DIVERSITY OF FUNGI ASSOCIATED WITH DIEBACK OF *ZIZIPHUS MUCRONATA* IN LIMPOPO PROVINCE, SOUTH AFRICA

BY

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Dissertation for Master of Science (MSc) degree in Agriculture (Plant Pathology) in

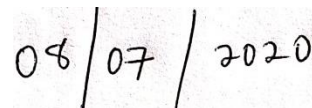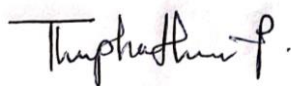**The Department of Plant Production,**

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DECLARATION

I, the undersigned declare that the dissertation, which I hereby submit to the University of Venda for the degree Master Science in Agriculture (Plant Pathology), is my own work and has not been previously submitted by me for a degree at this, or any other tertiary institution.

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As the supervisors/co-supervisor of the candidate, we agree to the submission of this dissertation.

09 July 2020

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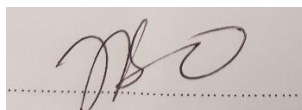   Signature   Date

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CONTENTS

PREFACE

Ziziphus mucronata (buffalo thorn: Rhamnaceae), is a valuable multipurpose fodder tree of considerable cultural importance in most of drier countries across the African continent such as South Africa. Various parts of the tree such as fruits and leaves are very nutritious and edible to both livestock and humans. Other parts of this tree such as wood and roots are used to make household implements (e.g., wooden spoons & and chairs) and as a source of medication for various infections (e.g., gonorrhoea, diarrhoea and dysentery) respectively. All these characteristics make *Z. mucronata* of great importance in semiarid to arid ecological areas of Africa.

Despite the uses of *Z. mucronata* by people in rural communities, the tree is faced by limiting factors such as diseases which affect the productivity of the tree. Among diseases found on this tree species, smut disease appears to be the only disease that is recorded thus far, therefore not much studies regarding diseases of this tree has been done. Smut disease is caused by a fungal pathogen *Coniodictyum chevalieri* and it was recorded for the first time in South Africa in the Kruger National Park.

The main aim for this study was to identify the fungal species associated with branch dieback of *Z. mucronata* in different locations of Limpopo Province; Buzzard Mountain Farm, Tshikundamalema and Wits Rural Facility. The further aim was to evaluate the diversity of Botryosphaeriaceae associated with *Z. mucronata* branch dieback in the three locations. The dissertation is composed of four chapters, of which two of them are research chapters. Chapter 1 is general introduction of the research topic combined with the literature review. The introduction section familiarises the reader with the study providing an overview of the tree of interest, usefulness of the tree and the aims of this study. The literature review provides information about the fungal diseases and their causal agents in the Rhamnaceae. Molecular and morphological methods used to identify fungal organisms are also reviewed in this chapter.

Chapter 2 is composed of detailed materials and methods that were used to carry out this study. This chapter describes all the study sites and explains how samples were collected from these sites. It further describes how the pathogens of interest ware isolated from infected plant parts and also how these pathogens were identified using molecular methods.

Chapter 3 is the first research chapter that dealt with the diversity of fungi found on branches of *Z. mucronata* showing dieback symptoms. This chapter provides a detailed report of the fungal species that were identified from trees in Buzzard Mountain Farm, Tshikundamalema

and Wits Rural Facility. The chapter also compares these fungi among the three locations and reports the frequently recorded fungi that could possibly be the causal agents of branch dieback on *Z. mucronata*. Fungal species that were collected, were identified and characterised based on comparison of DNA sequence data.

Chapter 4 is the second research chapter in which the diversity of fungi in the Botryosphaeriaceae associated with *Z. mucronata* in Limpopo Province is evaluated. Fungi in the Botryosphaeriaceae are well known as opportunistic endophytic plant pathogens, responsible for a number of symptoms such as dieback, wilting and cankers in both agricultural and undisturbed ecosystems. The aim of this chapter was to identify the fungal species from the Botryosphaeriaceae found on branch dieback of *Z. mucronata* in Buzzard Mountain Farm, Tshikundamalema and Wits Rural Facility. The chapter also provides a comparison of these fungi among the three locations. Fungal species in the Botryosphaeriaceae were identified based on comparison of multiple DNA sequence data.

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ABSTRACT

Ziziphus mucronata (buffalo thorn, Rhamnaceae) is an indigenous tree that serves multipurposes to rural communities and wildlife across Africa. The tree is considered important

because of its useful parts for various purposes. For example, leaves of this tree can be consumed as a vegetable by humans and wild animals such as antelopes and baboons feed on them. Fruits from *Z. mucronata* are edible and nutritious to both human and wild animals such as monkeys. Roots from this tree are used for medicinal purposes by people living in rural areas for treatment of wounds, snake bites, swelling glands as well as diarrhoea. However, the tree face diseases such as dieback that negatively affect its production and there is little research on diseases of *Z. mucronata* in South Africa. This study was conducted in Limpopo Province, in three different sites namely Tshikundamalema, Buzzard Mountain Farm and Wits Rural Facility to identify fungi from branches of *Z. mucronata* showing dieback symptoms. Symptomatic branches were collected from each site and processed in the laboratory before primary isolations. Isolates obtained from the samples collected were identified based on their morphology where isolates were grouped according to their morphological characteristics such as colour and structure of mycelia. The isolates were further identified based on DNA sequence data from multiple genome regions including the internal transcribed spacer (ITS), beta-tubulin (BT) and the translation elongation factor (TEF) genomic regions and phylogenetic analyses. Fungi identified in this study were from families Botryosphaeriaceae, Diaporthaceae, Cytosporaceae (=Valsaceae), Nectriaceae, Pleosporaceae and Didymellaceae. Fungi identified include *Dothiorella* (=*Spencermartinsia*), *Diplodia*, *Botryosphaeria*, *Neofusicoccum*, *Fusarium*, *Diaporthe* (=*Phomopsis*), *Cytospora*, *Didymella* (=*Phoma*) and *Alternaria*. Results obtained from this study showed the diversity of fungi associated with dieback of *Z. mucronata* in Limpopo Province. Studies in other parts of Limpopo Province are needed to further investigate the diversity of fungi found on branches of *Z. mucronata* with dieback.

Keywords: Dieback, DNA sequence data, fungi, ITS genomic region, BT genomic region, TEF genomic region, Phylogenetic analyses and *Ziziphus mucronata.*

CHAPTER 1: General introduction and literature review: Fungal pathogens associated with dieback and other diseases on Rhamnaceae

SECTION 1: General introduction

1.1 Background

Resource-poor rural communities depend on indigenous trees for their livelihoods. Among these widely utilized trees in Limpopo Province (South Africa), are the marula, brown ivory and buffalo thorn. *Ziziphus mucronata* (buffalo thorn) belongs to Family Rhamnaceae, which is a cosmopolitan family that includes trees, shrubs, climbers, and one herb, which make up approximately 50 genera and 900 species that are more common in the subtropical and tropical regions (Richardson *et al.,* 2000). Species in the Rhamnaceae exhibit xeromorphic adaptations that include reduced or absent leaves, crowding of leaves, shortening of branch axes and presence of thorns or spines (Richardson *et al.,* 2000). In Rhamnaceae, *Ziziphus* is probably one of the more widely studied genera that includes *Z. mucronata*, *Z. jujuba*, *Z. spina-christi* and *Z. mauritiana* (Mahajan and Chopda, 2009).

Ziziphus mucronata is an indigenous tree that grows up to 10m high, with a wide canopy of branches that contains thorns (Orwa *et al.,* 2009, Schmidt *et al.,* 2002). The tree is an important drought-tolerant species that is distributed across the African continent and occurs in various countries including Angola, Botswana, Ethiopia, Ghana, Kenya, Lesotho, Mozambique, Namibia, Niger, Senegal, Somalia, South Africa, Sudan, Eswatini, Tanzania, Uganda and Zimbabwe (Fig. 1.1 below) (Orwa *et al.,* 2009). The tree is considered important by people from rural communities in South Africa and other countries because parts of this tree are used for medicinal purposes as well as a source of food for humans and wild animals (Mazibuko, 2007).

Roots and stem extracts of *Z. mucronata* are used by traditional medical practitioners to treat bacterial infections such as gonorrhoea, syphilis, cholera, dysentery and boils (Mokgolodi *et al.,* 2011, Orwa *et al.,* 2009). Other parts of the tree such as leaves, wood and fruits are also useful to rural communities. The fruits are edible and in times of scarcity, they are used to prepare porridge and to make traditional beer through fermentation (Setshogo and Fenter, 2003). The leaves of *Z. mucronata* are important as a source of food as they are edible and nutritious when young and can be eaten as a vegetable when properly cooked. The tree also plays an important role in the food chain of wild animals such as birds and warthogs as they feed on leaves and the fruits (Mazibuko, 2007; Orwa *et al.,* 2009). In addition to being used as food and for medicinal purposes, wood from *Ziziphus* spp. is utilized for various purposes.

The timber of *Z. mucronata* contains 12 - 15% tannin which makes it resistant to termites and is used for fencing posts, wagons and for making a variety of household items such as tables, chairs, spoons, and dishes (Palmer and Pitman, 1972). The wood is dense and good for firewood and for making charcoal. It can also be used to manufacture agricultural implements (Orwa *et al.*, 2009). *Ziziphus mucronata* is a multipurpose tree of considerable cultural importance in Eastern and Southern Africa, with many traditions and cultural beliefs attached to it (Mazibuko, 2007). It is associated with tradition as the belief is that it protects from lightning when hiding under them.

Figure 1.1 Map showing the savannah biome distribution of *Ziziphus mucronata* in Africa (Countries coloured blue) (Maier *et al.*, 2006). Stars are indicating countries in which smut disease has been identified on *Z. mucronata*.

Among the Rhamnaceae, *Ziziphus jujuba* is the most notable economic fruit tree within the genus *Ziziphus* (Richardson *et al.*, 2000). *Ziziphus jujuba*, commonly known as Jujube, is indigenous to China with a history of over 4000 years, widely distributed in Europe, southern and eastern Asia, and Australia (Gao *et al.*, 2013). There are over 700 cultivars of jujube trees in China and this is the only country well known to be exporting jujube fruits to other countries, with over 1.5 million hectares planted with jujubes (Guo *et al.*, 2010). The fruits and leaves have been consumed by humans for thousands of years and it is believed that these consumptions prolong people's life-span by nourishing blood and regulating the digestive system (Chen *et al.*, 2017). Jujube fruits have a high content of vitamin C and therefore are considered a good source of vitamin C for human nutrition, and moreover, jujube fruits, although to a lesser extent, are a source of several other Vitamins, such as Thiamin, Riboflavin, Niacin, Vitamin B6, and Vitamin A (Table 1) (Gao *et al.*, 2013).

Table 1.1 Vitamin compositions of Jujube fresh fruits.

Vitamins	Content per (100g) fruit	Vitamin daily allowance for humans (Adults)	
		Males	**Female**
Vitamin C (mg)	69.0	90 mg	75 mg
Thiamin (mg)	0.02	1.2 mg	1.1 mg
Riboflavin (mg)	0.04	1.3 mg	1.1 mg
Niacin (mg)	0.9	17 mg	17 mg
Vitamin B6 (mg)	0.081	1.3 mg	1.3 mg
Vitamin A (mg)	26.8	14.74 mg	11.73 mg

Ziziphus trees are affected by fungal diseases that cause plant disfigurement, crop loss due to reductions in yield and quality, and plant death in highly severe cases (Mirzaee, 2014). Despite their importance, overall very little attention has been afforded to identify and study the fungal pathogens that cause diseases on these trees. In South Africa, smut disease is the only fungal disease that has been recorded to date on *Z. mucronata*, hence there is a need to document other diseases which affect the tree.

Ziziphus species are affected adversely by biotic (e.g. fungal diseases) and abiotic (e.g. unfavourable climate conditions) factors that reduce their productivity and this reduces their usefulness by people who largely depend on them as a source of food, income and traditional medicines. Fungi are diverse organisms that assemble in complex and dynamic communities in nature where they can act as saprophytes or pathogens of plants. Among the microbiota, fungi are considered as being the most important group that is responsible for plant diseases, even though only about 10% of the known fungi are capable of colonizing and infecting plants (Knogge, 1996). Fungal pathogens cause diseases that lead to catastrophic losses in agricultural crops, plantations and indigenous trees. For example, a financial loss of R 9.5 million ($708 995,22) was estimated on pine plantation per year due to *Sphaeropsis sapinea* in South Africa (Zwolinski *et al.*, 1990).

The effect of fungal pathogens on plants depends on their virulence. Highly virulent pathogens have a higher infection potential on healthy plants than those with lower virulence (Surico, 2013) and they also have the potential to cause more than one disease symptom, regardless of the health status of the plants. Pathogens with lower virulence are usually found in association with stressed plants (Viljoen *et al.*, 1992). For example, some member species of the Botryosphaeriaceae are considered highly virulent and will infect and cause disease in seemingly healthy plants. For example, *Neolfusicoccum mangroviorum*, *N. variabile* and *Pseudofusicoccum africanum* was found pathogenic to *Mimusops caffra* trees growing on the

east coast of South Africa (Jami *et al.*, 2018). However, members of this family are also considered opportunistic because they infect plants that are under stress due to drought and physical damage such as hail (Slippers and Wingfield, 2007, Smith *et al.*, 1994). For example, *N. australe* was identified as an opportunistic fungi responsible for crown dieback of *Agonis flexuosa* in Western Australia (Dakin *et al.*, 2010).

Apart from being plant pathogens, fungi present other forms of relationships with plants. These forms involve fungi occurring in plants as endophytes and as saprophytes decomposing dead plant material. Fungal endophytes have been known to inhabit every forest tree without showing any symptoms. However, some fungal endophytes are regarded opportunistic because of their capability of turning into pathogens and causing diseases when plants are stressed (Gimenez *et al.*, 2007). For example, *Lasiodiplodia theobromae* and *Diplodia* species have been found to occur endophytically in *Pinus* seeds (Cilliers *et al.*, 1995, Smith *et al.*, 1996), and later found causing cankers and dieback symptoms on *Pinus* trees (Úrbez-Torres *et al.*, 2016). Opportunistic endophytic fungi pose a particular threat to tree and plant health as they can easily, and unobtrusively, be moved around the world within seeds, cuttings and even fruits (Carroll, 1988). Saprophytic fungi play an important role in both natural and cultivated ecosystems in terms of nutrient recycling and the formation of humus in the soil (Berg, 2000). These microorganisms achieve all this through the decomposition of plant litter or dead plant material by attacking the lignocellulose matrix that other organisms are not able to assimilate (Adl, 2000 cited by Kubartová *et al.* (2009).

The introduction of plant pathogens from other countries into new areas has led to many plant disease epidemics globally. A study by Desprez-Loustau *et al.* (2007) mentioned that 65 - 85% of plant pathogens are non-native in the areas where they occur. The study by these authors further showed that invasions by non-native fungi may result in significant ecological, economic and social consequences. Well-known examples of introduced plant pathogens in South Africa include *Sphaeropsis sapinea* and *Rhizina undulata*, which can cause devastating damage to *Pinus* species after hail and fire (Zwolinski *et al.*, 1990). Another example of an introduced pathogen is *Phytophthora cinnamomi* in south-western Australia (Hardham, 2005). This pathogen substantially altered the native plant communities by killing dominant *Eucalyptus marginata* and most of Proteaceae species in Western Australia. The rapid spread of this pathogen in many countries has continued to cause extensive economic losses in agriculture, horticulture and forestry, and the pathogen is a major threat to natural ecosystems and biodiversity (Hardham, 2005).

Not much is known about fungal diseases affecting *Z. mucronata* except for smut caused by *Coniodictyum chevalieri*, which was reported by Maier *et al.* (2006) in South Africa for the first

time. The pathogen caused severe damage on fruits and branches of *Z. mucronata*. Diseases occurring on this important indigenous tree need to be given attention since they limit the productiveness and utilization of the tree. Therefore, this study aimed to determine the diversity of fungi associated with dieback on *Z. mucronata*. Dieback is a condition in plants where branches and shoots die from the tip progressing inwards. This condition is usually caused by biotic factors such as pathogenic microorganisms as well as by abiotic factors such as unfavourable environmental conditions (Abengmeneng, 2013). Dieback is one of the most significant diseases affecting forest trees and has been identified by several studies on many trees around the world.

1.2 Problem statement

Indigenous trees, like other plants, are affected by biotic and abiotic factors that negatively impact their growth and development, hence reducing their utilization (Haferkamp, 1987). Abiotic stress conditions such as drought, unfavourable temperatures and salinity are known to potentially influence the occurrence and spread of biotic agents such as pathogens, insects and weeds (Pandey *et al.*, 2017). *Ziziphus* trees are affected by fungal diseases that cause plant disfigurement, crop loss due to reductions in yield and quality, and plant death in highly severe cases (Mirzaee, 2014). Despite the social importance of *Z. mucronata*, very little attention has been afforded to identify and study the fungal pathogens that cause diseases on the tree. The only record is that of smut disease on trees in the Kruger National Park (South Africa) by Maier *et al.* (2006), which was the first report of a fungal disease associated with this tree. Hence there is a need to document other diseases that affect the tree. This study aimed to identify fungi associated with dieback on branches of *Z. mucronata* as this condition has the potential to negatively affect the health and productivity of these trees.

1.3 Research questions

- What is the diversity of fungal species found on branches of *Z. mucronata* with dieback at different locations in Limpopo Province?
- What is the diversity of species in the Botryosphaeriaceae found on *Z. mucronata* in Limpopo Province?

1.4. Aims and objectives

The first aim of this study was to identify fungal species that are associated with branches of *Z. mucronata* with dieback in different locations in Limpopo Province. The second aim was to evaluate the diversity of species in the Botryosphaeriaceae found on *Z. mucronata* branches with dieback in Limpopo Province since fungi in this family are mostly associated with this

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condition of many woody species in South Africa and globally and there is also no record on this from Limpopo Province.

SECTION 2: Review of fungal diseases on the Rhamnaceae

2.1 Introduction

Pathogenic fungi cause diseases that result in huge losses in yield and quality of crops, fruits and other edible plant material. This results in a negative impact on human livelihoods and the

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economy of the country (Yang *et al.,* 2017). Fungal pathogens causing dieback on trees in the natural and agricultural ecosystems where they attack vascular tissues, disrupt water, carbohydrates and mineral flow within the host, thereby interfering with plant health. Eventually, it may cause death of the entire tree (Hodel *et al.,* 2012).

In South Africa, some of well-known fungal diseases on trees include pitch canker that is caused by *Fusarium cricinatum* on *Pinus* species (Coutinho *et al.,* 2007), dieback caused by *Colletotrichum gloeosporioides* on *Eucalyptus* species (Smith *et al.,* 1998), *Armillaria* root rot caused by *Armillaria* species on botanical trees (*Virgilia oroboides*, *Ekebergia pterophylla*, *Leucadendron strobilium*, *Olea capensis*, *Widdringtonia schwarzii*) (Coetzee *et al.,* 2018) and black spot caused by *Guignardia citricarpa* on citrus species (Carstens *et al.,* 2012). Among the trees in the Rhamnaceae, there is not much documented information on fungal diseases affecting the tress. This review will discuss the fungal species and their hosts from the Rhamnaceae. The review will also cover morphological and molecular techniques used for fungal identification.

2.2 *Coniodictyum chevalieri*, a smut fungus infecting *Ziziphus mucronata*

Smut fungi belong to the Basidiomycota and are a group of specialized pathogens that attack any plant parts above the ground such as leaves, stems and flowers. Most of the smut pathogens are highly specialized on a few host species and are known to affect mainly members of the Poaceae (Begerow *et al.,* 2004). Examples of hosts in the Poaceae for smut fungi include *Zea mays*, *Z. mexicana*, *Tilletia elizabethae* and *T. ventenatae* (Pataky and Snetselaar, 2006, Denchev and Denchev, 2018). These fungi infect individual plant tissues causing abnormal growth of certain organs or tissues, such as galls or boils. In some cases, the entire plant is stunted and show giant growth of galls on branches, as in *Z. mucronata* (Piepenbring, 2009).

In South Africa, *Coniodictyum chevalieri* (Basidiomycota, Ustilaginomycetes, Exobasidiales, Cryptobasidiaceae) was confirmed to be the causal agent of smut on *Z. mucronata* (Maier *et al.,* 2006). *Coniodictyum chevalieri* is a rare fungus with a poorly known ecology and infection biology, belonging to a monotypic genus that resides in the Phylum Basidiomycota (Bauer *et al.,* 2001). The species combines two very unique features that make it easily recognisable: snow-white spore-producing galls breaking out of diverse organs of *Z. mucronata* and the shape of the multi-celled spores (Malençon, 1953) (Fig. 1.2). This fungus was first collected in Chad in 1909 by Chevalier and later found causing epidemics in Kruger National Park (KNP), South Africa (Maier *et al.,* 2006). Apart from South Africa and Chad, the presence of

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this fungal pathogen has been recorded in other countries such as Senegal and Zimbabwe (Maier *et al.*, 2006)(Fig.1.1 above).

In the KNP, Maier *et al.* (2006) suggested that smut infection caused a severe decline in vigour and consequently, flower and fruit production of *Z. mucronata*. The trees that were heavily infected in the previous year hardly produced fruits the following year. Furthermore, infected trees that were growing along the river seemed to have recovered well and produced fruit in abundance due to favourable soil conditions with higher water availability, which reduced the impact of the disease (Maier *et al.*, 2006). This implies that trees under stress due to unfavourable environment have very limited chances of recovery in such growing conditions, which may lead to tree death. The study by Maier *et al.* (2006) is the only documented record of fungal disease on *Z. mucronata*.
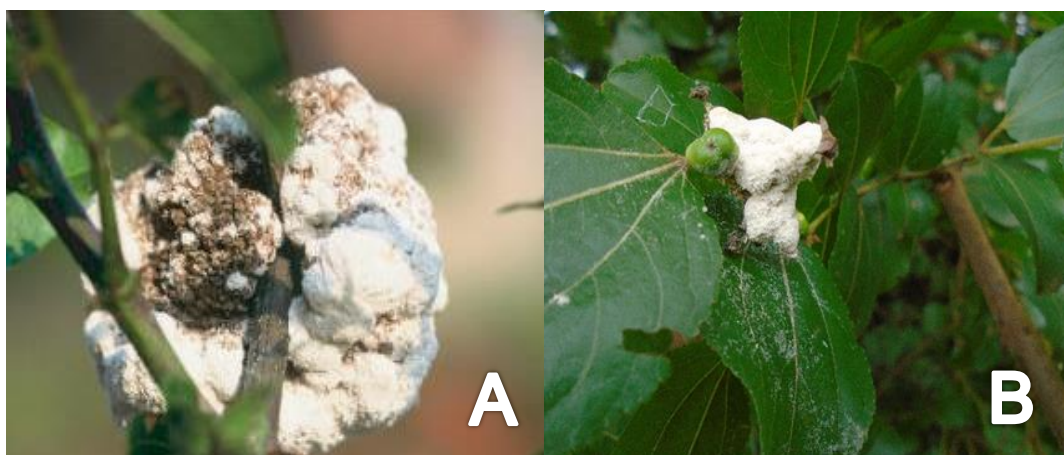
Figure 1.2 *Coniodictyum chevalieri* galls on leaves, branches, and fruits of *Z. mucronata* tree (A: Galls on branches; B: galls on fruits and leaves).

