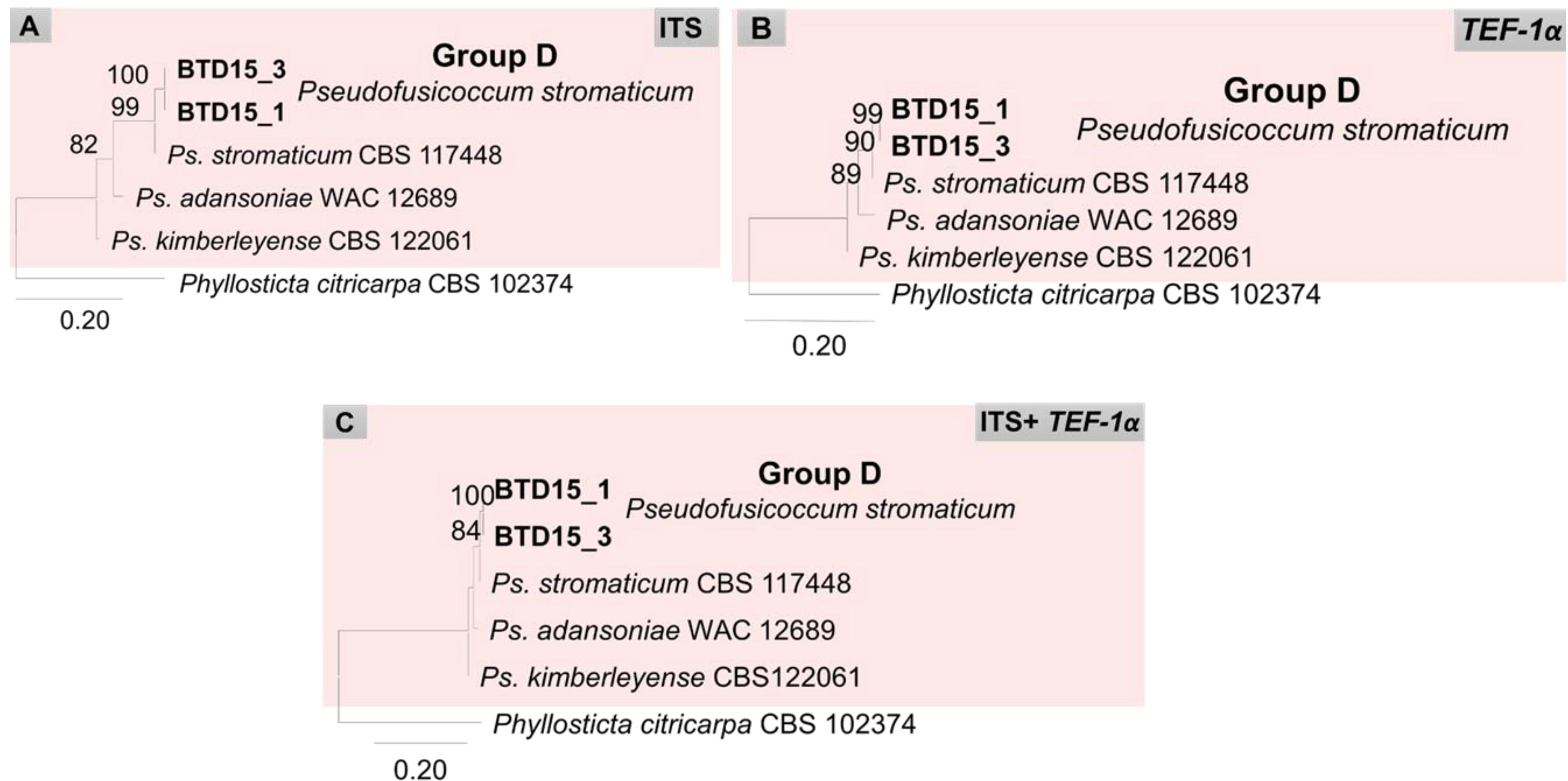