2.3 Other fungal diseases recorded from trees in the Rhamnaceae.

2.3.1 Botryosphaeriaceae species

Species of Botryosphaeriaceae are known to cause dieback on forestry and indigenous trees, as well as agricultural tree species such as mango (*Lasiodiplodia theobromae and L. iraniensis, L. pseudotheobromae*) and grapevine trees (*Botryosphaeria dothidea, Diplodia seriata, and Neofusicoccum parvum*) (Ismail *et al.*, 2012, Ammad *et al.*, 2014, Rodríguez-Gálvez *et al.*, 2017). Botryosphaeriaceae contains a wide range of morphologically diverse genera, with their member species occurring as either pathogens, endophytes or saprobes on woody hosts (Phillips *et al.*, 2013). These fungi are considered weak pathogens since they invade plants that are under stress and they have been shown to occur as latent pathogens in tree stems, branches, twigs and leaves and persist endophytically on woody hosts such as *Vachellia karroo* and *Eucalyptus* species, and produce symptoms during pathogenicity tests

(Jami *et al*., 2015, Smith *et al.,* 1994). Due to their endophytic occurrence on plants, these fungi can easily be moved around the world without knowing that they are present on the plants. This makes it very important to understand their characteristics and host range in order to employ effective quarantine strategies on plants that are moved across the world (Burgess and Wingfield, 2002, Smith *et al*., 1996). Members of the Botryosphaeriaceae are also capable of spreading and infecting both related and unrelated host plants, which increases the threat they pose as potential economic and ecologically important pathogens of native and cultivated trees globally (Mehl *et al*., 2017). In South Africa, *Diplodia africana and Lasiodiplodia plurivora* were isolated from symptomatic branches of *Prunus* species while *Botryosphaeria australis*, *B. lutea*, *B. obtusa*, *B. parva*, *L. theobromae* and *a Diplodia* species were isolated from diseased shoots of *Vitis* species (van Niekerk *et al*., 2004, Damm *et al*., 2007).

Species in the Botryosphaeriaceae gain access to their hosts through both natural openings (growth cracks, leaf scars, stomata and lenticels) and wounds, and they are associated with symptoms such as shoot blights, stem cankers, fruit rots, dieback and gummosis (Mehl *et al*., 2013, Slippers and Wingfield, 2007). The existence of these symptoms is then followed by extensive production of kino (a dark-red tree sap), and in severe cases, infected plants die (Jami *et al*., 2015, Mohali *et al*., 2007). Some members of the Botryosphaeriaceae infect the host from the stem of the inflorescence and colonize tissues progressing towards the stem, causing branch dieback, for example, *Dothiorella dominicana, Do. mangiferae, L. theobromae* on *Mangifera indica* (Johnson *et al*., 1992). Other species colonize dead branches and move down the branch into healthy sapwood and cause the death of the entire tree (Slippers and Wingfield, 2007).

There is no documentation of the Botryosphaeriaceae associated with *Ziziphus* trees. However, *Dothiorella* species (Botryosphaeriaceae) were identified from other Rhamnaceae trees; *Rhamnus alaternus* (Dissanayake *et al*., 2017b) and *Paliurus spina-christi* (Dissanayake *et al*., 2016a). *Dothiorella* species are found in a wide range of woody hosts, for example as pathogens on *Ostrya carpinifolia* (Pavlic-Zupanc *et al*., 2015) , endophytes on *Vachellia karroo* (Jami *et al*., 2012) and saprobes on *Rosa canina* (Dissanayake *et al*., 2017b, Crous *et al*., 2006). Dissanayake *et al*. (2016a) isolated *Do. sarmentorum* from diseased branches and twigs of *Paliurus spina-christi* in Italy and more recently, a study by Dissanayake *et al*. (2017b) recorded *Do. rhamni* form dead branches of *Rhamnus alaternus,* hence the fungal species was designated a saprophyte in their study.

2.3.2 *Fusarium* species

The genus *Fusarium* comprises filamentous fungi that are widely distributed, many of which are plant pathogens. Species of this genus are responsible for economically important diseases in agriculture and forestry (Gordon, 2006, Goswami and Kistler, 2004). Some *Fusarium* species have been reported from a variety of woody species, causing economic losses especially in pine plantations (Mitchell *et al.*, 2011, Wingfield, 1999), while in agriculture they cause yield losses and contaminate cereals with mycotoxins that are harmful to humans and animals (Halstensen *et al.*, 2006, Pasquali *et al.*, 2010). *Fusarium* species can also occur in plants as endophytes and only initiate infection when the host is under stress (Wingfield *et al.*, 2008). They also infect plant reproductive organs such as seeds without causing symptoms, which facilitate the spread of pathogens to new areas. For example, *F. circinatum* was introduced to South Africa through the importation of infected seeds and was first identified in 1990 from a pine production nursery in Mpumalanga, South Africa (Porter *et al.*, 2009). This fungus is one of the most significant fungal pathogens of pine species around the world (Wingfield *et al.*, 2008).

Dieback due to *Fusarium* species has not been reported on *Z. mucronata* anywhere in the world*,* however, it was reported on *Z. jujuba* in Iran for the first time by Mirzaee *et al.* (2011), where the causal agent was *F. solani.* Dieback is known to cause devastating losses that include the reduction of natural resources and the restriction of productivity in plants (Maloy, 2005). Dieback symptoms caused by *Fusarium* species observed by Mirzaee *et al.* (2011) include twig dieback, blackish discolouration of wood and foliage, and wilting followed by leaf shedding. *Fusarium solani* has also been reported causing dieback in other plant species such as palm and mango trees (Khanzada *et al.*, 2004). This pathogen has also been reported in North East India and West Bengal, causing one of the most destructive diseases on tea crops (Kumar *et al.*, 2016). *Fusarium solani* is frequently found associated with damage or stress events caused by biotic or abiotic factors (Mirzaee, 2014). Biotic or abiotic factors may include extended drought in combination with high temperatures and strong winds, vascular diseases, soil nutrient deficiencies, insect injury to the trunk or branches and excessive soil moisture (Anon, 2002).

Fusarium solani is also known to cause cankers on hardwood species in African countries such as Kenya and Tanzania and has been identified causing stem canker on *Maesopsis eminii* Engl. in Uganda (Brown, 1964). *Maesopsis eminii*, locally commonly known as musizi, is one of the most important indigenous hardwood and drought-tolerant species that belongs to the Rhamnaceae (Epila *et al.*, 2017). The tree is widely distributed across the African continent, and it is mainly planted and used for its good timber and crop shade services in

Uganda, Tanzania and Rwanda (Ani and Aminah, 2006, Orwa *et al.*, 2009). Canker symptoms caused by *F. solani* on *Maesopsis eminii* include yellowing and falling of leaves, small brownish patches on young bark, curling of the bark and exudation of a fermented fluid that attracts insects (Brown, 1964).

In China, *F. oxysporum was* found associated with wilt disease on *Z. jujuba* (Rhamnaceae) and this condition is known to be very closely related to dieback. This pathogen is known to cause wilt disease, damping off and crown and root rots of a wide variety of plants (Leslie and Summerell, 2008). *Fusarium oxysporum* is known to occur on host plants without producing any apparent symptoms. The fungus also causes diseases of important economic plants such as banana (Gordon and Martyn, 1997). The fungus is believed to occur in agricultural soils throughout the world as well as soils that have never been cultivated, which led to this fungal species being termed a global mycoflora (Parkinson, 1981). *Fusarium oxysporum* has been reported causing dieback on other tree species in other families including *Vachellia koa* and *Albizia julibrissin* (Anderson *et al.*, 2002) and was also recorded associated with cankers on *Cedrelinga cateniformis* in South America (Lombard *et al.*, 2008). The fungus exhibited symptoms that are similar to *F. solani* that were observed on *Z. jujuba* by Mirzaee *et al.* (2011), which shows that *F. oxysporum* has a wide host range. Zhang *et al.* (2012) reported fruit rot of jujube for the first time caused by *F. proliferatum* and Zhang *et al.* (2013) later reported *F. oxysporum* causing soft fruit rot of *Z. jujuba* for the first time in China as well.

Apart from causing diseases on trees in the Rhamnaceae, *Fusarium* species are also found on the trees in this family without showing any infection symptoms. A study by El-nagerabi *et al.* (2013) identified endophytic *Fusarium spp.* associated with healthy leaves on *Z. spina-christi* and *Z. hajanensis* from Saudi Arabia. The species isolated in their study were *F. chlamydosporum F. sambucinum* from both tree species and *F. lateritium*, *F. merismoides F. nivale*, *F. reticulatum* from *Z. hajanensis* only (El-nagerabi *et al.*, 2013). This may suggest that some of these species isolated as endophytes on *Ziziphus* species could be potential pathogens and this needs to be confirmed through pathogenicity trials.

2.3.3 *Diaporthe* species

Diaporthe species are known to occur as pathogens on a wide range of plant hosts, causing multiple diseases, some of which are economically important such as sunflower and citrus in Yugoslavia and China, respectively (Santos *et al.*, 2011, Huang *et al.*, 2013, Muntanola-Cvetkovic *et al.*, 1981). Furthermore, *Diaporthe* species are saprophytic on dead plant material such as decaying leaves, twigs and stem residues, but also colonize healthy plant parts as endophytes (Gomes *et al.*, 2013). These fungi have a worldwide distribution and wide host

ranges, such that, sometimes multiple species may occur on the same host species (Thompson *et al*., 2015). For example, Huang *et al.* (2013) identified *D. citri, D. citriasiana* and *D. citrichinensis* on citrus in China and were confirmed to be pathogenic during pathogenicity tests.

Diaporthe species cause several plant diseases such as dieback, cankers, leaf spots, blights, root and fruit rots (Van-Rensburg *et al*., 2006). A study by Zhang *et al.* (2018) reported canker disease on *Ziziphus jujuba* for the first time in China. Fungal cankers are among the most destructive and difficult to manage diseases of woody plants. Most canker fungi usually infect woody plants that are severely weakened and under stress by factors such as drought, floods, early spring low temperatures, extreme temperature fluctuations, chemical and mechanical injury (Wegulo and Gleason, 2001). On *Z. jujuba*, canker symptoms include brown, sunken, elongated, necrotic lesions on the twigs or shoots. Later symptoms involve the death of shoots that are girdled by canker lesion as well as the death of new twigs developing from infected buds (Zhang *et al.*, 2018). Zhang *et al.* (2018), identified the causal agent of the canker on *Z. jujuba* as *Diaporthe eres*, a fungal pathogen in the Ascomycota that has been previously reported as the causal agent of shoot blights and cankers on several plant species such as sycamore maple (*Acer pseudoplatanus*) and butternut tree (*Juglans cinerea*) (Anagnostakis, 2007). *Diaporthe eres* was also isolated from another Rhamnaceae species (*Rhamnus alpinus*) associated with dead areal branches in Forlì-Cesena, Italy (Dissanayake *et al.*, 2017a).

Diaporthe fungi have also been isolated as endophytic fungi from *Ziziphus* species. A study by Yang *et al.* (2015) reported *D. infecunda* as an endophyte from healthy fruits of *Z. jujuba* for the first time in Henan Province, China. A study by Yang *et al.* (2016) later identified a new endophytic species, *D. henanensis* from healthy fruits of *Z. jujuba* in China. *Diaporthe henanensis* was found to be morphologically and molecularly distinct from known species, hence it was isolated and described as new based on its distinctive morphology (Yang *et al.*, 2016). More recently, Suryanarayanan *et al.* (2018) identified *Diaporthe* species from healthy matured leaves of *Z. jujuba* and *Z. xylopyrus* from Masinagudi, India. These studies recorded *Diaporthe* species being endophytes on trees in the Rhamnaceae and other families. Moreover, *D. eres* was reported from healthy leaves of *Astragalus membranaceus* in Korea (Kim *et al.*, 2017). Identifying fungal species may present some challenges at times and they can easily be misidentified using morphological characteristics since some of the same fungal species can produce different growth structures when exposed to different conditions. It is, therefore, important to use both morphological and robust molecular identification tools to delineate fungal organisms.

2.4 Identification of fungi using morphological characteristics and molecular techniques.

The reliable identification of the organisms responsible for a plant disease is an essential prerequisite for the implementation of disease management strategies. For the diagnosis of plant-fungal infections, it is essential to carry out correct pathogen identification (Ray *et al.*, 2017). Diagnosis of diseases caused by fungus-like and fungal pathogens is based on certain characteristic symptoms induced by the pathogen (Narayanasamy, 2011). Both local and systemic symptoms may be produced following infection by fungi. Symptoms commonly produced on leaves include spots, blights, anthracnose, rusts and powdery mildews, whereas root and stem rot, stem canker, clubroot and galls/tumours are usually associated with root and stem infections (Narayanasamy, 2011).

Traditionally, fungal identification has been based on morphological, physiological and chemical characteristics of specimens. Most fungal species can be identified from the microscopic reproductive structures they produce (Pernezny *et al.*, 2014). However, these structures may not be sufficient for reliable identification since many species produce structures with similar morphology, or do not produce them at all in the laboratory. Also, certain fungi are obligate autotrophs and are therefore not culturable. As a result, molecular techniques are implemented for the identification and classification of fungal species (Bernreiter, 2017).

2.4.1 Morphological techniques

Traditionally fungi, similar to other organisms, were identified and classified based on their morphological features. This required the production of characteristic structures, such as spores, for differentiating species. Even today, with the use of different species concepts, a morphological description is still required by the Code (Pernezny *et al.*, 2014). Identification based on morphology holds several disadvantages and problems for taxonomists. Identifying fungal pathogens based on their morphological characteristics requires extensive knowledge of classical taxonomy and experience. It can be time-consuming as some fungi grow slowly such as *Coniodictyum chevalieri* (Maier *et al.*, 2006), and take a long time to develop the necessary characteristics for identification. Besides, morphological features of fungi may also change considerably depending on the environment and the conditions they are exposed to, which can also be a challenge when identifying them (Jiminez *et al.*, 1999). Hence, fungal species should be grown in an environment with uniform conditions such as temperature and ventilation. Some fungi cannot be grown in culture as they are obligate autotrophs (Kumhar *et al.*, 2015). Furthermore, some fungal species do not sporulate on growth media making it

impossible to describe the sexual phase of these fungi (Guo *et al.*, 2000). Another limitation is that many fungal species are morphologically similar and this leads to misidentifications, for example, species of *Dothiorella* are morphologically most similar to those of *Diplodia*, while they are phylogenetically closely related to *Neofusicoccum* species (Phillips *et al.*, 2005). These limitations have led to the introduction of molecular techniques for the identification and classification of fungi.

2.4.2 Molecular techniques

Molecular techniques have improved the understanding of the taxonomy, evolution and phylogeny of organisms within the past three decades in an unprecedented dimension (Spring and Thines, 2010). These techniques improve the accuracy of species identification and as a result, the number of known fungal species has significantly increased since the introduction of molecular-based techniques in fungal taxonomy (Capote *et al.*, 2012). The techniques have several advantages compared to morphological based techniques and are therefore commonly used in fungal taxonomy. Some of the PCR based techniques that have been widely used in plant pathology include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP).

Restriction fragment length polymorphisms (RFLP) are one of the earliest types of DNA based molecular markers that were developed and have been widely used for the detection and characterization of fungi (Langridge and Chalmers, 2004). These markers can be used to construct conventional genetic linkage maps, follow the inheritance of genetic diseases and examine variation between and within populations (Chang *et al.*, 1988). Jasalavich et al. (2000) successfully detected and identified fungal species that were causing wood-decaying of *Picea* species with the use of RFLP. Jacobs *et al.* (2007) also used RFLP to distinguish between members of the *Gibberella fujikuroi* complex and further confirmed the presence of *Fusarium circinatum* on isolates collected from cankered parts of *P. radiate* in Chile. Restriction fragment length polymorphisms (RFLP) is an inexpensive technique, does not require advanced instruments and can be easily designed and achieved using public available programs. However, RFLP consists of several steps that are time-consuming and is not suitable for high-throughput analysis (Rasmussen, 2012).

Random amplified polymorphic DNA (RAPD) is a technique used for identifying genetic variation and was first developed and introduced by (Welsh and McClelland, 1990). This technique is based on PCR amplification of pathogen fragments of the genome and is useful in distinguishing strains of fungal species (Capote *et al.*, 2012). The RAPD technique has been

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used to identify isolates of *Colletotrichum graminicola* from diseased parts of sorghum (Guthrie *et al.*, 1992), as well as to detect the genetic variation of *Magnaporthe poae* in USA (Huff *et al.,* 1994). The RAPD method is a fast and inexpensive method that does not require extensive knowledge of DNA sequence of the target organism, however, it has poor reproducibility and cannot differentiate non-homologous co-migrating bands (Capote *et al.,* 2012).

Amplified fragment length polymorphism (AFLP) is a PCR-based fingerprinting technique that was first introduced and described by Vos *et al.* (1995). This technique is a combination of RFLP and RAPD (Singh *et al.*, 2013), and rapidly generate large numbers of marker fragments for any organism, without prior knowledge of genomic sequence. Moreover, AFLP requires only small amounts of starting template and, in comparison with other techniques such as RAPD and RFLP, it exhibits much better results (Paun and Schönswetter, 2012). It has been used to differentiate *Monilinia laxa* from apples and isolates that infect other host plants in Slovenia (Gril *et al.*, 2008). The AFLP technique was also used by Belabid *et al.* (2004) for pathogenic and genetic characterization of *Fusarium oxyxsporum* isolates obtained from wilted *Lens culinaris* in Algeria. However, AFLP is relatively labour-intensive method and expensive (Paun and Schönswetter, 2012).

The strength of molecular-based methods lies in the fact that they can be universally applied to all fungi. Unlike morphological techniques, identifications are not influenced by growth conditions, because DNA from non-culturable fungi can be extracted and sequenced. Molecular techniques can be used whether sexual or other characteristics are produced or not (Borman *et al.*, 2008). These techniques are highly specific and can be used to detect and identify fungi from minute quantities of fungal DNA (Atkins and Clark, 2004). The choice of a molecular technique to use usually depends on the research question that is being addressed (Pereira *et al.*, 2008), for example, studying specific genomic regions or the whole genome of a fungal species. Some methods rely only on PCR reactions while others will use DNA sequence comparisons. For example, a PCR reaction using species-specific primers can be used if a researcher is only interested in knowing the presence or absence of specific species in a sample (White *et al.*, 1990). DNA sequence comparisons and phylogenetic methods are used to identify and characterise isolates with unknown identities.

Several genes are used in DNA sequence-based approaches. The internal transcribed spacer (ITS) region is commonly used for fungal DNA sequence comparisons as there is a large database of fungal ITS sequences. However, it is known that DNA sequence comparisons based only on this region often yield misidentification. For this reason, combinations of different genomic regions or genes are being used. Over time, genes such as largest (RPB1) and second-largest (RPB2) subunits of RNA polymerase, beta-tubulin (BT) and translation

elongation factor 1-alpha (TEF-1α) have been widely used for inferring phylogenetic relationships among fungi (Raja *et al.*, 2017). The regions of RPB1, RPB2, TEF-1α and BT are amplified and the DNA sequence is determined using Sanger sequencing and the sequence is compared with those of known species in public databases such as GenBank and applying phylogenetic methods.

2.5 Conclusions and objectives

This review discussed the tree of interest for this study, *Z. mucronata*. Since there is not much information about the fungal diseases associated with this tree, except one record of smut pathogen in the Kruger National Park, the focus of the review was shifted to fungal diseases that are found on other trees in the Rhamnaceae. *Ziziphus jujube* is generally recognised as the most important *Ziziphus* species for fruit production in the Rhamnaceae (Gao *et al.*, 2012). However, this tree is subject to diseases that limit its productivity which results in low fruit yield. Examples of these diseases include dieback and cankers which are caused by *Fusarium* and *Diaporthe* species (Mirzaee *et al.*, 2011, Zhang *et al.*, 2018), and these diseases are considered important for *Z. jujube* globally. Plant pathogens need to be identified with the aid of molecular techniques and studied to better understand the potential threats they can pose to indigenous trees. The first objective of this study was to identify the fungal species associated with dieback on *Z. mucronata* in Limpopo Province (Tshikundamalema, Wits Rural Facility and Buzzard Mountain Farm) since there is no documented information available. The second objective of the study was to determine the diversity of species in the Botryosphaeriaceae causing dieback on *Z. mucronata* in Limpopo Province since no study has been carried out on this aspect.

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CHAPTER 2: Materials and methods

2.1 Study sites

The samples for this study were collected from three sites in Limpopo Province: Tshikundamalema, Wits Rural Facility and Buzzard Mountain Farm.

A. Tshikundamalema

Tshikundamalema is located in Mutale Municipality, in Vhembe District, Limpopo Province, about 90 km north of Thohoyandou. The area is primarily comprised of sandy soils and is mountainous being situated between longitude 22°40.52'4 South and latitude 30°39.49'7 East.

The area is hot and dry with an average rainfall of 450mm per annum (Soil Classification Working Group cited by Mzezewa and Gwata (2012).

B. Wits Rural Facility

Wits Rural Facility is a 350 ha unique rural campus of the University of the Witwatersrand. It is situated in the far North-east of South Africa, in the central Lowveld of Limpopo Province, on the western boundary of Kruger National Park, about 35km from the Orpen Gate. The facility is situated at longitude 24º 56'386 South and latitude 31º 29'076 East. It is a granitic sandy soil area dominated by *Combretum*, *Vachellia* and *Terminalia* species. The area receives an average annual rainfall of 680 mm occurring mostly between October and May (Smith and Cain III, 2009).

C. Buzzard Mountain Farm

Buzzard Mountain Farm is a private farm located 20 km west of Louis Trichardt along Vivo Road. It is situated at longitude 29º 46'4 E and latitude 23º 1'3 S and altitude of 950 m above sea level. The area receives an average rainfall of 793 mm per annum and is characterised by semi-arid savanna vegetation dominated by *Vachellia* species in red well-drained clay-loamy soils.

2.2 Sample collection

A minimum of 30 trees with smut infection and 30 trees without smut infection were identified, selected and permanently marked with a GPS navigator (Model: GPSMAP 64 Handheld, Garmin, Johannesburg, South Africa) at all sites. All the trees were firstly assessed for branch dieback and branches showing dieback symptoms were collected. In total, samples were collected from 70 trees at Wits Rural Facility (32 smut-infected + 38 with no smut), 78 trees at Buzzard Mountain Farm (36 smut-infected + 42 with no smut symptoms) and 79 trees at Tshikundamalema (39 smut-infected + 40 with no smut symptoms). Plant samples were kept in separate sampling paper bags and taken to the laboratory for fungal isolations.

2.3 Primary and secondary fungal isolations

Fungal isolations were done under aseptic conditions where all laboratory tools such as dissecting needles, scissors and tweezers, were firstly sterilised by dipping them into 70% alcohol and passing them through a flame. Dieback branches were first surface-disinfected by wiping them with 3.5% household bleach. Small pieces (4 - 6 mm) were then cut from the branches and submerged in 70% alcohol for 30 seconds after which they were rinsed in sterile water for 1 minute twice. The disinfected pieces were then aseptically inoculated on 2% potato

dextrose agar (Mahajan and Chopda, 2009) in Petri dishes, which were then sealed and marked. The plates were then incubated at a temperature of 27 - 29°C for 7 - 10 days until the mycelial growth was apparent in the growth media. Secondary isolation and culture purification was achieved by single hyphal inoculation from the growing cultures onto 2% malt extract agar (Johnson *et al.*, 1992) under a laminar flow. The fungal cultures were allowed to grow for a period of 1 - 3 weeks in the dark. Cultures from each site were then viewed under a stereomicroscope and grouped based on morphological characteristics such as colour and structure of mycelia. Representative isolates were selected from each group and subjected to preliminary identifications using DNA sequence data.

2.4 DNA isolation

DNA was extracted following the protocol of Chang *et al.* (1993), with the following modifications. The extraction buffer contained: 100mM Tris-HCl (pH=8), 2M NaCl, 25mM EDTA and 2% CTAB. In brief, 100 - 150mg fresh mycelia were added to 2 ml homogenising tubes with 2% (w/v) polyvinylpyrrolidone (PVP), 650 µl extraction buffer, 500 mg/L spermidine (500mg/l), 2% (v/v) β-mercaptoethanol and one ceramic ball in each tube. The mycelia was homogenised at 4.0m/sec for 40 seconds to crush the cell walls. This was followed by incubation at 65 °C for 1 hour while gently turning the tubes upside down every 15 minutes for 5 seconds to make sure the cells were well mixed with the extraction buffer. The tubes were then centrifuged at 13400 G-force for 45 mins at room temperature to separate the cell debris from the supernatant.

The supernatants were transferred to new 1.5 ml Eppendorf tubes. A volume of 650 µl chloroform/isoamyl alcohol (24:1) was then added to each tube, vortexed for 30 sec and centrifuged at 4 °C for 20 minutes at 13400 G-force to remove the cell debris and proteins from the supernatants. This step was repeated one more time and the supernatant were then transferred into new 1.5 ml tubes. After transferring the supernatants, 1.5 volume of cold absolute ethanol was added to the tubes containing the supernatants and incubated at -20 °C overnight for DNA precipitation. The following day, samples were centrifuged at 4 °C for 1 hour at 13400 G-force to precipitate the DNA pellet. The supernatant was then carefully decanted and the pellet washed twice by adding 500 µl room temperature 70% ethanol and centrifuged at 13400 G-force for 10 mins twice. The pellet was dried (vacuum spin at 30 °C for 5-10 minutes), dissolved by adding 50 µl Sabax water and left to dissolve in room temperature for 2 - 3 hours. The DNA was quantified using a Thermo Scientific NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and adjusted to a working concentration of 50 ng/µL using sterile SABAX water.

2.5 PCR amplification and clean-up

PCR amplifications were performed on a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, U.S.A.) in a total volume of 25 µL containing 5 µl MyTaq reaction buffer (10 mM Tris-HCL [pH 8.3], 3.0 mM $MgCl_2$, 50 mM KCl, Roche Diagnostics, Mannheim, Germany), 0.2 µM of each primer, 2 µl template DNA (50ng/µl), 0.5 U MyTaq DNA polymerase and 16.5 µl sterile SABAX water with the following profile: 2 minutes denaturation at 94 °C and 30 cycles of 30 seconds denaturation at 94 °C, 1 min annealing at 52 - 54 °C (depending on the gene to be amplified), 1 minute extension at 72 °C, followed by a final extension at 72 °C for 7 minutes.

Three genomic regions, namely the internal transcribed spacer (ITS) region, translation elongation factor 1-α (TEF-1α) and β-tubulin (BT), were amplified. The ITS region of the ribosomal DNA (rDNA) was amplified using primers ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.*, 1990). Part of the translation elongation factor gene was amplified using the primers EF1F (5'-TCGGGTGGTATCGACAAGCGT-3') and EF2R (5'-AGCATGTTGTCGCCGTTGAAG-3') (Jacobs *et al.*, 2004). The BT gene was amplified for isolates that were preliminarily identified belonging to the Botryosphaeriaceae based on the ITS and TEF regions. The amplification was done using the primers Bt-2a (5'GGTAACCAAATCGGTGCTGCTTTC-3') and Bt-2b (5'-AACCTCAGTGTAGTGACCCTTGGC-3') (Glass and Donaldson, 1995).

PCR amplicons were purified using ExoSAP-IT™ PCR Product Cleanup Reagent. A total volume of 8 µl ExoSAP-IT was transferred into each tube containing the 23 µl PCR products, and a 31 µl reaction was ran for 30 minutes (15 mins at 37 °C and 15 mins at 80 °C). Purified PCR products were stored at –20 °C.

2.6 DNA sequencing

Using the same primers that were employed for PCR reactions, purified amplicons were sequenced in both directions for the ITS and other two gene regions. The reaction was carried out in a total volume of 12 µl composed of 4 µl sterile SABAX water, 2.5 µl sequencing buffer, 0.5 µl Big Dye (Kapa Biosystems, Cape Town, South Africa), 0.4 µM of each primer and 50-80 ng PCR product. Sequencing reactions were performed at the following conditions; 25 cycles of denaturation at 94 °C for 10 sec, annealing at 54 °C for ITS and TEF, and 56 °C for β-tubulin for 10 sec.

Sequencing products were then transferred to sequencing tubes and purified by adding sodium acetate (NaAc) master mix containing 8 µl sterile SABAX water, 2 µl sodium acetate (3 M, pH 4.6) and 50 µl cold absolute ethanol. The mixture was incubated at -20 °C for 10-15

minutes and centrifuged at 13400 G-force for 30 min at 4 °C. The pallet was then washed by adding 250 µl 70% ethanol and centrifuged at 13400 G-force for 20 minutes at 4 °C twice. Samples were then dried in a vacuum spin and submitted to the DNA sequencing facility at the University of Pretoria for Sanger sequencing.

2.7 DNA sequence and phylogenetic analyses

The sequences received from the sequencing facility were edited using CLC Main Workbench v8.0.1 (QIAGEN, Aarhus, Denmark) to correct for incorrect base calls during sequencing. Contig sequences from these sequences were constructed by assembling the forward and the reverse sequences for each gene region in CLC Main Workbench. Preliminary identifications of the strains were done by subjecting the sequences to BLASTn searches against sequences hosted in the National Centre for Biotechnology Information (GenBank, NCBI, http://www.ncbi.nlm.nih.gov) nucleotide database. Datasets for each gene region were constructed by combining the sequences from this study together with the sequences from GenBank that showed higher similarity after BLASTn searches. Datasets of sequences from this study, for each gene region were also constructed.

Sequences for each of the three gene regions were aligned using the online interface of MAFFT v. 5.667 (Katoh *et al.*, 2002) and edited manually using BioEdit (Hall, 1999). For each sequence dataset, the best fit nucleotide substitution model for constructing a maximum likelihood phylogenetic tree was determined using jModelTest v0.1.1 (Posada, 2008). Phylogenetic analyses of sequence data for Maximum Likelihood (ML) were done using RAxML v8.2 (Stamatakis, 2016). For the combined phylogenetic tree of the three genomic regions, the sequence data was concatenated using FASconCAT-G-master v1.04 (Kück and Longo, 2014).

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CHAPTER 3: Diversity of fungi found on branches of *Ziziphus mucronata* showing dieback at different locations of Limpopo Province

ABSTRACT

Dieback is initiated in trees as a response to poor growing conditions, physical injury to the tree as well as pest and pathogen attack. Fungi have been frequently reported causing dieback and other diseases on many woody species around the world. There is little information available about fungal diseases attacking *Ziziphus mucronata* (Rhamnaceae) and their causal agents, except smut disease that is caused by *Coniodictyum chevalieri*. The aim of this study was to identify fungal species associated with *Z. mucronata* branches showing

dieback at three different locations within the Limpopo Province that included Buzzard Mountain Farm, Tshikundamalema and Wits Rural Facility. Isolates were obtained from branches showing dieback and delineated based on DNA sequence data of two genome regions. Genomic regions that were amplified and sequenced were the internal transcribed spacer (ITS) and translation elongation factor (TEF-1α). DNA sequence data were used to construct phylogenetic trees to different taxa. Results from the analyses showed that the isolates belonged to six fungal families; Botryosphaeriaceae, Diaporthaceae, Cytosporaceae (=Valsaceae), Nectriaceae, Pleosporaceae and Didymellaceae. Among the six families, species in the Botryosphaeriaceae were most frequently identified among the three sampling locations followed by species residing in the Diaporthaceae. These results suggested that species in the Botryosphaeriaceae are associated with dieback on *Z. mucronata* in Limpopo Province.

Keywords: *Ziziphus mucronata*, ITS, TEF-1α, DNA, dieback, phylogenetic analysis, fungal families.

3.1 Introduction

Extensive dieback has been reported from trees in many forest types around the world since the early 1960s (Huettl and Mueller-Dombois, 2012). The condition affects trees of all ages, but in most cases, large and old trees are more frequently affected producing symptoms that occur slowly and subtly (Heimann and Worf, 1999). The symptoms include thinning of the tree crown, limited terminal branch growth and branch mortality beginning from the top of the tree progressing downwards. These symptoms can be caused by visible factors such as insect attack and parasitic plants, and underlying factors such as lack of available soil moisture, waterlogging, high soil salinity, imbalanced soil nutrition, soil compaction and plant pathogens (DEH, 2005).

Dieback occurs in plants that are growing in both natural and managed ecosystems and has most commonly been attributed to pathogenic fungi (Agrios, 2005). Fungi from the

Botryosphaeriaceae have been associated with dieback in different ecosystems. These fungi are well known important environmental and agricultural pathogens of angiosperms and gymnosperms (Slippers and Wingfield, 2007). Members of the family such as *Neofusicoccum*, *Diplodia* and *Lasiodiplodia* species have been shown to cause dieback of temperate and forestry trees including those in the genera *Quercus*, *Vachellia*, *Pinus* and *Eucalyptus* (Slippers *et al.*, 2009, Lynch *et al.*, 2013, Mohali *et al.*, 2007). Members of this family are also known to remain latent in plant parts and take advantage of a host when it is under stress from abiotic or biotic factors, causing symptoms such as cankers, brown fruit rot, loss of canopy and damping off (Slippers and Wingfield, 2007). Therefore, an increase in environmental stress experienced by potential hosts due to climate change are likely to increase the prevalence of diseases caused by species in the Botryosphaeriaceae (Pitt *et al.*, 2010).

In addition to the Botryosphaeriaceae, there are multiple dieback-associated fungal pathogens. Fungi in the genera *Fusarium* (Nectriaceae) and *Etypa* (Diatrypaceae) have previously been associated with dieback (Al-Mahmooli *et al.*, 2013). For example, the pathogen *Fusarium euwallaceae* has been recorded as a problem in Israel (Mendel *et al.*, 2012) and California in the United States where it is causing dieback on avocado (Eskalen *et al.*, 2013). The fungus is vectored by an ambrosia beetle (polyphagous shot hole borer) native to Asia, which has a symbiotic relationship with *Fusarium* species that invade vascular tissue, causing necrosis of the cambium that result in dieback and tree death (Eskalen *et al.*, 2013). In South Africa, an association between the ambrosia beetle and *F. euwallaceae* was first reported by Paap *et al.* (2018) causing *Fusarium* dieback on *Platanus* x *acerifolia* (London Plane) in the KwaZulu-Natal National Botanical Gardens, Pietermaritzburg. *Fusarium euwallaceae* was later reported on *Persea americana* in Sandton (Gauteng Province, South Africa) with the ambrosia beetle causing necrotic lesions (van den Berg *et al.*, 2019). Qi *et al.* (2013) also recorded dieback caused by *Fusarium* species on *Mangifera indica* in China. The authors did their isolations from the infected petioles and twigs and the causal agent was confirmed as *F. decemcellulare*. Pathogenicity tests were carried out and the fungal species was found being pathogenic (Johnston, 1966). Although this fungus has been previously reported causing dieback on mango in Indonesia, this was the first report of its pathogenicity towards mango in China.

Dieback has been observed on *Z. mucronata* in Limpopo Province but the causal agent is not known. There is also no information available on fungi associated with dieback on *Z. mucronata*. However, dieback was reported in eastern Iran on *Z. jujube* being caused by *F. solani* (Mirzaee *et al.*, 2011). *Ziziphus jujube* seems to be the only *Ziziphus* species for which the occurrence of dieback has been reported around the world. Hence, this raised a question about the diversity of fungal species associated with dieback on *Z. mucronata* in Limpopo

Province, South Africa. The aim of this study was to identify fungi found on dieback branches of *Z. mucronata* in different locations of the Limpopo Province.

Research questions:

- What are the fungal species associated with dieback on branches of *Z. mucronata* in Limpopo Province?
- Is the diversity of these fungal species influenced by location?

3.2 Materials and methods

3.2.1 Study sites and sample collection

Samples were collected from three study areas, namely Tshikundamalema, Buzzard Mountain Farm and Wits Rural Facility. *Ziziphus mucronata* trees were examined for dieback on branches and their location recorded using a GPS navigator (Model: GPSMAP 64 Handheld, Garmin, Johannesburg, South Africa). Branches showing dieback were collected for primary fungal isolations as described in Chapter 2.

3.2.2 Primary fungal Isolations and culture purification

Primary and secondary fungal isolations were done on Potato Dextrose Agar (Mahajan and Chopda, 2009) and 2% Malt Extract Agar media, respectively. Branches were initially surface disinfected by wiping them with 1% sodium hydrochloride and small pieces, approximately 2 - 4 mm^2, were then cut using a sterile scalpel from the area between the dead and healthy parts of the branches after removing the bark (Fig.3.1). The small pieces of the branch were then incubated on PDA at 25 °C for 1 to 2 weeks (Fig.3.1). Secondary isolation was done by transferring single hyphal tips from the growing primary isolates onto 2% MEA and incubated at a growing temperature of 25 °C for 1 - 2 weeks. Pure cultures were then arranged into morphological groups and representative isolates were selected for DNA extraction and sequencing.
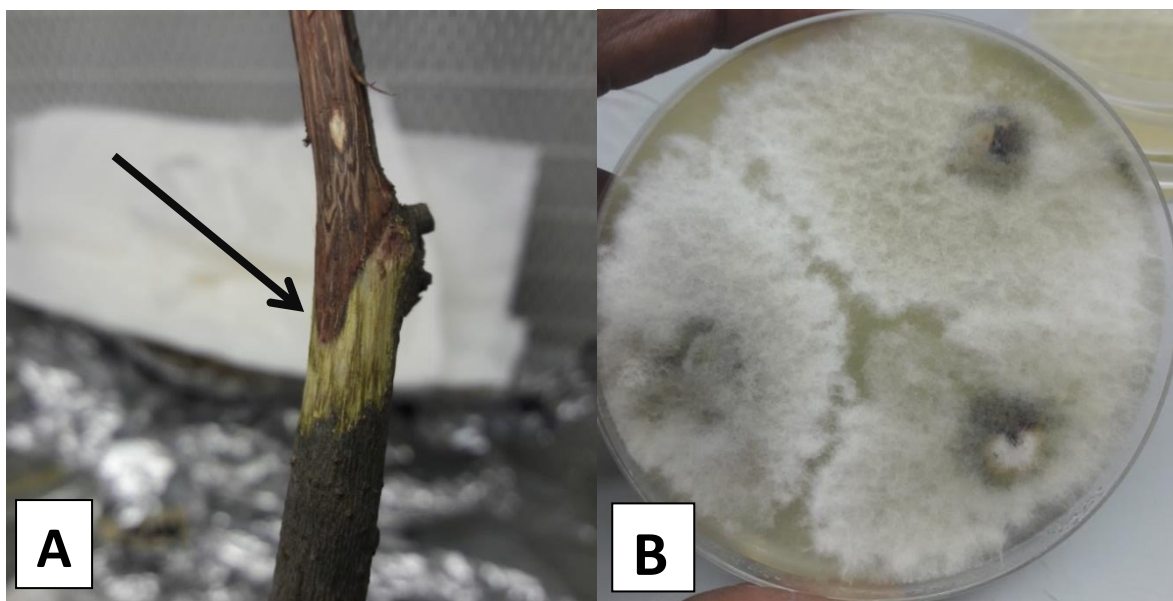
Figure 3.1: Primary isolations: A. Necrotic dieback on a branch of *Z. mucronata* displaying the intersection between dead and live part of the branch (see arrow); **B.** Fungal growth on PDA media, following the primary isolations.

3.2.3 DNA extraction, PCR amplification and sequencing

Sequences of the representative isolates were generated through genomic DNA extraction, PCR amplification and sequencing following the laboratory protocols and procedures described in Chapter 2. Two genomic regions, the internal transcribed spacer (ITS) and a portion of the translation elongation factor (TEF-1α) gene were successfully amplified from the extracted DNA. The primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.*, 1990) were used to amplify the ITS region. The TEF-1α region was amplified with primers EF1F (5′-TGCGGTGGTATCGACAAGCGT-3') and EF2R (5′-AGCATGTTGTCGCCGTTGAAG-3') (Jacobs *et al.*, 2004). Using the same primers that were used for PCR, the resulting amplicons were sequenced in both directions as descried in Chapter 2, and sent to the DNA sequencing facility of the Faculty of Natural and Agricultural Sciences (NAS), at the University of Pretoria for sequencing.

3.2.4 Sequence and phylogenetic analyses

Sequences obtained from the sequencing facility were assembled with CLC Main Workbench v8.0.1 (QIAGEN, Aarhus, Denmark) to construct contigs as described in Chapter 2. The consensus sequences were then subjected to BLASTn searches to obtain preliminary identities for the isolates. Sequences from GenBank that showed high similarities to sequences of the isolates from *Z. mucronata* were downloaded (Table 3.2). The sequence datasets were then aligned using an online version of MAFFT v5.667 (Katoh *et al.*, 2002) and

best fit nucleotide substitution models for each dataset was determined using jModelTest v0.1.1 (Posada, 2008) for each sequence dataset. Maximum likelihood trees were constructed using RAxML v8.2 (Stamatakis, 2016) and the trees were rooted to selected outgroups species.

3.3 Results

3.3.1 Sample collection and isolations

In this study, 227 trees were surveyed that include 79 trees from Tshikundamalema, 78 trees from Buzzard Mountain Farm and 70 trees from Wits Rural Facility. Most of the trees showed dieback symptoms such as wilted leaves and dead shoots, twigs and branches. We obtained a total of 350 isolates from dieback branches of *Z. mucronata*. A total of 181 isolates were obtained from 79 trees sampled in Tshikundamalema, 128 from 78 trees in Buzzard Mountain Farm and 41 isolates from 70 trees sampled at Wits Rural Facility. Isolates were grouped according to their morphological characteristics such as the colour of mycelia and their growth form, which resulted into a total of 34 morphological groups (Fig. 3.2).
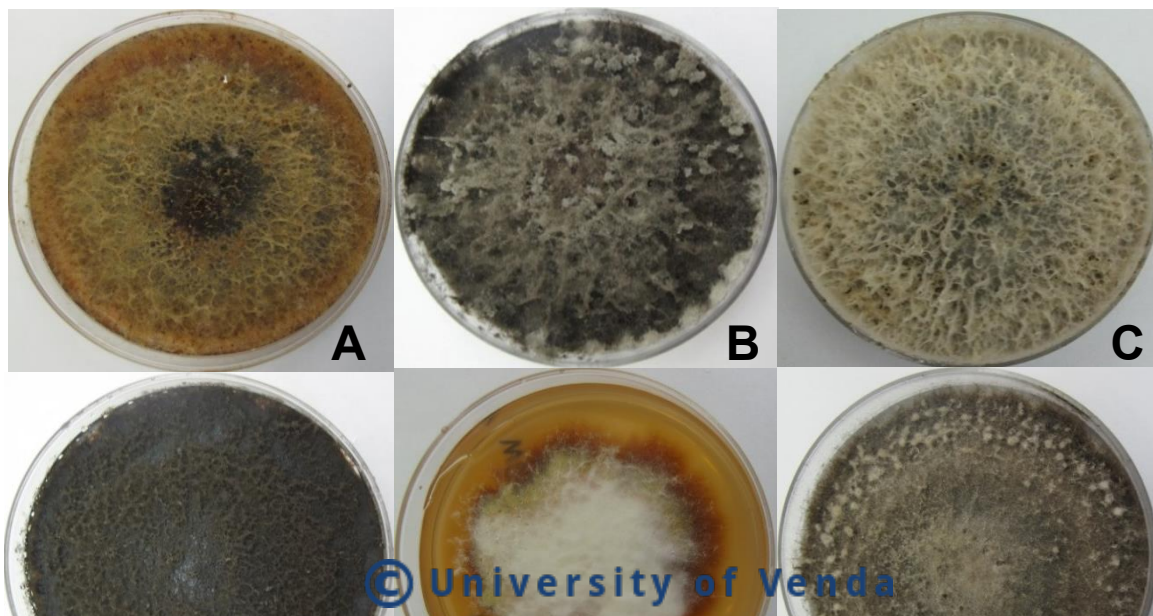
Figure 3.2: Culture morphology of some of the representative isolates from different morphological groups observed in this study.

The majority of the isolates resembled morphological characteristics of the Botryosphaeriaceae. These characteristics were light-grey, light-brown and light to dark-black mycelia. In total, 26 morphological groups resembling this family were distinguished. A total of 86 isolates were selected representing each of the groups for further identification through DNA sequencing.

3.3.2 DNA extraction, PCR amplification and sequencing

DNA extraction and PCR amplification were successfully performed on the 86 representative isolates as described in Chapter 2. The band size of the ITS amplicons was approximately 500bp. The TEF-1α amplicons yielded a band size of approximately 600bp.

3.3.3 Sequence and phylogenetic analysis

Nucleotide sequences for the two gene regions sequenced were subjected to BLASTn searches against sequences in GenBank nucleotide database to determine the identity of the fungi isolated from *Z. mucronata.*

Table 3.1: ITS and TEF-1α BLASTn results for isolates obtained from diseased _Z. mucronata._

Location	Isolate code	ITS BLAST	TEF BLAST
Buzzard M. Farm	ZBM45.3	_Diplodia pseudoseriata_	_Diplodia_ species
	ZBM29.4,ZBM8.5	_Diplodia pseudoseriata_	_Phialemonium dimorphosporum_
	ZBM5.3,ZBM9.1,ZBM63.2,ZBM80.6,ZBM27.2A	_Dothiorella acacicola_	Botryosphaeriaceae sp.
	ZBM70.3	_Dothirella viticola_	_Specermartinsia viticola_
	ZBM12.3,ZBM5.2, ZBM78.1	_Specermartinsia viticola_	_Specermartinsia viticola_
	ZBM66.10	_Diaporthe foeniculina_	_Diaporthe baccae_
	ZBM27.2B	_Diaporthe velutina_	_Diaporthe baccae_
	ZBM13.2	_Diaporthe_ sp.	_Diaporthe baccae_
	ZBM77.4B	_Diaporthe baccae_	_Diaporthe baccae_
	ZBM8.1	_Diaporthe raonikayaporum_	_Diaporthe raonikayaporum_
	ZBM79.3	_Diaporthe_ sp.	_Stegonsporium acerophilum_
	ZBM64.1	_Diaporthe_ sp.	
	ZBM12.7	_Diaporthe_ sp.	_Diaporthe psoraleae_
Tshikundamalema	ZT10.3	_Diaporthe_ sp.	
	ZT8.3	_Diaporthe_ sp.	_Diaporthe betulae_
	ZT57.1,ZT43.1	_Cytospora_ sp.	_Stegonsporium acerophilum_
	ZT23.3,ZT3.4	_Cytospora_ sp.	_Stegonsporium pseudopyriforme_
	ZT36.2	_Fusarium_ sp.	_Fusarium lateritium_
	ZT43.2,ZT11.1	_Didymella_ spp (=_Phoma_ spp)	_Boeremia exigua_
	ZT5.2,ZT46.1,ZT4.2	_Alternaria alternata_	
	ZT18.2,ZT57.3, ZT44.1,ZT17.6	_Dothiorella longicollis_	_Dothiorella omnivora_
	ZT31.2	_Diplodia pseudoseriata_	_Diplodia alatafructa_
	ZT13.4		Botryosphaeriaceae sp
	ZT54.3,ZT33.3,ZT45.1	_Diplodia pinea_	_Diplodia seriata_
Wits rural Facility	WRZ24.2,WRZ36.2, WRZ22.2		
	WRZ33.1	_Botryosphaeria dothidea_	_Neofusicoccum_ sp.
	WRZ60.1,WRZ67.2		_Botryosphaeria dothidea_
	WRZ23.1	_Dothiorella longicollis_	_Dothiorella omnivora_
	WRZ26B.1	_Dothiorella oblonga_	_Dothiorella omnivora_
	WRZ65.1	_Dothirella viticola_	_Specermartinsia viticola_
	WZR1.1	_Fusarium decemcellulare_	_Fusarium decemcellulare_
	WRZ36.3	_Fusarium equiseti_	_Fusarium equiseti_
Tshikundamalema	ZT17.8	_Botryosphaeria dothidea_	_Botryosphaeria dothidea_

Based on BLASTn searches, isolates from the three sampling sites showed high similarities with species in the Botryosphaeriaceae from the following genera; *Dothiorella*, *Diplodia*, *Boryosphaeria* and *Neofusicoccum*. (Table 3.1). Isolates also showed high similarity with species in *Diaporthe*, *Cytospora*, *Fusarium*, *Alternaria* and *Didymella*. The TEF-1α and ITS sequence alignments were analysed individually. The ITS sequence matrix had a total of 710 characters, of which 582 were variable. The TEF-1α sequence matrix had 1076 total characters with 698 characters being variable.