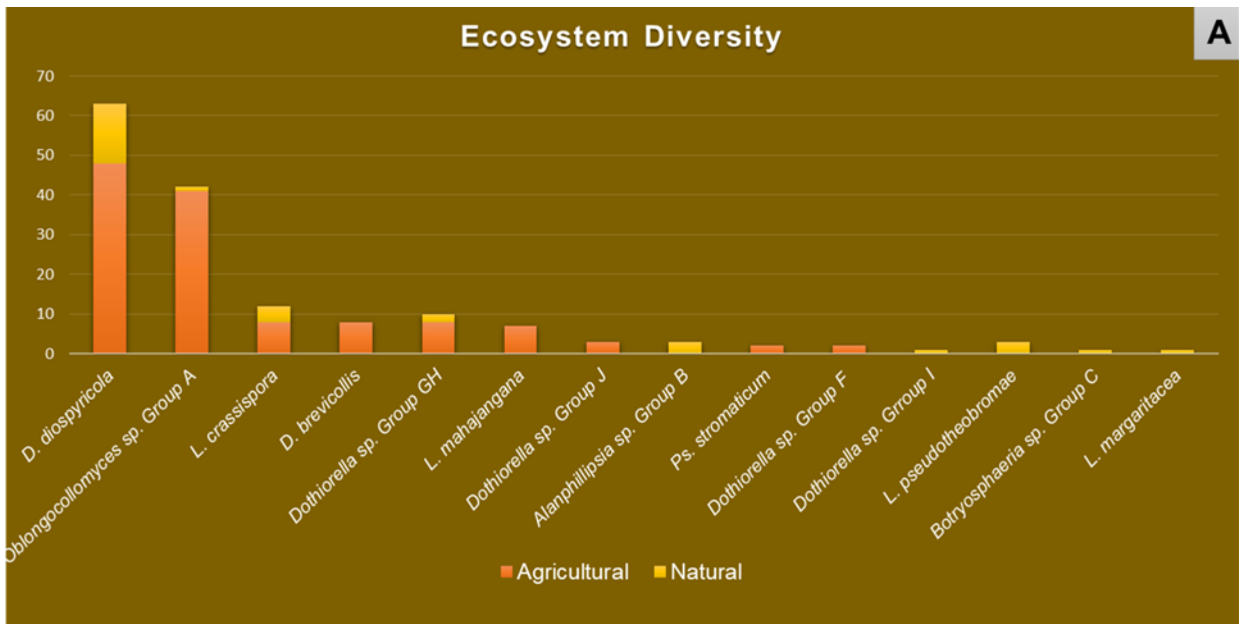