3.3.3.1 ITS phylogeny that included isolates from *Z. mucronata* and sequences obtained from GenBank.

The ITS dataset comprised a total of 194 sequences, of which 61 sequences were from this study and 133 sequences were sequences from GenBank that had high similarity with the isolates used in this study. The phylogenetic tree generated placed the sequences in six fungal families; Botryosphaeriaceae, Diaporthaceae, Cytosporaceae, Nectriaceae, Didymellaceae and Pleoporaceae (Fig. 3.5).

3.3.3.1.1 Botryosphaeriaceae

Within the Botryosphaerriaceae, isolates were accommodated in three genera; *Dothiorella*, *Diplodia* and *Botryosphaeria* (Fig. 3.5). The genus *Dothiorella* included a total of 21 isolates, 10 from Buzzard Mountain Farm, eight from Tshikundamalema and three isolates from Wits Rural Facility (Fig. 3.5). Five isolates from Buzzard Mountain Farm and one from Tshikundamalema were placed within the genus *Dothiorella* close to *Do. viticolla*, *Do. rosulata*, *Do. plurivora* and *Do. westrale* supported with a 97% bootstrap support (BS) on the ITS phylogenetic tree (Fig. 3.5). The phylogenetic tree also placed five isolates from Buzzard Mountain Farm, two from Tshikundamalema and one isolate from Wits Rural Facility close to *Do. acacicola* and *Do. iberica* (BS<60%), while two isolates from Tshikundamalema and Wits Rural Facility respectively, were placed close to *Do. oblonga* and *Do. dulcispinae* (BS=79%). The last six isolates in this genus grouped with *Do. brevicollis* and *Do. longicollis* with a supporting value of 71%.

The genus *Diplodia* included 12 isolates, six from Tshikundamalema, three from Buzzard Mountain Farm and three from Wits Rural Facility. Three isolates from Buzzard Mountain Farm and three from Tshikundamalema grouped close to *D. pseudoseriata*, *D. seriata* and *Diplodia* species (BS=100%). The last six isolates in this genus, three from Tshikundamalema and three from Wits Rural Facility grouped close to *D. pinea* and *D. allocellula* on the ITS phylogeny supported with a 97% bootstrap support (Fig. 3.5). In the *Botryosphaeria,* three isolates from

38

Wits Rural Facility and one from Tshikundamalema were placed close to *B. dothidea* and *B. fusispora* (Fig. 3.5). The grouping of the isolates was supported with a 75% bootstrap support.

3.3.3.1.2 Diaporthaceae

The Diaporthaceae was the second largest family with 11 isolates from all three locations sampled. Isolates resided in the genera *Diaporthe* (Fig. 3.5). Four isolates from Buzzard Mountain Farm and two isolates from Tshikundamalema grouped close to *D. foeniculina* and *Diaporthe* species (BS<60%). One isolate from Tshikundamalema and one from Buzzard Mountain Farm were placed close to *D. parapterocarpi* and *D. raonikayaporum* respectively, with bootstrap support values above 85%. The last two isolates from Buzzard Mountain Farm and one isolate from Wits Rural Facility grouped close to *D. velutina*, *D. macintoshii* and *Diaporthe* species with support values below 60% (Fig. 3.5).

ZBM63 2

ZBM80 6

ZBM27 2A

ZT13 4

ZT57 2

ZBM5 3

NR 145255 *Dothiorella acacicola* T

WRZ64 1

ZBM9 1

64

NR 111165 *Do. iberica* T

EU331075 *B. iberica*

90

JQ239401 *D. dulcispinae*

80

NR 111702 *Do. dulcispinae*

NR 137689 *Do. oblonga*

79

KF766163 *Do. oblonga*

WRZ26B 1

ZT14 2

ZT18 2

ZT44 1

ZT57 3

JQ239404 *Dothiorella brevicollis*

NR 111703 *Dothiorella brevicollis* T

ZT52 2

71

ZT17 6

WRZ23 1

KF766162 *Do. longicollis*

NR 136999 *Do. longicollis* T

83

KF766227 *S. rosulata*

NR 136991 *Do. rosulata* T

KX464126 *Do. plurivora*

WRZ65 1

97

KT240299 *Spencermartinsia* sp

ZBM78 1

MG198185 *Do. viticola*

60

NR 111186 *Do. viticola* T

MH236148 *Do. viticola*

KP692191 *S. viticola*

KT240296 *Do. viticola*

80

HM009376 *S. westrale*

ZBM70 3

EF445361 *Do. viticola*

ZBM5 2

KM103250 *S. viticola*

ZBM12 3

ZBM12 4

*Dothiorella/
Spencermartinsia*

Botryosphaeriaceae

67

KX342943 *P. crystallifera*

GU237910 *P. aliena*

KY790596 *Phoma* sp

68

ZT43 2

MG967669 *Didymella* sp

81

ZT11 1

KF128801 *Phoma* sp

GU237810 *P. insulana*

96

GU237870 *P. costarricencis*

FJ427066 *P. schachtii*

FJ426981 *P. betae*

95

98

KX828180 *A. tenuissima*

MF693801 *A. alternata*

ZT46 1

ZT4 2

100

MF958649 *A. alternata*

93

MH730190 *Alternaria* sp.

ZT5 2

MH716004 *A. alternata*

MG209668 *Alternaria* sp.

Didymellaceae

Pleosporaceae

41

Figure 3.5 Maximum likelihood phylogeny constructed based on the ITS region showing relationships between isolates obtained from *Z. mucronata* and known sequence from GenBank. The tree is rooted to *Arthrobotrys vermicola* and isolates marked in bold are from this study and "T" represent the known type-material sequences. Bootstrap values greater than 60 % from 1000 replications ML analysis are indicated on the nodes.

3.3.3.1.3 Cytosporaceae and Nectriaceae

In the Cytosporaceae (=Valsaceae), there were only four isolates from Tshikundamalema. The sequences of these isolates showed higher similarity to *Cytospora* species on BLASTn search results (Table 3.1). These isolates grouped with a sequence of *Cytospora* sp. from GenBank on the ITS phylogenetic tree with the supporting value of 90% (Fig. 3.5). Isolates from *Z. mucronata* also formed a sister clade with *C. rhizophorae*, *C. eucalyptina* and *C. magnoliae* (Fig.3.5).

In the Nectriaceae, two isolates and one isolate from Wits Rural Facility and Tshikundamalema respectively, grouped with *Fusarium* species (Fig. 3.5). The isolates were furthermore placed in three different subclades. Isolate ZT36.2 grouped with *Fusarium* sp. and *F. xylariodes*, isolate WRZ1.1 grouped with *Fusarium* sp. and *F. decermcellulare*, and isolate WRZ36.3 grouped close to *F. equiseti* and *F. verticillioides*. The sub-clades were supported by bootstrap values above 80% (Fig. 3.5).

3.3.3.1.4 Pleosporaceae and Didymellaceae

The last two families to which some of the isolates belonged were Pleosporaceae and Didymellaceae. In this case, isolates representing these families originated from Tshikundamalema only. In the Pleosporaceae, three isolates were placed in the genus *Alternaria* close to *A. alternata* and *A. tenussima* and *Alternaria* sp. with a supporting value of 98% (Fig. 3.5). BLASTn results of these isolates showed higher similarity to *A. alternata*. The Dedymellaceae consisted of two isolates that grouped with *Didymella aliena* (=*Phoma aliena*), *D. crystallifera* and *Didymella* spp. in one clade (BS=81%) (Fig. 3.5). This group formed a sister clade with *D. insulana* and *P. costarriencencis*. The genus *Didymella* is believed to be a sexual reproductive stage of *Phoma*.

3.3.3.2 TEF phylogeny that included isolates from *Z. mucronata* and sequences obtained from GenBank.

Representative isolates that were identified through ITS phylogenetic analysis were further characterized using a portion of the translation elongation factor (TEF-1α). The TEF-1α sequence data consisted of 112 sequences in total, of which 61 are from this study and 51 were retrieved from GenBank. The phylogenetic tree generated from this data also revealed six fungal families; Botryosphaeriaceae, Diaporthaceae, Cytosporaceae, Nectriaceae, Didymellaceae and Pleosporaceae.

3.3.3.2.1 Botryosphaeriaceae

Five isolates from Buzzard Mountain Farm and two from Wits Rural Facility grouped with *Dothiorella viticola* (=*Spencermartinsia viticola*) on Clade **A** (BS=91%) (Fig. 3.6). These isolates also grouped closely related to *Dothiorella viticola* on the ITS phylogenetic tree (Fig. 3.5). However, within *Dothiorella* there were six isolates from Tshikundamalema and two from Wits Rural Facility that did not group with any sequence from GenBank but formed sister clade with *Do. omnivore*, *Do. samentorum* and *Do. iberica*. These isolates grouped close to *Do. oblonga*, *Do. brevicollis* and *Do. acacicola* on the ITS phylogenetic tree, but TEF-1α sequences for these three fungal species were not available in GenBank. Furthermore, seven isolates grouped with sequences from a member of the Botryosphaeriaceae on the TEF-1α phylogenetic tree with a supporting value of 68% (Fig. 3.6). These isolates grouped with *Do. acacicola* on the ITS phylogenetic tree, but TEF-1α sequence for this species was also not available in GenBank.

Clade **B** included species in *Diplodia* where isolates from *Z. mucronata* grouped with *D. alatafuctra, D. seriata* and *Diplodia* sp. (BS=87%) (Fig. 3.6). These isolates also grouped with the species on the ITS phylogeny, as well as with *D. pseudoseriata, D. pinea*, *D. intermedia* and *D. allocellula*. The translation elongation factor (TEF-1α) sequences for these fungal species were not available in GenBank. However, two isolates that were identified belonging to *Diplodia* (ZBM8.5, ZBM29.4) and one belonging to *Dothiorella* (ZBM12.4) on the ITS phylogeny formed a separate Clade **C** on the TEF-1α maximum likelihood tree (Fig. 3.6). Lastly, two isolates from Tshikundamalema and Wits Rural Facility grouped with *Botryosphaeria dothidea* supported by a 99% bootstrap value and one isolate (WRZ33.1) that was placed close to *Botryosphaeria* species on the ITS phylogenetic tree grouped with *Neofusicoccum kwambonambiense* on the TEF-1α phylogenetic tree (BS=100%).

ZBM5 2
ZBM70 3
ZBM80 2
ZBM12 3
KC769865 *Spencermartinsia viticola*
ZBM78 1
WRZ65 1
WRZ64 1
WRZ26B 1
ZT14 2
ZT46 1
ZT57 3
ZT44 1
WRZ23 1
ZT52 2
ZT17 6

A: *Dothiorella/ Spencermartinsia*

80
91
99
96
97

KY681037 *Dothiorella omnivora*
KY681038 *Do. omnivora*
JQ512104 *B.dothidea*
JQ512133 *Do. sarmentorum*
KP828802 *Do. iberica*
KP828801 *Do. iberica*
HM176514 Botryosphaeriaceae sp
ZBM80 6
ZBM5 3
ZT57 2
ZBM63 2
ZBM27 2A
ZT13 4
ZBM9 1
KR611910 *Do. ulmicola*

100
97
96
76
68
97
69

GU134939 *Botryosphaeria mamane*
GU134941 *B. mamane*
FJ888457 *Cophinforma atrovirens*
ZT17 8
KU997256 *B. dothidea*
WRZ60 1
FJ900655 *Neofusicoccum* sp.
WRZ33 1
KY024656 *N. kwambonambiense*

74
100
76
99
99
100
64

ZT33 3
WRZ36 2
ZT45 1
WRZ22 2
WRZ24 2
ZT54 3
KX029225 *D. seriata*
ZBM45 3
HM176511 *Diplodia* sp.
HM176508 *Diplodia* sp.
FJ888446 *D. alatafructa*
ZT13 1
ZT31 2
ZT55 1

B: *Diplodia*

Botryosphaeriaceae

62
72
99
68
87
87
90
86

Figure 3.6: Maximum likelihood tree based on the TEF-1α region showing relationships between isolates obtained from *Z. mucronata* and known sequences from GenBank. Isolates marked in bold are from this study. Bootstrap values greater than 60 % from 1000 replications of ML analysis are indicated on the nodes. The tree is rooted to *Arthrobotrys vermicola*.

3.3.3.2.2 Diaporthaceae

The TEF-1α phylogenetic tree placed 10 isolates (Buzzard Mountain Farm = seven, Tshikundamalema = two, Wits Rural Facility = one) in the Diaporthaceae within *Diaporthe*, same as on the ITS phylogenetic tree (BS=94%) (Fig. 3.5). The isolates grouped close to *D. baccae*, *D. macintoshii*, *D. raonikayoporum* and *D. psoraleae*. Although there were few isolates that grouped close to *Phomopsis* species in the ITS maximum likelihood tree, *Diaporthe* is well known as the sexual reproductive stage of *Phomopsis.*

3.3.3.2.3 Cytosporaceae and Nectriaceae

The Cytosporaceae (=Valsaceae) included four isolates from Tshikundamalema that were placed within *Cytospora* and were phylogenetically closely related to *C. sacchari* and *C. acacia* on the TEF-1α phylogeny (BS=68%) (Fig. 3.6). These isolates also grouped as sister with *C. rhizophorae*, *C. nitschikii* and *C. cerdri*. In the Nectriaceae, two isolates from Wits Rural Facility and one isolate from Tshikundamalema formed three sub-clades grouping with *Fusarium* species same as in the ITS-based phylogenetic tree. Isolates obtained from *Z. mucronata* grouped with *F. decemcellulare, F. lateritium*, *F. lacertarum* and *F. equiseti* and the sub-clades were supported by bootstrap values above 80% (Fig. 3.6).

3.3.3.2.4 Pleosporaceae and Didymellaceae

In the Didymellaceae, two isolates from Tshikundamalema formed a sub-clade grouping with *Boeremia exigua* (syn. *Didymella exigua*) with a supporting value of 99% (Fig. 3.6). The TEF-1α phylogeny of Pleoporaceae also included isolates from Tshikundamalema only. The

isolates grouped close to *Alternaria alternate* and *Alternaria* species (Fig. 3.6), same as in the ITS phylogenetic tree (Fig. 3.5).

3.3.4 Fungi identified on *Z. mucronata* showing smut (*Coniodictyum chevalieri*)

Isolates in *Diplodia*, *Dothiorella* and *Diaporthe* were identified from branches of *Z. mucronata* showing both dieback and smut infection, as well as from trees that had no visible smut symptoms at all. These isolates originated from Tshikundamalema, Buzzard Mountain Farm and Wits Rural Facility. Isolates identified as *Botryosphaeria* and *Fusarium* species collected from Tshikundamalema and Wits Rural Facility were also from smut-infected trees and trees that showed no smut symptom. *Didymella* and *Alternaria* isolates were identified only from Tshikundalema. However, *Didymella* isolates were obtained from *Z. mucronata* trees with smut and isolates belonging to *Alternaria* were obtained from both trees with smut and trees that had no visible smut infection.

Figure 3.7: Pictures showing a branch with both dieback and smut (A), and a branch with dieback only (B).

3.3.5 Fungal occurrence in different locations

Phylogenetic results showed overlapping identities of the fungal isolates among the three study locations; Tshikundamalema, Buzzard Mountain Farm and Wits Rural Facility. However, there are isolates that were identified from only one location. *Diplodia* and *Dothiorella* (=*Spencermarticia*) isolates were frequently identified from samples collected in all the three locations, and were the major genera identified in the Botryosphaeriaceae (Fig.3.8).

Buzzard Mountain Farm

Wits Rural Facility

Tshikundamalema

Diaporthe

Diplodia
Dothiorella

Botryosphaeria

Fusarium

Cytospora

Alternaria

Didymella
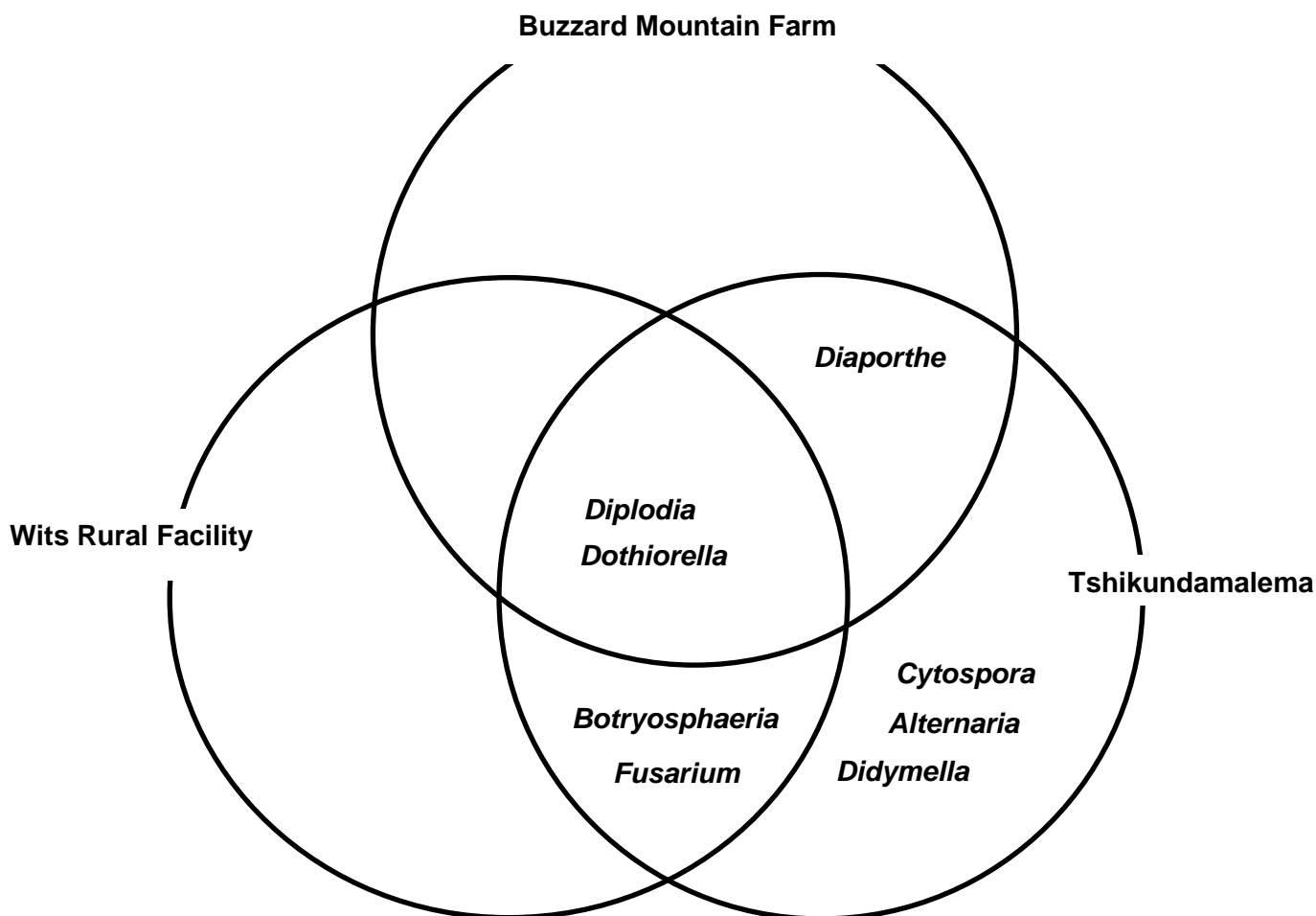
Figure 3.8: Diagram showing different genera identified on isolates from *Z. mucronata* branches with dieback collected at Buzzard Mountain Farm, Tshikundamalema and Wits Rural Facility.

Isolates belonging to *Diaporthe* were identified from Buzzard Mountain Farm and Tshikundamalema only, while *Botryosphaeria* and *Fusarium* isolates were only identified from Wits Rural Facility and Tshikundamalema. *Cytospora*, *Didymella* and *Alternaria* isolates were identified from Tshikundamalema only (Fig. 3.8).

3.4 Discussion

In this study, fungal species from six different families that are associated with dieback on branches of *Z. mucronata* from different locations in the Limpopo Province (South Africa) were identified. These fungal families were Botryosphaeriaceae, Diaporthaceae, Cytosporaceae, Nectriaceae, Pleoporaceae and Didymelaceae. Identifications of the isolates were based on the internal transcribed spacer and a portion of the translation elongation factor gene.

Dieback is one of the most common diseases of woody plants in both natural and agricultural ecosystems across the world. For example, in South Africa this condition has been reported on woody species in the genera *Eucalyptus* (Smith *et al.*, 1998), *Vitis* (Ferreira *et al.*, 1989) and *Schizolobium* (Mehl *et al.*, 2017). In all cases, fungi were the causal agents of infection. In this study, branch dieback of *Z. mucronata* was manifested by defoliation and/or wilting of the leaves, as well as dying of branch tips in the three study locations.

A comparatively larger number of isolates in this study belonged to the Botryosphaeriaceae. Isolates from this family represented three genera which were *Dothiorella*, *Diplodia* and *Botryosphaeria* based on the ITS region. *Dothiorella* was the most frequently occurring genus, followed by *Diplodia* and *Botryosphaeria*. Species in these genera have been reported causing dieback on several woody species around the world (Slippers and Wingfield, 2007). For example, *Diplodia seriata*, *D. sapinea* and *D. mutila*, *Dothiorella sarmentorum*, *Botryosphaeria dothidea* and *Dothiorella* spp. were reported causing dieback on ornamental trees (*Chamaecyparis lawsoniana*; *Abies concolor*; *Cedrus atlantica*; *Sequoiadendron giganteum*) in Western Balkans (Zlatković *et al.*, 2016). These species also showed high similarity to isolates obtained from *Z. mucronata* and grouped close to them on the phylogenetic trees. The isolates also showed similarity with *D. allocellula*, *Do. dulcispinae* and *Do. brevicollis* which were first identified by Jami *et al.* (2012) and being associated with dieback on *Vachelia karroo* (=*Acacia karoo*) in South Africa (Pretoria).

Neofusicoccum species have been reported in several studies causing dieback and cankers on woody plants in both natural and agricultural ecosystems (Amponsah *et al.*, 2009, Iturritxa *et al.*, 2011, Mehl *et al.*, 2014). In this study, one isolate grouped with a *Neofusicoccum* species (previously known as *Botryosphaeria* spp.) on the TEF-1α phylogenetic tree. *Neofusicoccum* species may survive as endophytes. For example, Smith *et al.* (2001) reported *N. eucalyptorum* in South Africa on *Eucalyptus grandis* and *Eucalyptus nitens* from cankered branches and twigs with dieback. This fungal species was later reported by Pérez *et al.* (2009) being present on trees growing in regions of healthy native forest surrounding *Eucalyptus* plantations. This suggests that *Neofusicoccum* species can occur as endophytes on trees that do not show any signs of infection. Similarly, the *Neofusicoccum* species identified from *Z. mucronata* might occur as endophytes, but have caused dieback when the tree was under stress.

Species in *Diaporthe* cause diseases such as dieback, cankers, leaf spots, blights, root and fruit rots, decay and wilt on various plants hosts in South Africa (Mostert *et al.*, 2001, Van-Rensburg *et al.*, 2006). In our study, Diaporthaceae included the largest number of isolates after the Botryosphaeriaceae. Ten isolates, seven from Buzzard Mountain Farm, two from

Tshikundamalema and one from Wits Rural Facility were identified as *Diaporthe* species. Some of the *Diaporthe* species in this study were collected from the same branches that were infected with species in the Botryosphaeriaceae. The presence of both Botryosphaeriaceae and Diaporthaceae was also recorded by Chen *et al.* (2014) on *Pistacia vera* causing shoot blight in California, USA. A study by Chebil *et al.* (2017) also identified species from these two families on grapevine (*Vitis* sp.) in Tunisia.

Alternaria is a cosmopolitan genus that consists of several saprophytic and pathogenic species (Woudenberg *et al.*, 2015). Species in this genus are well known to cause leaf and fruit spot on ornamental and fruit trees. In our study, only three isolates were identified as belonging to genus *Alternaria*. Ferreira *et al.* (1989) Identified *A. alternata*, *Sphaeropsis* sp., *Fusarium oxysporum, Eutypa lata, Pestalotia quepini* and *Botrytis cinerea* associated with dieback of grapevines (*Vitis* sp.) in South Africa. In their study, *A. alternata* was identified as a saprophyte and the other fungi were established being parasitic fungi on the grapevines. Chebil *et al.* (2017) also identified *A. alternata* together with *Diplodia seriata*, *Neofusicoccum australe*, *N. vitifusiforme* and *Diaporthe neotheicola* associated with dieback and other symptoms on grapevine in Tunisia. In the current study, *Alternaria* species are regarded being saprophytes since most of these fungal species are well known to cause black spots on leaves and fruits of hosts such as *Coriandrum sativum* (Mangwende *et al.*, 2018), *Spinacia oleracea* (Czajka *et al.*, 2015) and *Allium cepa* (Bihon *et al.*, 2015), and they have mostly been recorded on tree branches as a saprophytes.

Three isolates, two from Wits Rural Facility and one from Tshikundamalema were identified as *Fusarium* species based on both ITS and TEF-1α phylogenetic analysis. The isolates grouped closely related to *F. decemcellulare*, *F. equisetii, F. lateritium*, *F. lacertarum* with a supporting value above 80% in the phylogenetic trees. This is the first report of *Fusarium* species occurring on *Z. mucronata* branches in South Africa. Species in *Fusarium* are known to be cosmopolitan and inhabit a wide range of substrates such as soil, decaying plant materials, healthy plant parts and are also plant pathogens (Nelson *et al.*, 1994). Although there is no report of dieback on *Z. mucronata* available, Mirzaee *et al.* (2011) identified *F. solani* for the first time in Iran associated with dieback on *Z. jujube*. Qi *et al.* (2013) reported *F. decemcellulare* being the causal agent of dieback on mango in China. *Fusarium decemcellulare* was also identified with other two *Fusarium* species by Lombard *et al.* (2008) causing canker bark and dieback on *Cedrelinga cateniformis* in Ecuador. In our study, one isolate from Wits Rural Facility grouped closely with this fungal species, suggesting that this species related to *F. decemcellulare* might be responsible for branch dieback on the particular tree at this study site. *Fusarium equiseti* and *F. lateritium* with which some isolates in this study showed an evolutionary affinity, have been reported being associated with wilt and dieback of

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Aquilaria malaccensis and *Fraxinus excelsior* respectively (Kowalski *et al*., 2016, Pandey *et al*., 2019), while *F. lacertarum* is known to cause cladode rot as well as damping-off on *Nopalea cochenellifera* and *Casuarina equisetifolia* in Brazil, respectively (Poletto *et al*., 2015, Santiago *et al.*, 2018). However, this fungal species have not yet been reported in South Africa.

Cytospora species (Diaporthales, Cytosporaceae) are known as causal agents of cankers and dieback on hardwoods and coniferous trees (Adams *et al*., 2006). They are considered facultative parasites that attack trees that are stressed and some are strictly saprobic on dying trees (Christensen, 1940, Adams *et al*., 2006). In the current study, four isolates from Tshikundamalema were identified as *Cytospora* species based on the ITS and TEF-1α phylogenetic analysis. *Cytospora* species isolated from *Z. mucronata* might have attacked the trees when they were stressed or parasitize on the dead part of the branch after primary infection by other fungal pathogens. Recently, Jami *et al*. (2018) identified a new *Cytospora* species (*C. carpobroti*) on *Carpobrotus edulis* in areas close to Cape Town, South Africa. The fungus was collected from wilted leaves and dead woody stems of *C. edulis* and pathogenicity test confirmed that the fungal species is pathogenic to the host species (Jami *et al*., 2018). However, Adams *et al*. (2006) identified *Cytospora* species from dead branches of trees around South Africa where the fungi occurred as saprophytes. In accordance with the results from these studies, we suggest that *Cytospora* species identified from *Z. mucronata* might act as saprobes or secondary pathogens on this tree species. Further studies are needed to assign species rank to the isolates and to determine if they are indeed pathogens of *Z. mucronata*.

Didymella (=*Phoma*) species were isolated from the same branches of *Z. mucronata* with dieback from which *Cytospora* species were isolated. A *Didymella* species was also recorded by Schreuder (1988) on *Vachellia maillfera* (blackthorn) in South Africa (Limpopo, North West and Eastern Cape) associated with dieback, cankers and defoliation. Species in *Didymella* are cosmopolitan in nature and they are known plant pathogens of a wide range of hosts, as well as being saprophytes (Irinyi *et al*., 2007). Taieb *et al*. (2014) identified *P. fungicola* in branches of olive trees with dieback in Tunisia. More recently, Moral *et al*. (2017) identified *Didymella*-like fungi together with *Neofusicoccum mediterraneum*, *Botryosphaeria dothidea*, *Cytospora* species and *Diaporthe* species associated with dieback of olive branches in Spain. Forbes and Pearson (1987) also identified a *Didymella* species associated with dieback, stem canker and anthracnose of *Coprosma* spp. in New Zealand. However, *Didymella* species did not show any significant infection symptoms during pathogenicity tests and, as such, the authors suggested that *Didymella* species isolated from their collected plant material are secondary pathogens or saprophytes. Although pathogenicity trials were not performed in the current

study, it is reasonable to assume that *Didymella* species identified from *Z. mucronata* could either be saprophytes or secondary pathogens due to their lower occurrence on *Z. mucronata.* Pathogenicity trials are, however, needed to confirm this.

Comparison of the genera present at the different collection sites revealed that site location does not affect the presence of the Botryosphaeriaceae on *Z. mucronata*. This is because species belonging to this family were identified from all the three locations. However, some species in Cytosporaceae, Nectriaceae, Didymellaceae and Pleoporaceae were only identified in one location, while other species in these families were collected at two locations. *Cytospora*, *Didymella*, and *Alternaria* species were only recorded from Tshikundamalema. *Fusarium* species were also identified from Wits Rural Facility and Tshikundamalema only. The difference in the occurrence of these fungal species could be due to differences in climatic conditions among our study locations or the effect of sampling strategy. Hence sample size needs to be increased and further research is needed to fully investigate the effect of geography on the species associated with dieback on *Z. mucronata.*

3.5 Conclusions

Results of this study revealed diversity of fungal species associated with branch dieback on the native tree species, *Z. mucronata* growing at different locations in the Limpopo Province. This study is the first to investigate the diversity of fungi associated with this tree species. Fungi belonging to the families Botryosphaeriaceae, Nectriaceae and Diaporthaceae, Cytosporaceae, Pleoporaceae and Didymellaceae were identified, among which the Botryosphaeriaceae represented the largest family. Species from this family have been reported being associated with dieback of woody plants in both agricultural and natural ecosystem in South Africa and other countries by several researchers (Pavlic *et al.*, 2007, Rodríguez-Gálvez *et al.*, 2017, Zlatković *et al.*, 2016). As such, we suggest that species in the Botryosphaeriaceae could be potential primary pathogens responsible for branch dieback on *Z. mucronata*, while other fungal species identified in this study act as secondary pathogens or saprophytes. Future studies should focus on confirming the identity of isolates through multiple gene sequencing and phylogenetic analysis, and to conduct pathogenicity studies to determine which fungal species are indeed pathogens of *Z. mucronata*

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CHAPTER 4: Diversity of Botryosphaeriaceae associated with branch dieback of *Ziziphus mucronata* in Limpopo Province.

ABSTRACT

Dieback of woody species has been associated with fungi in the Botryosphaeriaceae in South Africa and other countries. Before this study, there were no records of these fungi causing dieback on *Ziziphus mucronata* (Rhamnaceae). The aim of this study was to investigate the species of the Botryosphaeriaceae associated with branches of *Z. mucronata* that showed dieback symptoms from different locations in Limpopo Province, South Africa. Samples were collected from Tshikundamalema, Buzzard Mountain Farm and Wits Rural Facility. Sequence data from three genome regions, namely the internal transcribed spacer (ITS), beta-tubulin gene and translation elongation factor (TEF-1α) gene were obtained and used in phylogenetic analyses of the collections. The phylogenetic studies clustered the samples with *Dothiorella brevicollis*, *Do. plurivora*, *Dothiorella* spp., *Diplodia allocellula*, *D. pseudoseriata* and *Botryosphaeria fusispora* from the three study locations. *Dothiorella yunnana* and *Neofusicoccum* sp. were only identified from Buzzard Mountain Farm and Wits Rural Facility. This is the first study to give a report on the diversity of Botryosphaeriaceae fungi associated with dieback on *Z. mucronata*.

Keywords: Botryosphaeriaceae, BT, Dieback, ITS, TEF-1α, *Ziziphus mucronata*

4.1 Introduction

Botryosphaeriaceae (Dothideales) comprises a wide range of morphologically diverse fungal species that can either be pathogens, endophytes or saprobes (Phillips *et al.* 2013). Fungi in this family have a cosmopolitan distribution and occur on a wide range of host plants that include commercial fruit and forest trees as well as natural woody ecosystems (Slippers and Wingfield 2007; Mehl *et al.* 2017). Fungi in the Botryosphaeriaceae are considered being weak pathogens since they invade plants that are under stress (Jami *et al.* 2012). They have also been shown to occur in asymptomatic tissues, including woody tissues of stems, branches, twigs and leaves as latent pathogens and may persist endophytically (Smith *et al.* 1994; Jami *et al.* 2015). Their occurrence as endophytes makes them very important as they may be spread from one area to another without being detected, causing potentially serious damage to hosts that might not have co-evolved resistance (Slippers and Wingfield 2007).

The Botryosphaeriaceae was first described by Theissen and Sydow (1918) and included three genera namely *Botryosphaeria*, *Phaeobotryon* and *Dibotryon*. Since then, the taxonomy of the Botryosphaeriaceae based on morphology has been problematic for many years. In the recent past, the taxonomy of the family has been reviewed and updated based on phylogenetic analyses. Now, the Botryosphaeriaceae represents a diverse family of more than 78 genera (including separate names for sexual and asexual genera) with over 2000 species (Slippers and Wingfield 2007; Dissanayake *et al.* 2016b). The most commonly known genera include *Diplodia*, *Lasiodiplodia*, *Neofusicoccum*, *Pseudofusicoccum*, *Dothiorella*, *Botryosphaeria* and *Sphaeropsis* (Slippers *et al.* 2005; Dissanayake *et al.* 2016b).

Members of the Botryosphaeriaceae gain access to their hosts through natural openings and wounds and are associated with several plant disease symptoms that include shoot blights, stem cankers, fruit rots, dieback and gummosis (Slippers and Wingfield 2007; Jami *et al.* 2015). Sometimes these symptoms are followed by extensive production of kino, a dark-red tree sap, and in severe cases, tree mortality (Mohali *et al.* 2007). Most of the Botryosphaeriaceae have been isolated from plant parts showing dieback symptoms, others only from asymptomatic tissues and some have been found in both tissue types (Slippers and Wingfield 2007).

In South Africa, species of Botryosphaeriaceae occur widely and they have been found almost on every tree species that has been sampled (Smith *et al.* 1994; Smith *et al.* 2001; Jami *et al.* 2014). These fungi have been reported on native and non-native plants showing common symptoms such as dieback and cankers associated with them. Examples of native hosts include the following tree species; *Terminalia catappa* (Begoude *et al.* 2010), *Pterocarpus*

angolensis (Mehl *et al.* 2011), *Vachellia mellifera* (Slippers *et al.* 2013) and *V. karroo* (Jami *et al.* 2012). Non-native hosts in South Africa include *Pinus* spp., *Eucalyptus* spp. and *Prunus* spp. (Jami *et al.* 2014). Seven species of Botryosphaeriaceae were identified from branches and twigs of *Pterocarpus angolensis* with dieback, as well as from healthy plant parts by Mehl *et al.* (2011). The species that have been identified were *Pseudofusicoccum violaceum*, *P. olivaceum*, *Diplodia alatafructa*, *Fusicoccum atrovirens*, *Lasiodiplodia theobromae*, *L. pseudotheobromae* and *L. crassispora*, and this was the first study to consider the role of the Botryosphaeriaceae in the decline and dieback of *P. angolensis* trees in South Africa.

There is currently no information available regarding Botryosphaeriaceae associated with *Z. mucronata*. Only one pathogen, *Coniodictyum chevalieri* (the cause of smut) which was reported from *Z. mucronata* in the Kruger National Park, South Africa (Maier *et al.* 2006) is known to affect the tree species. *Ziziphus mucronata* (Rhamnaceae) commonly known as buffalo thorn is a multipurpose tree for people living in the rural areas in African countries where it is found. The tree serves medicinal purposes, for example, treatment of stomach aches using the roots and is used as source of food where leaves are eaten by humans when are cooked (Mazibuko, 2007). The current study aimed to investigate the diversity of Botryosphaeriaceae found on dieback branches of *Z. mucronata* in three different locations of Limpopo Province (Tshikundamalema, Buzzard Mountain Farm and Wits Rural Facility) since there is no documented record of this information.

Research question:

- What is the diversity of species in the Botryosphaeriaceae that are associated with branches of *Z. mucronata* showing dieback and smut infection?

4.2 Materials and methods

4.2.1 Isolate collection and Morphological groups

Samples were collected from three locations in Limpopo Province namely Tshikundamalema (longitude: 22°40.52'4 South; latitude: 30°39.49`7 East), Buzzard Mountain Farm (longitude: 23°1`3 S; latitude: 29°46`4 E) and Wits Rural Facility (longitude: 24° 56`386 S; latitude: 31° 29`076 E) that were described in Chapter 2 of this dissertation. Botryosphaeriaceae isolates were among the fungal species associated with branch dieback of *Z. mucronata* as described in the Chapter 3. Isolates resembling morphological characteristics of the Botryosphaeriaceae were grouped based on the colour and structure of their mycelia as described in Chapter 2.

4.2.2 DNA extraction

Genomic DNA was extracted from fresh mycelia using a protocol published by Chang *et al.* (1993) as described in Chapter 2. The quality and the presence of the extracted DNA were determined using a Thermo Scientific NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and DNA concentrations higher than 100 ŋg/μL were diluted to a working concentration of 50 ŋg/μL using sterile SABAX water.

4.2.3 PCR amplification and sequencing

Three gene regions, namely the internal transcribed spacer (ITS), portions of the translation elongation factor (TEF-1α) and beta-tubulin (BT) genes were amplified from the extracted fungal DNA to serve as templates for Sanger sequencing. The ITS and TEF-1α region were amplified using the primers ITS-1F and ITS-4R (White *et al.* 1990) and EF1F and EF2R (Jacobs *et al.* 2004) respectively, while the partial beta-tubulin was amplified using BT-2a and BT-2b (Glass and Donaldson 1995), as described in Chapter 2. Amplifications were confirmed by performing electrophoresis on 1% agarose gel in TAE (tris-acetate-EDTA) buffer for 30 minutes at 80 volts. Amplicons were then visualised under UV light (Biotium Inc., Hayward, CA, U.S.A.). Primers that were employed for amplification were used to sequence the purified amplicons in both directions (reverse and forward) for the gene regions and the sequencing products were then precipitated and cleaned-up using the methods described in Chapter 2. Samples were submitted to the DNA sequencing facility in the Faculty of Natural and Agricultural Sciences (NAS), at the University of Pretoria for Sanger sequencing.

4.2.4 Sequence and phylogenetic analyses

The quality of the resulting sequences was assessed with CLC Main Workbench v8.0.1 (QIAGEN, Aarhus, Denmark). Incorrect base calls during sequencing were corrected as described in Chapter 2. Contig sequences were created using this software. The sequences were then subjected to BLASTn searches to obtain their preliminary identifications.

Sequence matrices for each gene region were generated by combining the sequences from the isolates considered in this study with GenBank sequences that showed higher similarity. Sequence alignments were obtained with the online version of MAFFT v. 5.667 (Katoh *et al*. 2002) and edited using BioEdit (Hall 1999). Best fit nucleotide substitution models for each dataset was determined using jModelTest v0.1.1 (Posada 2008). Maximum Likelihood phylogenetic trees were generated with RAxML (Stamatakis 2016) as described in Chapter 2. Phylogenetic trees in this study were rooted to selected outgroup species.

4.3 Results

4.3.1 Isolates and DNA extraction

A total of 32 isolates resembling Botryosphaeriaceae were obtained from the morphological groups and they were 17 in total (Table 4.2 below). Fourteen representative isolates were collected from Tshikundamalema, nine from Buzzard Mountain Farm and nine isolates were collected from Wits Rural Facility. Morphological groups were differentiated based on their distinctive characteristics; colour and texture of mycelia (Fig. 4.1). Morphological group A included isolates with black to grey mycelia that is fluffy, while group B comprised isolates with flat brown mycelia with less melanin (Fig. 4.1). Isolates in group C had semi-fluffy mycelia that is white with brown hyphal tips and group D consisted of isolates that had black mycelia that is fluffy with abundant melanin (Fig. 4.1) Group E included isolates with black and flat mycelia, while group F comprised isolates that had white and fluffy mycelia with less melanin (Fig 4.1). DNA was successfully extracted from all the isolates. The quality of the extracted DNA was above 100ng/µl and the purity was an average of 1.9 ratio of absorbance (260/280) for all the isolates.
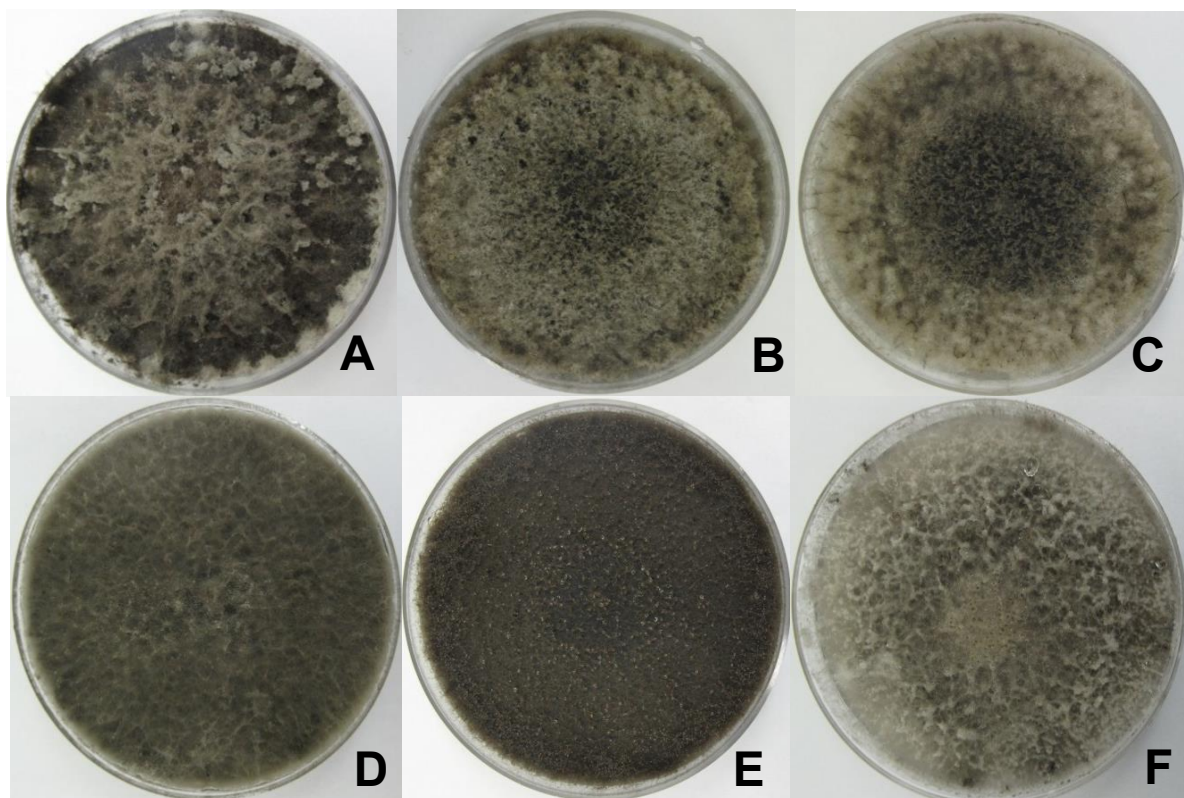
Figure 4.1: Culture morphology of representative isolates from the different morphological groups of Botryosphaeriaceae observed in this study. A: Black-grey and fluffy mycelia; B: brown and flat mycelia; C: white with brown hyphal tips; D: Black and fluffy mycelia; E: black and flat mycelia; F: white and fluffy mycelia.

4.3.2 PCR amplification and sequencing

PCR amplification of the internal transcribed spacer (ITS), translation elongation factor (TEF-1α) and beta-tubulin (BT) was successfully done on the extracted DNA using primers as described above. Band sizes were 500bp, 600bp and 400bp for the ITS, TEF-1α and BT genomic regions respectively. All isolates yielded similar band sizes for the respective genomic regions.

4.3.3 Sequences and phylogenetic analysis

BLASTn results showed that Botryosphaeriaceae isolates from *Z. mucronata* belong to four genera namely *Dothiorella*, *Diplodia*, *Botryosphaeria* and *Neofusicoccum* (Table 4.1).

Table 4.1: ITS, TEF-1α and BT BLASTn results for isolates obtained from infected *Z. mucronata.*

Location	Isolate code	ITS BLAST	TEF BLAST	BT Blast
Buzzard M. Farm	ZBM5.3,ZBM9.1, ZBM63.2, ZBM80.6,ZBM27.2A	*Dothiorella acacicola*	Botryosphaeriaceae sp.	*Dothiorella* sp.
	ZBM70.3,ZBM12.3, ZBM5.2, ZBM78.1	*Dothirella viticola*	*S. viticola*	*Do. plurivora*
	ZT18.2,ZT57.3, ZT44.1,ZT17.6	*Dothiorella longicollis*	*Do. omnivora*	*Do. longicollis*
	ZT31.2	*Diplodia pseudoseriata*	*D. alatafructa*	*Diplodia* sp.
	ZT13.4		Botryosphaeriaceae sp.	
	ZT54.3,ZT33.3, ZT45.1	*Diplodia pinea*	*D. seriata*	*D. allocellula*
	ZBM45.3	*Diplodia pseudoseriata*	*D.* species	*Diplodia* sp.
	ZBM29.4,ZBM8.5	*Diplodia pseudoseriata*	*Phialemonium dimorphosporum*	*Diplodia* sp.
Wits rural Facility	WRZ24.2,WRZ36.2, WRZ22.2	*Diplodia pinea*	*D. seriata*	*D. allocellula*
	WRZ33.1	*Botryosphaeria dothidea*	*Neofusicoccum* sp.	*N. kwambonambiense*
	WRZ60.1,WRZ67.2		*B. dothidea*	*Botryosphaeria* sp.
	WRZ23.1	*Dothiorella longicollis*	*Do. omnivora*	*Do. omnivore*
	WRZ26B.1	*Dothiorella oblonga*	*Do. omnivora*	*Do. oblonga*
	WRZ65.1	*Dothirella viticola*	*S. viticola*	*Do. plurivora*
Tshikundamalema	ZT17.8	*B. dothidea*	*B. dothidea*	*Botryosphaeria* sp.