Figure 8 A-C. Maximum Likelihood (ML) tree of *Pseudofusicoccum* species obtained from ITS, *TEF-1α* and combined ITS+*TEF-1α* sequence data, showing the phylogenetic placement of isolates obtained from *B. discolor* branches. Bootstrap values above 60% are given at the nodes. Isolates in this study are shown in bold and the tree was rooted to *Phyllostica citricarpa* (CBS102374).



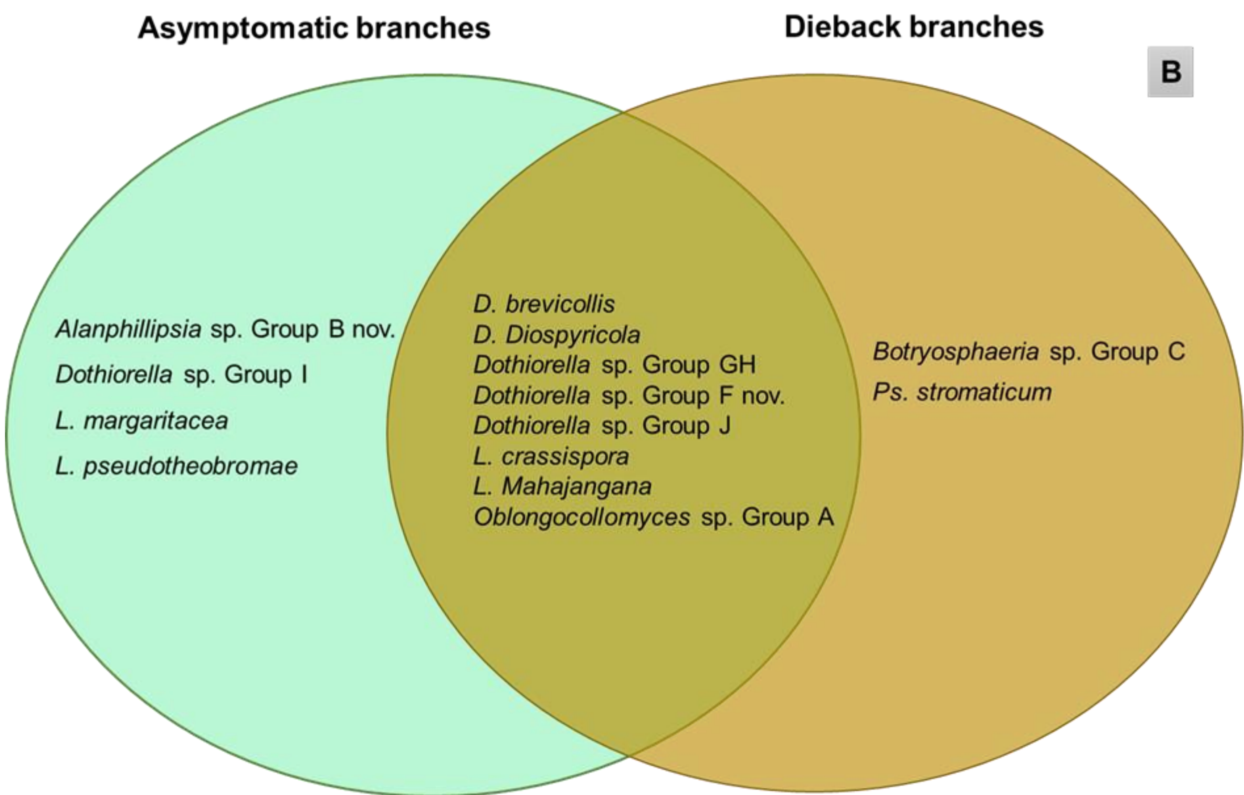


Figure 9. *Botryosphaeriaceae* species associated with branches of *B. discolor*. **A**: Bar chart showing the number of isolates representing a species in the agricultural and natural ecosystems; **B:** Venn diagram showing the species that occurred only on asymptomatic or on branches with dieback, and those that occurred on both the diseased and healthy branches.

SUMMARY

Previously, studies on *Berchemia discolor* trees focused on their pharmacological and nutritional properties. Despite the ecological, economical and pharmaceutical importance, little is known regarding the diseases affecting the species. One recently published paper dealing with fungi associated with *B. discolor* in Kenya reported 12 ascomycete species that cause dieback and cankers. Thus, the studies presented in this dissertation are the first that attempted to identify fungi associated with *B. discolor* in South Africa. A total of 29 species were tentatively identified from samples of *B. discolor* collected at different collection sites in the Limpopo Province of South Africa. These species were classified based on multi-gene DNA sequencing and the species belonged to the 17 genera within 10 families. out of the 29 species, eight species are potentially new to science. *Botryosphaeriaceae* species were the most notable and predominant in the natural ecosystems. Of the 29 species, 12 species overlapped on branches with dieback and asymptomatic branches, nine were isolated from branches with dieback, while 8 species were obtained from asymptomatic branches. No conclusive evidence could be found that the species obtained from the branches of *B. discolor* are the causal agents of the dieback disease, as Koch's postulate was not applied in this study through pathogenicity trials. This study, however, should be seen as a foundational study as limited samples were collected from one province. The results from the chapters presented in this dissertation warrant further research in which the sampling areas and the number of sampled trees should be expanded in order to realise the full extent of the fungal species diversity on *B. discolor* and their potential impact on the health of these trees in South Africa.