The ITS sequence matrix included 32 isolates from *Ziziphus mucronata* and 61 sequences from GenBank. The sequence matrix comprised a total of 617 characters of which 255 were variable. In the TEF-1α sequence matrix, there were 32 isolates from this study and 61 sequences from GenBank that made up a total of 853 characters with 379 being variable. The BT sequence matrix comprised 32 isolates from *Z. mucronata* and 56 GenBank sequences that had a total of 456 characters, with 306 being variable. A combined ML tree for the concatenated sequence data of ITS, TEF-1α and BT was also successfully constructed to further confirm the identity of our isolates. The concatenated sequence data comprised of 1557 characters of which 860 were variable characters. Maximum likelihood trees which were constructed from these three genomic regions confirmed the results of BLASTn, such that all isolates were accommodated in the four genera namely *Dothiorella*, *Diplodia*, *Botryosphaeria* and *Neofusicoccum*.

4.3.3.1 ITS phylogeny

The maximum likelihood tree constructed from all isolates in this study and those obtained from GenBank placed them in four genera, i.e *Diplodia*, *Botryosphaeria*, *Dothiorella* and *Neofusicoccum* (Fig. 4.2). Based on the ITS phylogeny, three isolates from Wits Rural Facility (WRZ) and three isolates from Tshikundamalema (ZT) grouped with *Diplodia allocelula* and *D. estuarina* in Clade **A** with a supporting value greater than 60 % (Fig. 4.2). Clade **B** included

three isolates from Tshikundamalema and one isolate from Buzzard Mountain Farm (ZBM), which grouped with *D. pseudoseriata* and *D. alatafuctra* supported by 97% bootstrap value.

Within the *Botryosphaeria* Clade **C** (Fig. 4.2)**,** one isolate from WRZ grouped with *B. ramosum*. Two isolates, one from ZT and one from WRZ, were placed with *B. fusispora* and *B. cortis* but with a supporting value lower than 60%. The identity of these two isolates was therefore not clear based on the ITS sequence data alone. In the genus *Dothiorella* (Clade **D**), two isolates from WRZ and ZT grouped with *Do. dulcispinae*, *Do. thailandica* and *Do. oblonga*. The grouping was supported with a value of 82%. Based on the ITS phylogenetic tree, the identity of these isolate was also not clear. Clade **E** included five isolates from ZBM, two from ZT and one from WRZ. The isolates grouped closely to *Do. acacicola* and *Do. causari* but with no support. This may suggest these isolates could be new Dothiorella species. Clade **F** included four isolates from ZT and one isolate from WRZ. These isolates were placed close to *Do. brevicollis*. The grouping was supported with 65% bootstrap support. Clade **G** included two isolates from ZT and WRZ. The clade also included *Do. plurivora* and *Do. rosulata.* Clade **H** consisted of two isolates from ZT that grouped close to *Do. yannana* but with no bootstrap support. This clade formed a sub-clade with Clade **G** (84% bootsrap support). The close association of the two isolates suggest that they belong to *Do. yunnana*.
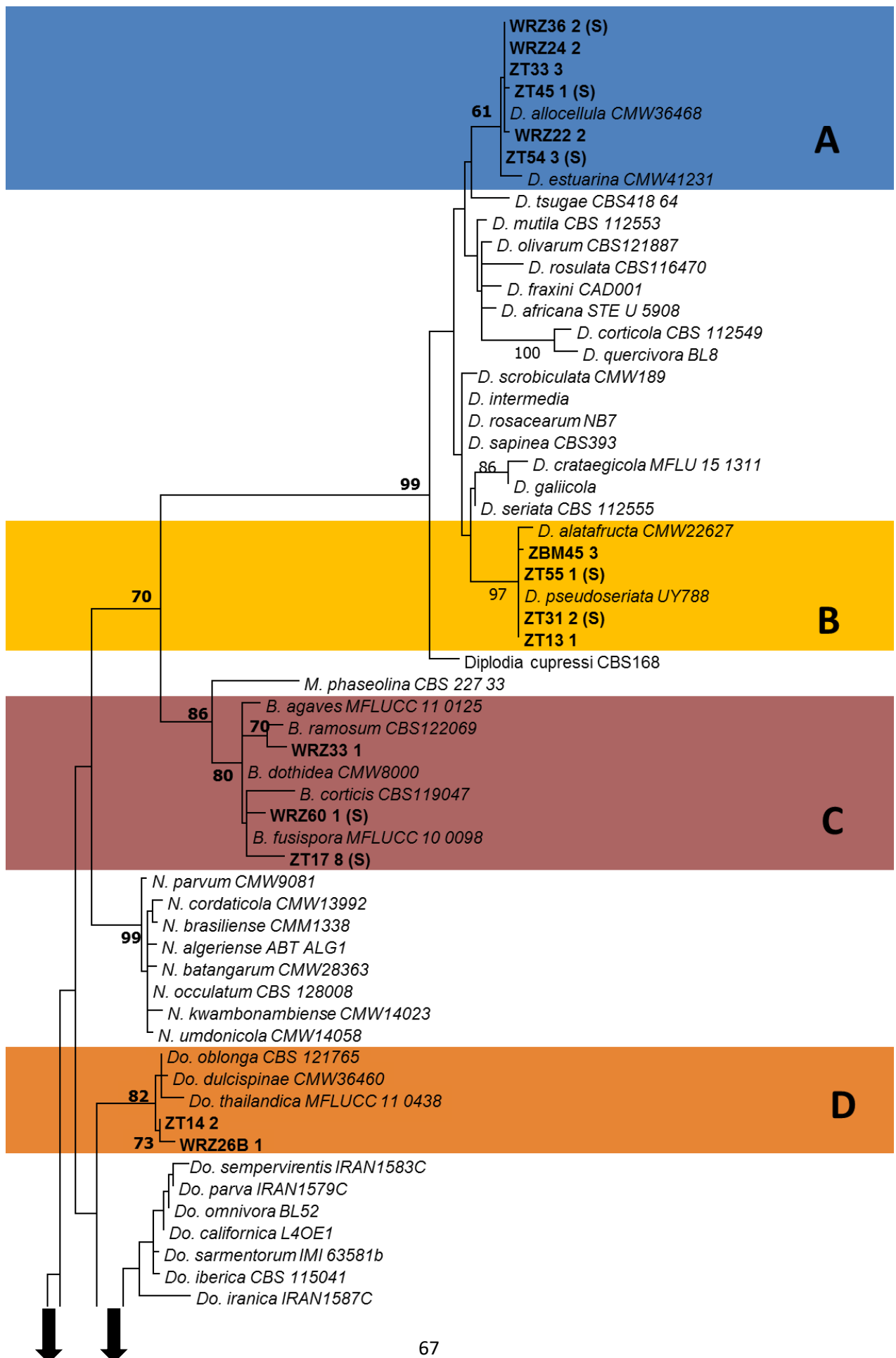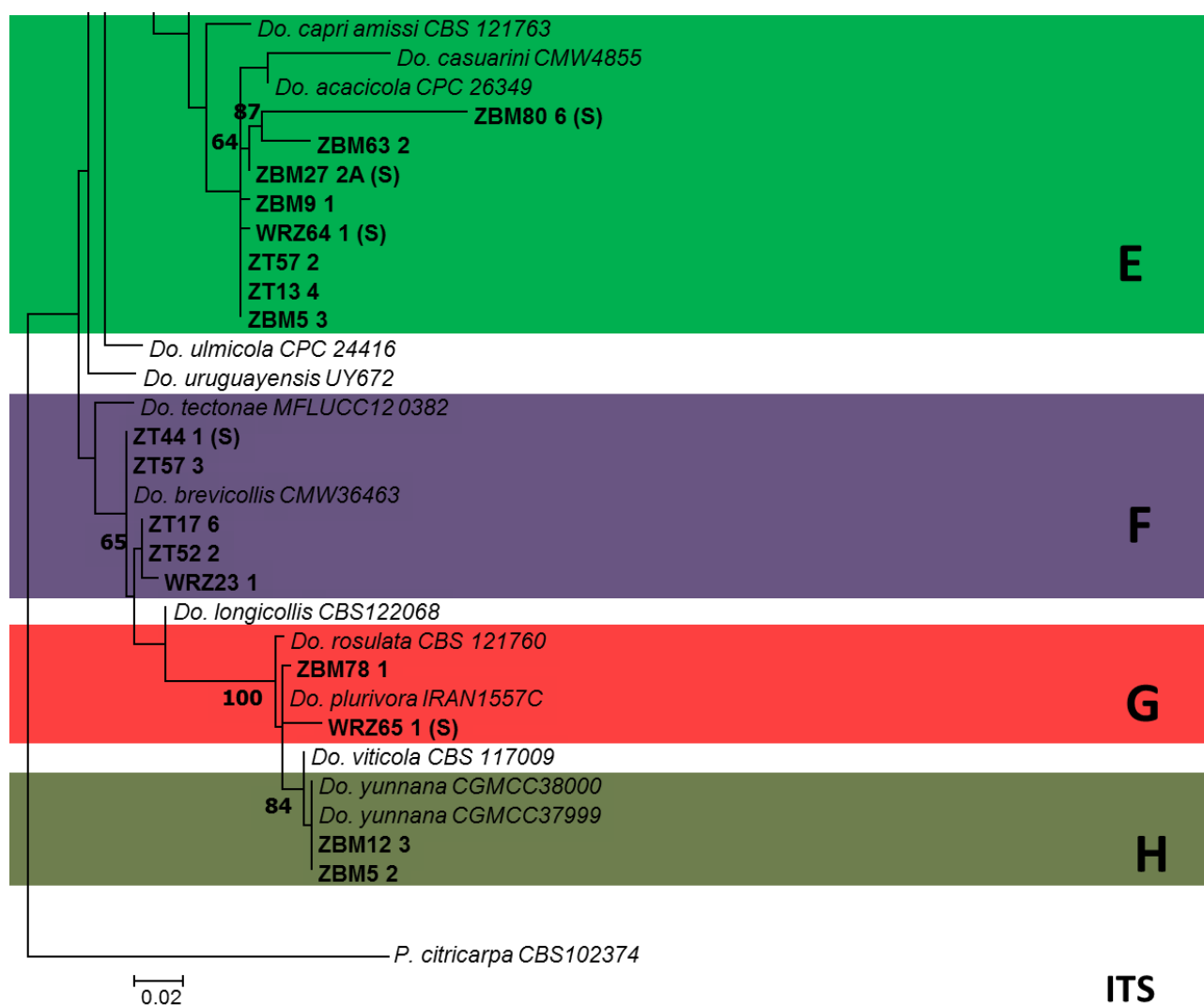
Figure 4.2. Maximum likelihood phylogenetic tree constructed based on the ITS region, showing relationships between isolates obtained from *Z. mucronata* and sequences retrieved from GenBank. Bootstrap values greater than 60 % are indicated on the nodes. Isolates marked in bold are from *Z. mucronata*, of which those with (S) were collected from trees with smut infection. The tree is rooted to *Phyllosticta citricarpa*.

4.3.3.2 TEF phylogeny

The maximum likelihood tree generated from the TEF-1α sequence data placed isolates from *Z. mucronata* within four genera which are *Diplodia, Dothiorella, Botryosphaeria* and *Neofusicoccum*. Three isolates from Tshikundamalema and three isolates from Wits Rural Facility grouped with *Diplodia allocellula* on Clade **A** with a supporting value of 94% (Fig. 4.3). Clade **B** included four isolates from this study that grouped with *D. pseudoseriata* and *D. alatafructa* with a 84% bootsrap support (Fig. 4.3). The result of this clade is congruent with the ITS maximum likelihood phylogenetic analysis (Fig. 4.2). Clade **C** comprised *Botryosphaeria* isolates with two isolates each respectively from Tshikundamalema and Wits Rural Facility. These isolates were placed close to *B. fusispora*, *B. cortis* and *B. dothidea.*

However, one isolate from Wits Rural Facility (WRZ33.1) that was placed within *Botryosphaeria* close to *B. ramosum* on the ITS maximum likelihood tree, grouped within the *Neofusicoccum* group with a bootstrap support of 83% (Fig. 4.3; Clade **C1**). The isolate grouped close to *N. kwambonambiense* and *N. brasiliense* on the TEF-1α phylogeny and it also grouped close to these two fungal species on the BT maximum likelihood tree.

C

- 61 — B. ramosum CBS122069
- 68 — B. agaves MFLUCC 11 0125
- 69 — B. dothidea CMW8000
  - B. corticis CBS119047
- 96 — B.fusispora MFLUCC 10 0098
- 67 — **WRZ60 1 (S)**
  - **ZT17 8 (S)**
- M. phaseolina CBS 227 33
- N. algeriense ABT ALG1
- N. occulatum CBS 128008
- N. parvum CMW9081
- 83 — N. batangarum CMW28363
  - N. cordaticola CMW13992
- 62 — N. umdonicola CMW14058

C1

- N. kwambonambiense CMW14023
- **WRZ33 1**
- N. brasiliense CMM1338

E

- **ZBM9 1**
- **ZBM63 2**
- 67 — **ZBM27 2A (S)**
- 100 — **ZT57 2**
  - **ZT13 4**
  - **ZBM5 3**
  - **ZBM80 6 (S)**
- 74 — 62 — Do. casuarini CMW4855
  - Do. capri amissi CBS 121763
  - Do. pretoriensis CMW36480
  - 83 — Do. moneti MUCC505
    - 93 — Do. santali MUCC509
  - Do. acacicola CPC 26349
- Do. sempervirentis IRAN1583C
- Do. californica L4OE1
- Do. omnivora BL52
- 98 — Do. parva IRAN1579C
  - Do. iberica CBS 115041
- Do. sarmentorum IMI 63581b
- Do. iranica IRAN1587C

H

- 68 — **ZBM5 2**
  - **ZBM70 3 (S)**
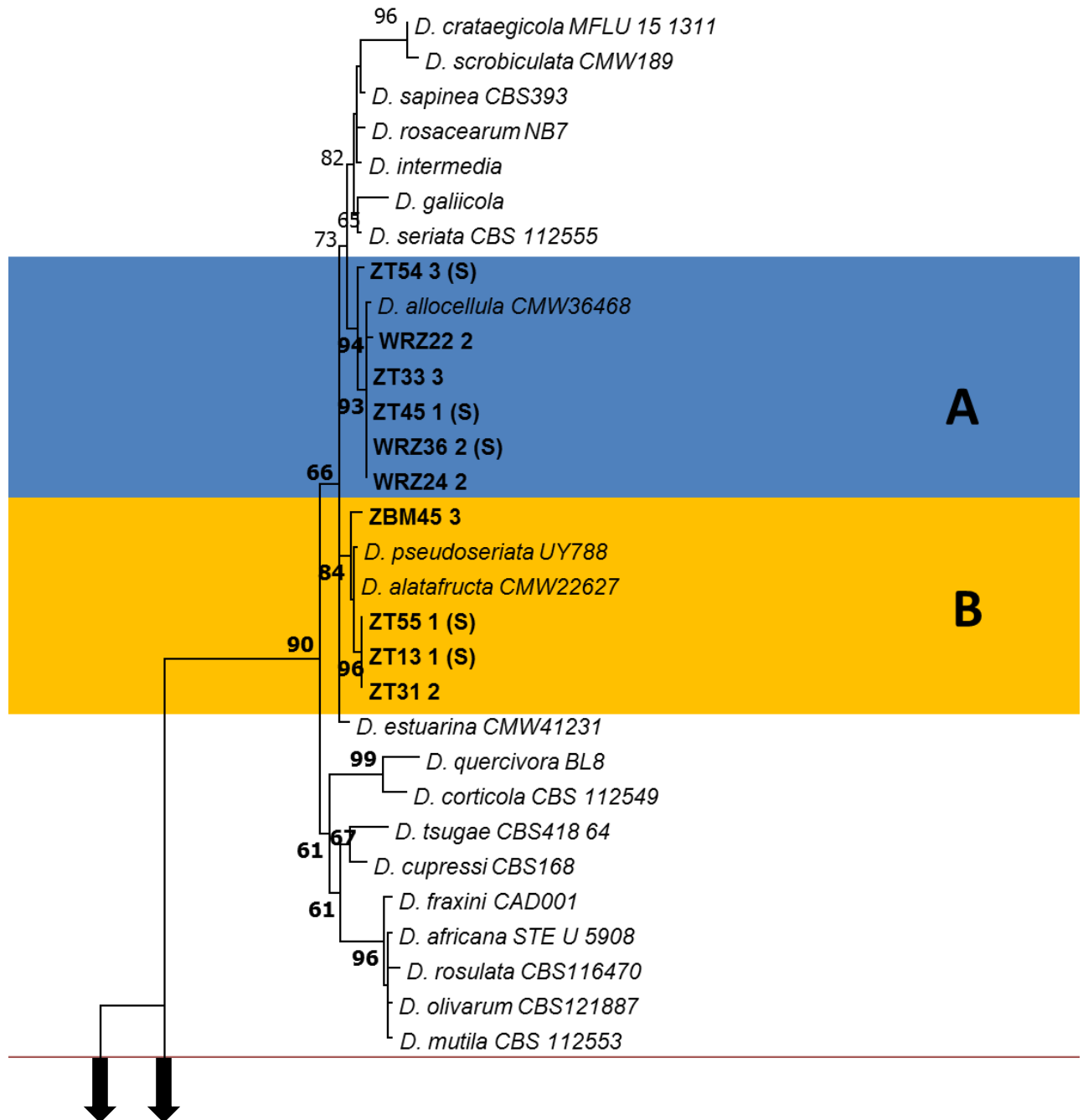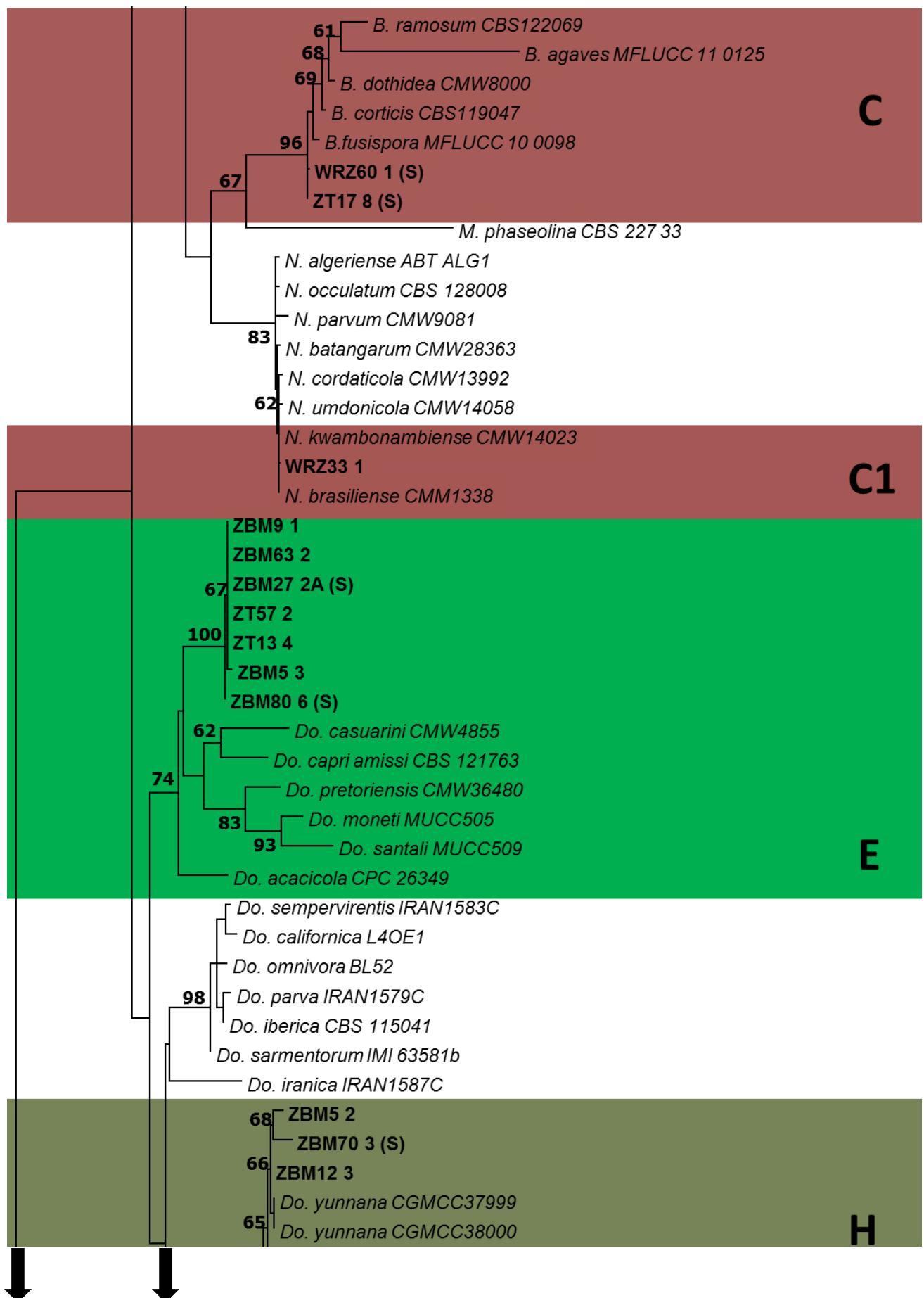- 66 — **ZBM12 3**
  - Do. yunnana CGMCC37999
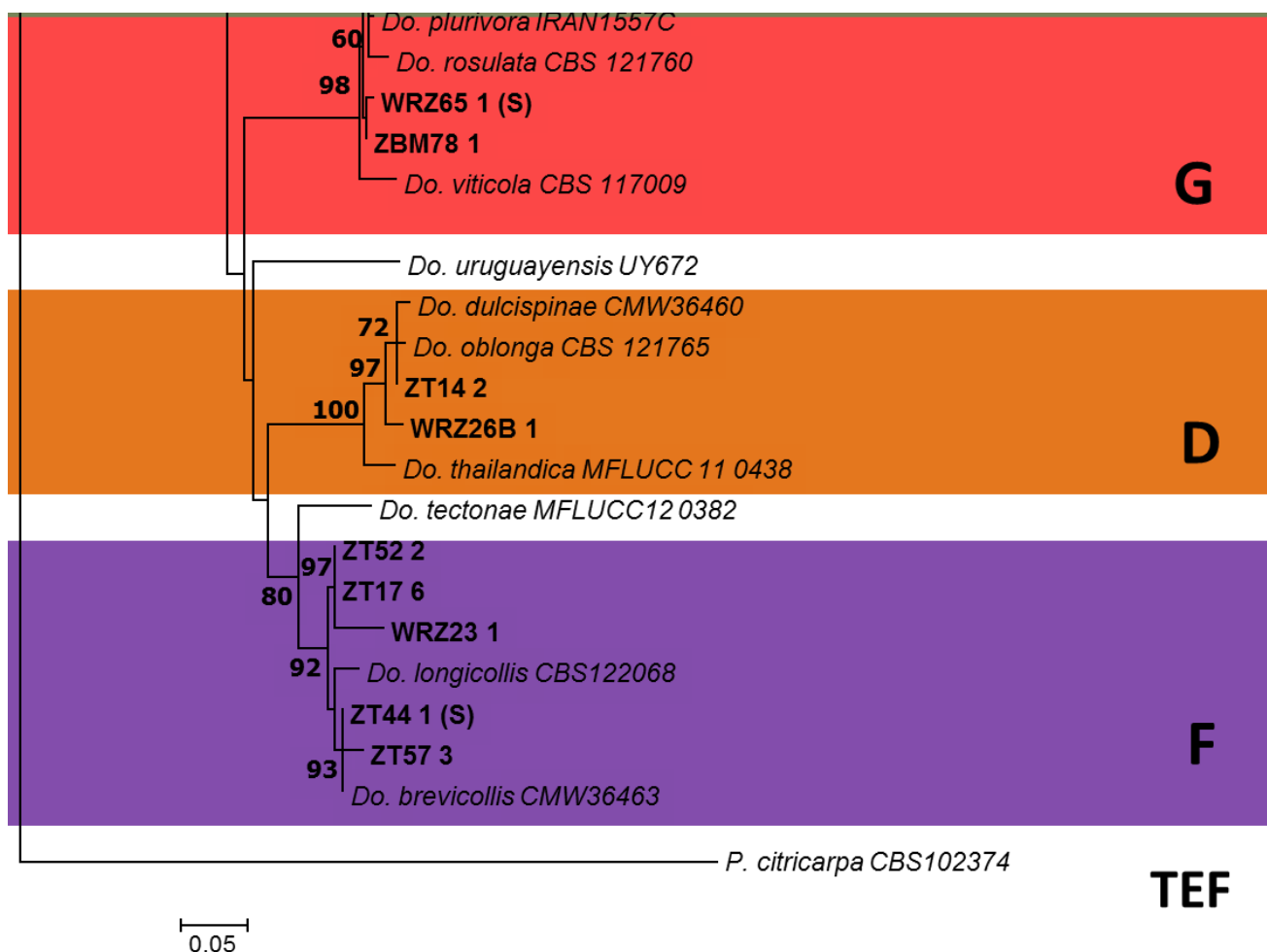- 65 — Do. yunnana CGMCC38000

70

Figure 4.3: Maximum likelihood tree based on the TEF-1α region, showing relationships between isolates obtained from *Z. mucronata* and known sequences of the Botryosphaeriaceae species from GenBank. Isolates marked in bold are from *Z. mucronata* and those with (S) were collected from trees with smut infection. Supporting values greater than 60 % from 1000 replications of maximum likelihood analysis are indicated on the branch nodes. The phylogenetic tree was rooted to *Phyllosticta citricarpa*.

Most of isolates from the current study were placed in the genus *Dothiorella*. Seven isolates, five from Buzzard Mountain Farm and two from Tshikundamalema, formed a separate sub-clade within *Dothiorella* and did not group with any sequence from GenBank (Fig. 4.3; Clade **E**). However, these isolates formed a sister clade with *Do. casuarini* and *Do. capri-amissi* and they also grouped with these fungal species on the ITS and BT phylogenetic trees. In Clade **F**, isolates from *Z. mucronata* formed two sub-clades together with two sequences from GenBank, these are *Do. longicollis* and *Do. brevicollis*. The isolates were also grouped with these fungal species on the ITS phylogenetic tree. Two isolates from Buzzard Mountain Farm and Wits Rural Facility grouped close to *Do. viticola* in sub-clade **G** while three isolates from Buzzard Mountain Farm were placed close to *Do. yunnana* in Clade **H**. The last two isolates in *Dothiorella*, one each from Tshikundamalema and Wits Rural Facility grouped with *Do.*

oblonga, *Do. dulcispinae* and *Do. thailandica* with the supporting value of 100% (Fig. 4.3; Clade **D**).

4.3.3.3 Beta-tubulin phylogeny

Clade **A** showed minimum genetic variability and included three isolates from Tshikundamalema and three isolates from Wits Rural Facility that were placed close to *Diplodia allocellula* from GenBank strongly supported by a bootstrap value of 94% (Fig. 4.4). In Clade **B**, four isolates from *Z. mucronata* grouped with *D. pseudoseriata*. These isolates consistently grouped with *D. pseudoseriata* and *D. alatafructa* on the ITS and TEF-1α trees, however, *D. alatafructa* beta-tubulin sequence was not available on GenBank. The maximum likelihood analysis of the BT genomic region (Fig. 4.4) showed an additional clade, referred to as Clade **C2,** in comparison to the TEF-1α tree (Fig. 4.3). This clade consisted of one isolate from Wits Rural Facility and two sequences representing *Neofusicoccum kwambonambiense* and *N. brasiliense* from GenBank.

73
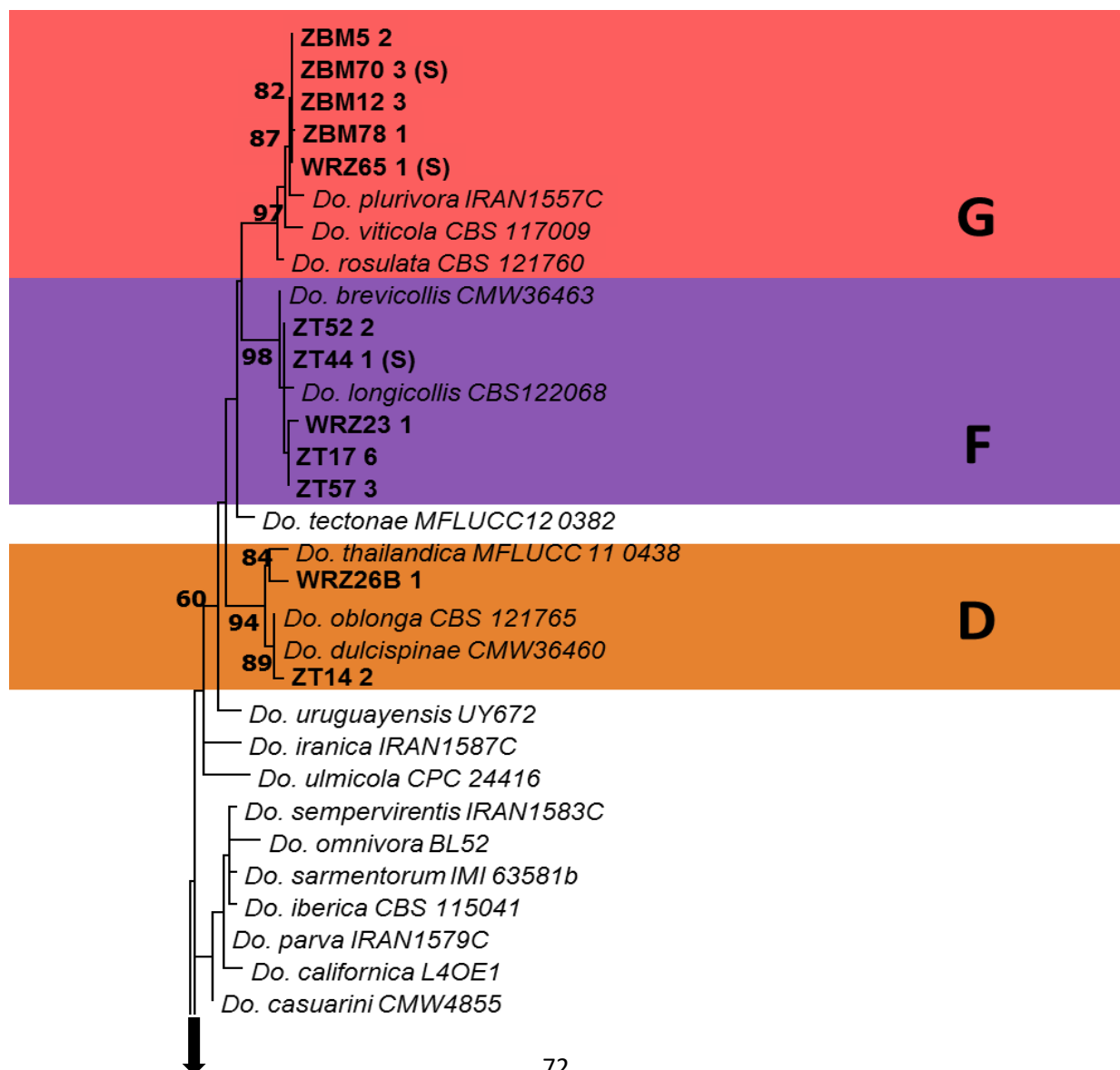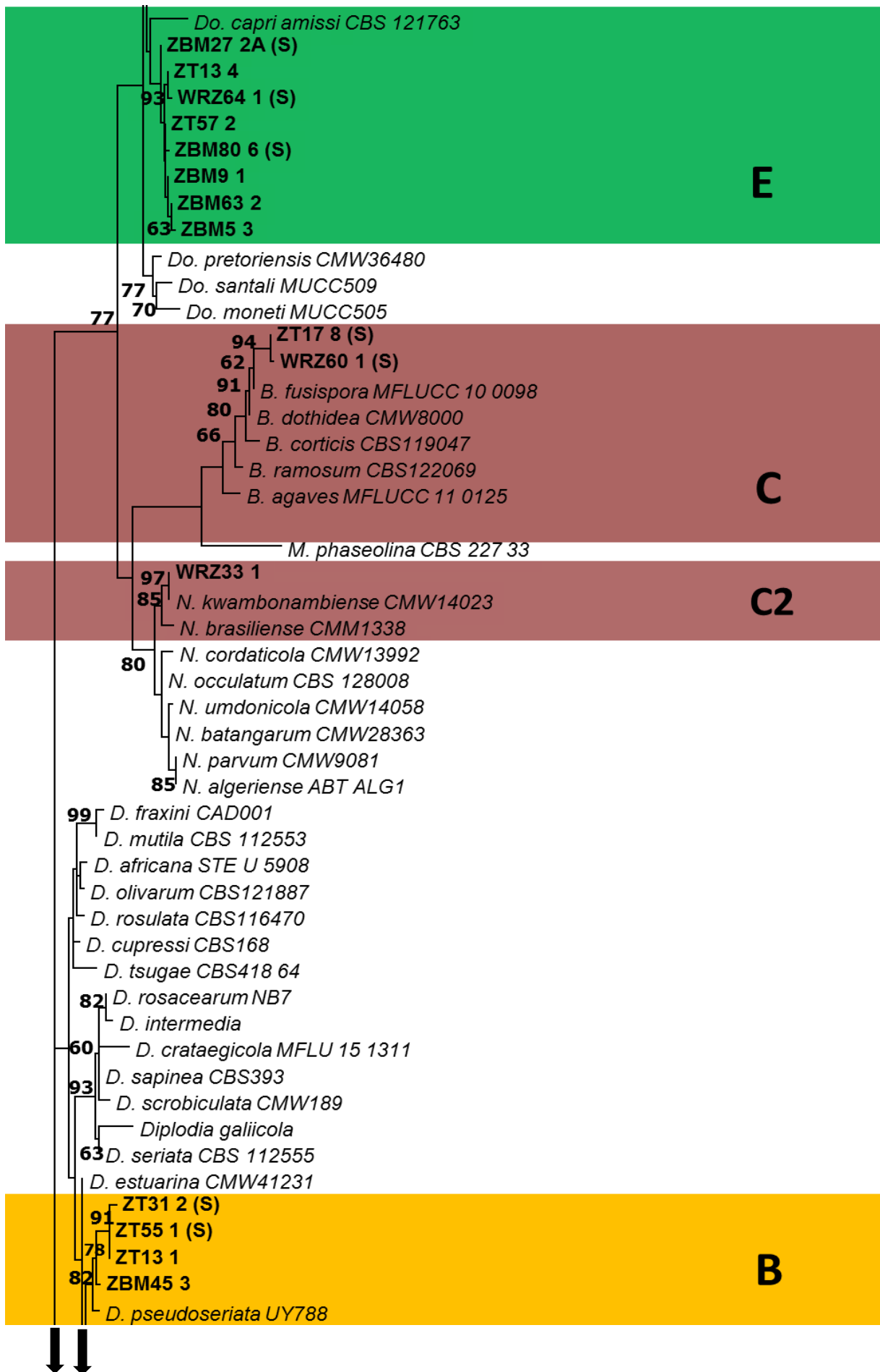
Figure 4.4: Maximum likelihood phylogenetic tree constructed based on the beta-tubulin showing relationships between isolates from *Z. mucronata* and the Botryosphaeriaceae sequences from GenBank. Isolates marked in bold are from *Z. mucronata* of which those with (S) were collected from trees with smut infection. Supporting values greater than 60 % of maximum likelihood analysis are indicated on the nodes. The tree is rooted to *Phyllosticta citricarpa*.
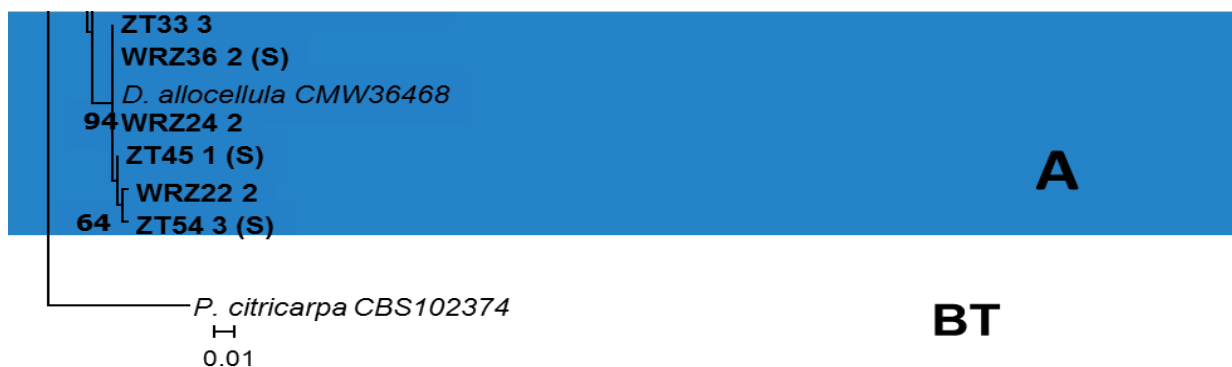
Clade **C** included only two isolates from our study and five species belonging to *Botryosphaeria*. This is in contrast to the ITS phylogeny that included three isolates from *Z. mucronata* (Fig.4.2). In the beta-tubulin phylogenetic tree (Fig. 4.4), Clade **D** included two isolates collected from *Z. mucronata* that formed two sub-clades grouping with *Dothiorella oblonga*, *Do. dulcispinae* and *Do. thailandica*. The two isolates grouped with the three fungal species in both ITS (Fig. 4.2) and TEF-1α (Fig. 4.3) maximum likelihood trees with the supporting values above 90%. Five isolates from Buzzard Mountain Farm, two from Tshikundamalema and one isolate from Wits Rural Facility formed Clade **E,** grouping as sister species with *Do. capri-amissi isolate* from GenBank. Clade **F** consisted of five isolates from *Z. mucronata* that consistently grouped with *Do. longicollis* and *Do. brevicollis* in all the maximum likelihood trees of the three genomic regions sequenced in this study. Clade **G** included four isolates from Buzzard Mountain Farm and one isolate from Wits Rural Facility place close to *Do. plurivora*, *Do. viticola* and *Do. rosulata*. In this clade, two isolates from Buzzard Mountain Farm (ZBM5.2 and ZBM12.3) were placed close to *Do. yunnana* in both ITS and beta-tubulin phylogenetic trees.

4.3.3.4 Phylogenetic analysis of the concatenated sequences of ITS, TEF and BT genomic regions

Phylogenetic analysis of the combined sequence data identified isolates from *Z. mucronata* as *Dothiorella*, *Diplodia*, *Botryosphaeria* and *Neofusicoccum* genera (Fig. 4.5). Clade **A** included six isolates from Tshikundamalema and Wits Rural Facility that were identified as *Dothiorella allocellula* supported by a bootstrap value of 100%. In Clade **B**, isolates ZBM45.3, ZT31.2, ZT13.1 and ZT55.1 were identified as *D. pseudoseriata* with a strong support value of 100%. Clade **C** formed a strongly supported group (bootstrap = 96%) with two isolates being

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placed close to *Botryosphaeria fusispora*. This clade included a sub-clade, referred to as Clade **C3**, that comprised one isolate from Wits Rural Facility was placed within *Neofusicoccum* and grouped sister to *Neofusicoccum* species. This isolate was identified as *N. kwambonambiense* based on BT (Fig. 4.4) and TEF-1α (Fig. 4.3) analysis but grouped with *Botryosphaeria* on the ITS phylogeny (Fig. 4.2).
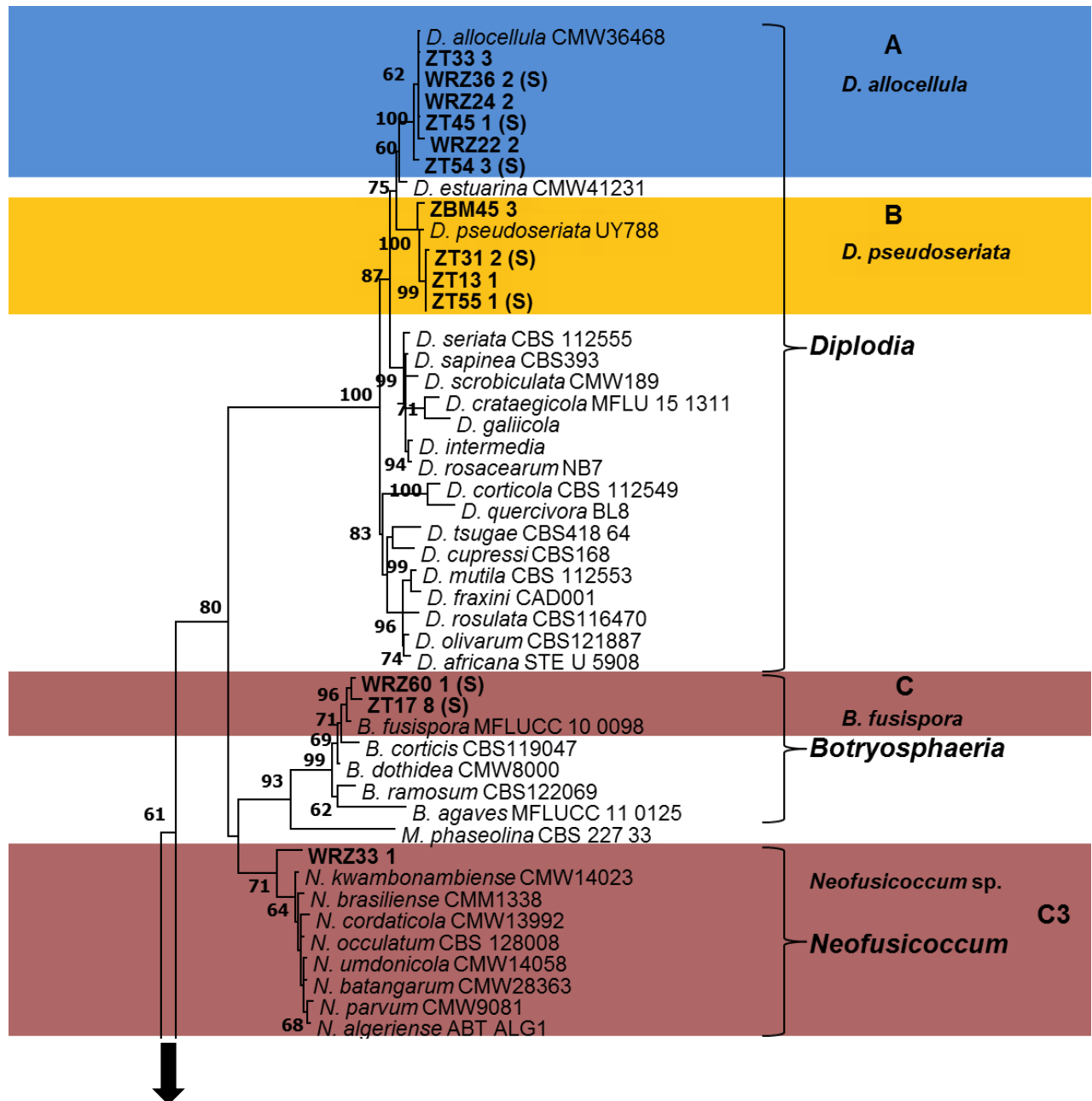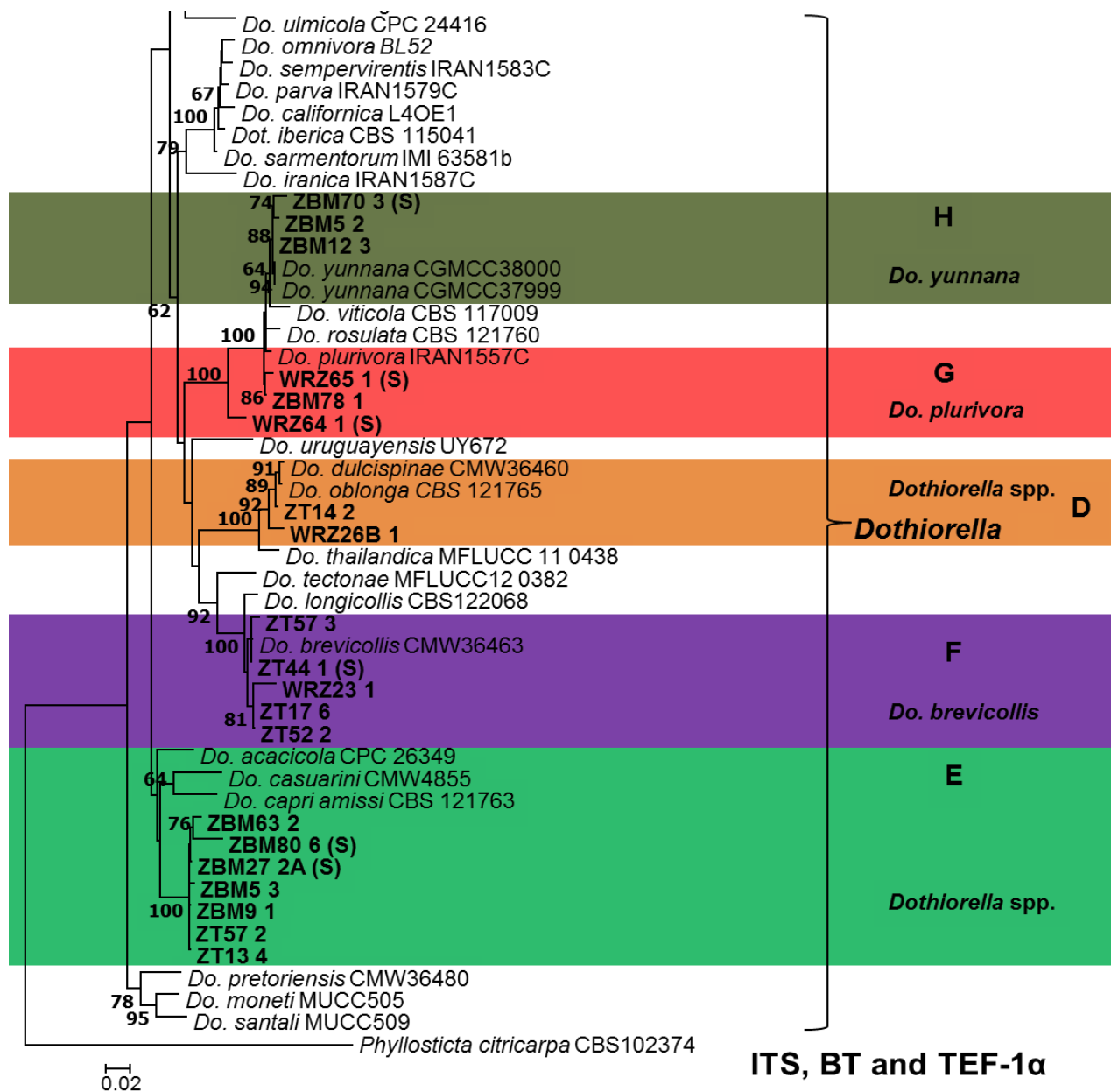
Figure 4.5: Concatenated phylogenetic tree obtained from Maximum Likelihood analysis of the ITS, TEF-1α and BT sequence data of the representative taxa of the Botryosphaeriaceae. Bootstrap support values above 60% from 1,000 replications are given on the branches. Isolates marked in bold represent those obtained from *Z. mucronata* and those with (S) were collected from trees with smut infection. The concatenated tree is rooted to *Phyllosticta citricarpa*.

Isolates belonging to Clade **D** grouped with *Do. oblonga* and *Do. dulcispinae* supported by 100% bootstrap, while Clade **E** included seven isolates from *Z. mucronata* that formed a strongly supported group (100%) and were separated from the sequences from GenBank (Fig. 4.4). These isolates were consistently placed within *Dothiorella* in the ITS (Fig. 4.2), BT (Fig. 4.3) and TEF-1α (Fig. 4.4) maximum likelihood tree, which suggests they may be new *Dothiorella* species. Clade **F** included four isolates from Tshikundamalema and one isolate from Buzzard Mountain Farm grouped with *Do. brevicollis* (100% bootstrap support). Isolates

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WRZ65.1, WRZ64.1 and ZBM78.1 grouped close to *Do. plurivora* in Clade **G** with a bootstrap support of 100% while three isolates from Buzzard Mountain Farm were placed close to *Dothiorella yunnana* in Clade **H** (94% bootstrap support).

4.3.5 Isolate morphological groups and their phylogenetic identities

Phylogenetic analysis and morphological grouping showed correspondence since most of the representative isolates from the same morphological group were placed together on the phylogenetic trees. Five isolates from Buzzard Mountain Farm that were from morphological group **A**, were placed together in one clade on the combined phylogenetic tree (Clade E, Fig. 4.5). However, one isolate from this clade (ZBM 80.6) was from a different morphological group (group **B**) (Table 4.2). Isolates WRZ33.1 and WRZ60.1 were from the same morphological group but one was placed within *Neofusicoccum* clade and the second isolate was placed in the *Botryosphaeria* clade (Fig. 4.5). Nevertheless, other morphological groups were congruent with their isolate identities based on the phylogenetic analysis.

Table 4.2: Representative isolates in this study and their morphological groups.

Site	Isolate code	Morphological group
Buzzard Mountain Farm	ZBM5.3, ZBM27.2A, ZBM63.2, ZBM9.1,	A
Buzzard Mountain Farm	ZBM80.6	B
Buzzard Mountain Farm	ZBM12.3, ZBM70.3, ZBM5.2	C
Buzzard Mountain Farm	ZBM78.1	D
Buzzard Mountain Farm	ZBM45.3	E
Tshikundamalema	ZT57.3, ZT44.1	F
Tshikundamalema	ZT17.6, ZT52.2	G
Tshikundamalema	ZT18.2	H
Tshikundamalema	ZT31.2, ZT55.1, ZT13.2	I
Tshikundamalema	ZT13.4, ZT57.2	J
Tshikundamalema	ZT54.3, ZT45.1, ZT33.3	K
Tshikundamalema	ZT17.8	L
Wits Rural Facility	WRZ24.2, WRZ36.2, WRZ22.2	M
Wits Rural Facility	WRZ33.1, WRZ60.1	N
Wits Rural Facility	WRZ23.1	O
Wits Rural Facility	WRZ26B.1	P
Wits Rural Facility	WRZ65.1, WRZ64.1	Q

4.3.4 Prevalence of species identified across the three study locations.

Phylogenetic results revealed nine species from the Botryosphaeriaceae showing high similarity to isolates collected from Tshikundamalema, Buzzard Mountain Farm and Wits Rural Facility. *Botryosphaeria fusispora* and *Diplodia allocellula* were identified from Tshikundamalema and Wits Rural Facility, while *D. pseudoseriata* was only isolated from Buzzard Mountain Farm and Tshikundamalema (Table 4.3.). *Dothiorella brevicollis* and *Dothiorella* species (close to *Do. dulcispinae*, *Do. oblonga*) were isolated from *Z. mucronata* in Tshikundamalema and Wits Rural Facility, while *Do. yunnana* was only isolated from Buzzard Mountain Farm. *Dothiorella plurivora* was identified from cultures collected from Buzzard Mountain Farm and Wits Rural Facility, and *Neofusicoccum* sp. was only identified from Wits Rural Facility.

Table 4.3: Prevalence of Botryosphaeriaceae fungi identified from isolates obtained from *Z. mucronata*.

Key: Red – present; White – absent.

Species	Location			Total number of isolates
	Buzzard Mountain Farm	Tshikundamalema	Wits Rural Facility	
Botryosphaeria fusispora		Red	Red	2
Diplodia allocellula		Red	Red	6
Diplodia pseudoseriata	Red	Red		4
Dothiorella brevicollis		Red	Red	5
Dothiorella spp. (*Do. dulcispinae*, *Do. oblonga*)		Red	Red	2
Dothiorella spp.	Red	Red		7
Dothiorella plurivora	Red		Red	3
Dothiorella yunnana	Red			3
Neofusicoccum sp.			Red	1

4.4 Discussion

Species in the Botryosphaeriaceae are believed to have a cosmopolitan distribution and are found on a wide range of host plants (Slippers and Wingfield 2007). In this study, species in the Botryosphaeriaceae were identified for the first time associated with dieback of *Z. mucronata* in Limpopo Province, South Africa. Species that were isolated in this study belong to four genera: *Dothiorella*, *Diplodia*, *Botryosphaeria* and *Neofusicoccum*. These identifications were based on phylogenetic analyses of the ITS, BT, and TEF-1α genomic regions. Based on the ML tree constructed from each sequence data and the combined

sequence data of the three genomic regions, isolates were identified as *Diplodia allocellula*, *D. pseudoseriata*, *Dothiorella brevicollis*, *Do. plurivora*, *Do. yunnana*, *Dothiorella* spp., *Botryosphaeria fusispora* and *Neofusicoccum* species.

The fungal species identified in this study showed variable distribution in Limpopo Province. *Dothiorella brevicollis*, *Dothiorella* spp. (*Do. dulcispinae*, *Do. oblonga*), *B. fusispora* and *D. allocellula* were isolated from Tshikundamalema and Wits Rural Facility which are far apart from each other (approximately 250km), hence these species are considered widely distributed in Limpopo Province. *Dothiorella plurivora* and *D. pseudoseriata* are also widely distributed since they were collected from Buzzard Mountain Farm, Tshikundamalema and Wits Rural Facility that are separated from each other. However, *Do. yunnana* and *Neofusicoccum* sp. were exclusively isolated from Buzzard Mountain Farm and Wits Rural Facility respectively. This could be due to limited number of samples collected and if the sample size was to be increased, these two species might be found among the three study locations.

Dothiorella species (Botryosphaeriaceae) are known saprophytes, pathogens and endophytes associated with various woody plants (Abdollahzadeh *et al.* 2014). For example, *Do. brevicollis* and *Do. dulcispinae* were reported being associated with diseased and healthy branches of *Vachellia karroo* by Jami *et al*. (2012), and Dissanayake *et al*. (2017) identified *Do. italica* from dead aerial branches of *Rosa canina* in Italy. *Dothiorella* species were frequently identified from *Z. mucronata* in this study with eight isolates identified as *Dothiorella* spp., five isolates as *Do. brevicollis*, three as *Do. yunnana*, two isolates as *Do. plurivora* and the last two isolates grouped close to *Do. oblonga* and *Do. dulcispinae*. Fungi in *Dothiorella* represented 58% of all the Botryosphaeriaceae isolates obtained from *Z. mucronata* and were found in all the study locations surveyed with *Do. brevicollis* and *Dothiorella* spp. being the most prevalent. These fungal species have not been reported on *Z. mucronata* in South Africa, however, they have been isolated from other native host such as *Vachellia karroo* from South Africa and *Quercus castaneifolia* from Iran associated with dieback and cankers (Jami *et al.* 2012; Chakusary *et al.* 2019). Isolates that were identified as *Dothiorella* spp. did not group close to any isolate for which sequences are present in GenBank but consistently formed a sister clade with *Do. capri-amisii*, *Do. acacicola* and *Do. casuarini* in the ITS, BT, TEF-1α, and combined sequence data phylogenies. These isolates were from the same morphological group except for one isolate (ZBM80.6) and they could represent a new *Dothiorella* spp. Therefore, further analyses using sequence data for additional gene regions and morphological studies will be required to clearly characterize potential cryptic species in this group in future.

In South Africa, *Dothiorella brevicollis* was first identified on a commonly occurring native tree, *Vachellia karroo* by Jami *et al*. (2012) associated with asymptomatic branches. *Dothiorella brevicollis* is an endophytic fungus that is believed to be a potential latent pathogen (Jami *et al*. 2012). Due to this kind of a relationship between the fungi and host plants, our results could suggest that *Do. brevicollis* isolated from *Z. mucronata* was inhabiting the tree species endophytically and possibly turned to a pathogen when the tree was under stress. Our study is the second report to identify this fungal species associated with dieback of a native tree after a study by Zhang *et al*. (2017). *Dothiorella yunnana* was first identified and described from Yunnan Province in China associated with dead and dying branches and stems from a wide range of woody species including *Camellia* sp.,*Ternstroemia gymnanthera* (Theaceae), *Acer buergerianum* (Sapindaceae) and *Poncirus trifoliate* (Rutaceae) (Zhang *et al.* 2017). Only Zhang *et al*. (2017) have reported this species, hence our study expands the geographic range of this species. This is the first report on a native host plant in South Africa and second worldwide, which suggest that this fungal species remains to be discovered on other woody species in Southern Africa and other countries.

One isolate from Wits Rural Facility and one isolates from Buzzard Mountain Farm were identified as *Do. plurivora* based on the three genomic regions analyzed (Fig. 4.4). This species was first described by Abdollahzadeh *et al*. (2014) associated with woody plants (*Citrus* sp., *Casuarina equisetifolia*, *Malus domestica* and *Eucalyptus* sp.) in Iran, New Zealand, Portugal and Spain, but strains of the fungus were first identified by Luque *et al*. (2005), in conjunction with the generic type, *Do. viticola* from *Vitis vinifera*. A study by Pitt *et al*. (2015) also reported *Do. plurivolla* for the first time associated with diseased branches of *Vitis* species in Australia. The fungus was recently identified together with other fungi in the Botryosphaeriaceae such as *D. malorum*, *D. olivarum*, *D. seriata*, *D. pseudoseriata*/*D. alatafructa*, *Do. sarmentorum*, *Do.* viticola, *N. mediterraneum* and *N. parvum* causing dieback on branches of *Eriobotrya japonica* in Spain (González-Domínguez *et al.* 2017), which suggests that the fungus is pathogenic to most woody species worldwide.

Two isolates from *Z. mucronata* showed higher relatedness to *Dothiorella oblonga* and *Do. dulcispinae* on the concatenated phylogenetic tree and these isolates consistently grouped close to these fungal species in the rest of the phylogenetic trees with supporting values above 80%. *Dothiorella dulcispinae* was first identified and described from *Vachellia karroo* in South Africa associated with healthy branches and branches showing dieback (Jami *et al*. 2012), and *Do. oblonga* was later described for the first time by Slippers *et al*. (2014) from *Vachellia* species in South Africa. In the study by Slippers *et al*. (2014), the authors identified *Do. oblonga* together with *Do. dulcispinae* from healthy branch tips and diseased branch tips showing symptoms such as lesions on branches, black pith in the branches, cankers and tip

dieback. In addition, species such as *Botryosphaeria dothidea*, *Lasiodiplodia pseudotheobromae* and *Dothiorella viticola* and *Sphaeropsis variabilis* were also described and identified by these authors. Therefore, our results provide support for the finding that *Do. oblonga and Do. dulcispinae* are associated with dieback of woody plant species.

In this study, *Diplodia* was the second most abundant genus after *Dothiorella* associated with *Z. mucronata*. Based on the concatenated phylogenetic tree of the three genomic regions (Fig. 4.4), six isolates from Tshikundamalema and Wits Rural Facility were identified as *D. allocellula*, while four isolates from Tshikundamalema and Buzzard Mountain Farm were identified as *D. pseudoseriata*. Isolates from *Z. mucronata* also grouped close with these two fungal species based on the ITS, TEF-1α and BT maximum likelihood trees. *Diplodia allocellula* was first identified and described in a study by Jami *et al*. (2012) from *Vachellia karroo* associated with asymptomatic branches in South Africa. The study by Jami *et al*. (2012) seems to be the only one that identified this fungal species in South Africa and other countries. Therefore, our study is the second to report *D. allocellula* from a native species in South Africa. However, our isolates were identified from branches showing dieback symptoms, while Jami *et al*. (2012) identified *D. allocellula* from healthy branches, which suggest that this fungal species might be a pathogen as well as a latent pathogen that might have initiated the infection when *Z. mucronata* was under stress.

Diplodia pseudoseriata was described for the first time by Pérez *et al*. (2010) from native Myrtaceae trees in Uruguay associated with cankers. The fungus was later identified for the first time from *Prunus persica* by Sessa *et al*. (2016) from branches showing dieback, discolouration and cankers in Uruguay. In South Africa, *D. pseudoseriata* was recorded from healthy branches of *V. karroo* and three commonly occurring and surrounding tree species, namely *Celtisa fricana, Searsia lancea*, and *Gymnosporia buxifoli* (Jami *et al*. 2014). *Diplodia pseudoseriata* was isolated from branches showing dieback in our study, and these results contribute to the knowledge that most species in the Botryosphaeriaceae are opportunistic fungi that attack plant hosts that are stressed (Slippers and Wingfield 2007). González-Domínguez *et al*. (2017) also identified *D. pseudoseriata* associated with branches of *Eriobotrya japonica* showing dieback in Spain. This fungus was isolated together with *Do. plurivora* that was also identified from *Z. mucronata* in our study.

Neofusicoccum is a genus that comprises numerous species that occur on a wide range of plant hosts from agricultural, forestry and natural ecosystems (Slippers and Wingfield 2007; Slippers *et al*. 2013). Species of *Neofusicoccum* colonize healthy plant parts as endophytes without producing any visible symptoms but can become pathogenic due to unfavourable conditions such as drought and extreme temperature fluctuations (Slippers and Wingfield

2007). In this study, isolate WRZ33.1 from Wits Rural Facility was identified as a *Neofusicoccum* species. The isolate nested with *Neofusicoccum* species on the concatenated maximum likelihood tree and was also placed within *Neofusicoccum* on the TEF-1α and BT phylogenetic trees grouping close to *N. kwambonambiense* and *N. brasiliense.* However, this isolate was placed in the *Botryosphaeria* close to *B. ramosum* on the ITS phylogenetic tree (Fig. 4.2), which is known as a synonym of *Fusicoccum ramosum* that resides in *Neofusicoccum* (Crous *et al.* 2006), hence this isolate could be regarded as a *Neofusicoccum* species.

In South Africa, *N. kwambonambiense* was first isolated and described from dying twigs and asymptomatic healthy twigs of *Syzygium cordatum* growing in close proximity with *Eucalyptus* plantations (Pavlic *et al.* 2009). Based on their pathogenicity trial results, *N. kwambonambiense* was most aggressive to *S. cordatum* among the five species tested. This fungus, for the first time, was later identified in South Africa from healthy parts of *Eucalyptus grandis* (Pillay *et al.* 2013). It was also identified from healthy parts of *V. karroo* and the surrounding vegetation (Jami *et al.* 2014). Based on these records and many others, *Neofusicoccum* species isolated in our study is one of the potential pathogens that caused dieback on *Z. mucronata. Neofusicoccum brasiliense* has also been reported being associated with dieback, cankers and stem rot of woody plants such as *Mangifera indica*, *Anacardium occidentale* and *Psidium guajava* in Brazil (Marques *et al.* 2013; Coutinho *et al.* 2018).

Species in the *Botryosphaeria* have a cosmopolitan distribution, occurring on a wide range of hosts as saprophytes, parasites and as endophytes (Smith *et al.* 1996). *Botryosphaeria* species occur on plant parts such as woody branches, twigs, herbaceous leaves and stems of grasses, and they cause dieback and cankers on most woody species (Denman *et al.* 2000; Beckman *et al.* 2003; van Niekerk *et al.* 2004). Two of our isolates from Tshikundamalema and Wits Rural Facility were identified as *B. fusispora* based on the analysis of the three genomic regions sequenced. *Botryosphaeria fusispora* was first described by Liu *et al.* (2012) from dried bark of *Entada* species in Thailand. The fungus was recently reported from *Eucalyptus* associated with conditions such as dieback, stem canker, branch canker and twig blight in China (Li *et al.* 2018). Although there were other fungi in the Botryosphaeriaceae identified, *B. fusispora* was the most frequently identified fungus from diseased parts of *Eucalyptus* (Li *et al.* 2018). There is currently insufficient information available for *B. fusispora* and there is no record of this fungus in South Africa yet. Thus, our study is the first report of this fungal species occurring in South Africa.

4.5 Conclusions

In this chapter, we investigated, for the first time, the diversity of species in the Botryosphaeriaceae associated with dieback of *Z. mucronata* in Limpopo Province, South Africa. Fungal species from four genera namely *Diplodia*, *Dothiorella*, *Botryosphaeria* and *Neofusicoccum* were identified from *Z. mucronata* in three locations, which indicates that species in the Botryosphaeriaceae associated with this tree are diverse in Limpopo Province. This study also confirms that species in this family are associated with most woody native trees and are also associated with dieback on these tree species. Some of the fungal species that we isolated such as *Neofusicoccum kwambonambiense* and *D. pseudoseriata* have been previously identified from asymptomatic parts of woody species (Pérez *et al.* 2009; Jami *et al.* 2014). Therefore, in future these fungi need to be examined from healthy branches of *Z. mucronata* and pathogenicity tests will be needed to confirm if they are pathogenic and also to better understand their relationship with the tree.

CHAPTER 5: General discussion, conclusion and recommendations

5.1 General discussion

Indigenous trees play a vital role in both natural ecosystems and in livelihoods of people living in rural communities. These trees are however faced by limiting factors such as adverse environmental conditions and fungal diseases that potentially reduce their productivity, hence reduce their usefulness. Fungal pathogens have reported from diseased parts of indigenous trees across the world, however there is still little information on the diversity of fungi on indigenous trees and the diseases that they cause on these trees. Therefore, this study identified fungal species that associated with dieback of an important indigenous tree species, *Ziziphus mucrona*ta found in different parts of Limpopo Province, South Africa.

Ziziphus mucronata (buffalo thorn) is an indigenous tree species that is widely distributed across the African continent (Orwa *et al.*, 2009). This tree is regarded as one of important indigenous trees by people living in rural areas of Limpopo Province including Tshikundamalema, where some of the samples for this study were collected. The tree is used for various purposes that include the use of leaves, roots and the bark as medicine to cure various infections, and consumption of fruits and leaves by humans as they are palatable and nutritious (Mazibuko, 2007; Mokgolodi *et al.*, 2011). Despite the usefulness of this tree, it is however faced by dieback that occurs on branches and is usually caused by plant pathogens. The results of this study revealed fungal species belonging to six families associated with dieback of *Z. mucronata*, namely the Botryosphaeriaceae, Nectriaceae, Diaporthaceae, Cytosporaceae, Didymellaceae and Pleosporaceae.

Diversity of fungi on indigenous trees is comprised of different fungal communities that include endophytes, pathogens and saprophytes. In the current study, fungal species that were isolated from diseased branches of *Z. mucronata* have been previously reported as endophytes and more well as potential pathogens from other hosts than for this tree. For example, Jami *et al.* (2012) identified *Diplodia allocellula* associated with healthy branches of *Vachellia karroo* in South Africa. Furthermore, *D. pseudoseriata* was also recorded from healthy branches of *V. karroo* and surrounding tree species, namely *Celtisa fricana* and *Searsia lancea* (Jami *et al.* 2014). However, these fungal species were isolated from diseased branches of *Z. mucronata* in our study. Hence further studies need to be carried out to identify fungal species from healthy branches of *Z. mucronata* in the very same study sites as this will help determine if fungal species that were identified in this study are latent pathogens.

Fungal pathogens are capable of co-existing in the same host plant and cause similar disease symptoms. In this study, *Diaporthe*, *Fusarium, Alternaria* and *Didymella* species were also

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isolated from branches of *Z. mucronata* showing dieback, of which some of these fungi were found in the same trees as the members of the Botryosphaeriaceae and some were isolated alone. *Diaporthe* and *Fusarium* species that were isolated have also been reported before causing dieback on trees, which suggests that they may also be responsible for dieback of *Z. mucronata*. *Alternaria* and *Didymella* species are usually isolated as saprophytes from diseased plant parts, however they have also been reported to be pathogenic to some plant species. Our results showed that species in these two genera were isolated together with species in the Botryosphaeriaceae from the same trees. Therefore, further studies need to be carried out to confirm the fungal species that cause dieback on Z*. mucronata.*

The results of this study further showed that most of the isolates obtained from *Z. mucronata* were identified belonging to the Botryosphaeriaceae and the least isolates were from the Didymellaceae. Botryosphaeriaceae is well known to be a cosmopolitan family with opportunistic fungal species occurring in a wide range of hosts in agriculture and undisturbed natural ecosystems (Mehl *et al.* 2017; Phillips *et al.* 2013). Botryosphaeriaceae fungi that were identified in the current study include *Dothiorella*, *Diplodia*, *Neofusicoccum*, and *Botryosphaeria* species. This study is the first to report on members of the Botryosphaeriaceae associated with dieback of *Z. mucronata*, however these fungal species have been reported from other host plants/trees causing dieback and related infections. For example, studies by Jami *et al.* (2012) reported the same fungus that was identified from *Z. mucronata, Do. brevicollis* causing dieback and cankers on *Vachellia karoo* in South Africa which confirms that these fungal species cause dieback of many woody plants.

5.2 General conclusion

This study investigated and showed the diversity of fungi found on branches of *Z. mucronata* showing dieback in different parts of Limpopo Province and it is the first study to carry out this survey in South Africa. Among the fungal species isolated, members of the Botryosphaeriaceae were the most frequently identified from the three study locations, which suggest that dieback of *Z. mucronata* is mostly caused these fungal species or they could be the primary pathogens that initiate the infection on the trees and as such, the other fungi identified are regarded secondary pathogens or saprophytes. The results of this study contribute to the evidence that fungi in the Botryosphaeriaceae are associated with dieback of most trees in both agricultural and natural ecosystem in South Africa and other countries. This study also make a good and a relevant contribution to the study of fungal phylogenetic in indigenous trees.

5.3 Recommendations

This study brought relevant information on the diversity of fungi associated with *Z. mucronata* results are a good addition to the knowledge of potential pathogen in this tree species and it has formed a basis in which further studies can be undertaken. However, the study was conducted in only three sites of the province where only diseased branches were collected from the trees and pathogenicity trials were not carried out, which makes it difficult to know the fungal species that are pathogenic to *Z. mucronata*. Therefore, pathogenecity trials are required to confirm the pathogenic fungi responsible for dieback on this woody species and fungal species need to be identified on healthy plants parts of *Z. mucronata*. Furthermore, the identity of the isolates needs to be confirmed through multiple gene sequencing and phylogenetic analysis and possible new species should be described.

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APPENDICES

Appendix 1: Isolates used for phylogenetic analysis

Species	host	GenBank accession no	
		ITS	**TEF**
Botryosphaeriaceae sp	*Acacia* spp. (= *Vachellia* spp.)	-	HM176514
Botryosphaeria aterrima	Unknown	KU359182	-
B. auasmontanum	*Vachellia mellifera*	NR 136992	-
B. auasmontanum	Unknown	MF398863	-
B. dothidea	*Pyrus* sp.	MG595271	-
B. dothidea	Unknown	KU997558	KU997256
B. dothidea	Unknown	NR 111146	-
B. dothidea	Unknown	MF398856	JQ512104
B. eucalyptorum	Unknown	DQ131570	-
B. fusispora	Unknown	KX631723	-
B. fusispora	Unknown	NR 121552	-
B. iberica	Unknown	EU331075	-
B. protearum	*Eucalyptus gomphocephala*	EF591912	-
B. quercuum	Unknown	AF383949	-
B. ramose	*Vachellia* sp.	KX197073	-
B. ramose	Unknown	NR 151841	-
B. rhodina	Unknown	AY612337	-
B. ribis	*Ribes* sp.	AY236936	-
B. dothidea	*Aucuba japonica*	MH393519	
Diplodia intermedia	*Vitis* sp.	KT595692	-
D. pinea	*Rhizophora* sp.	KP860828	-
D. pinea	*Parkinsonia aculeata*	KT699869	-
D. pseudoseriata	Unknown	NR 121336	-
D. pseudoseriata	*Eriobotrya japonica*	KT240361	-
D. sapinea	Unknown	NR 152452	-
D. sapinea	*Pinus mugo*	JF440618	-
D. seriata	*Citrus* sp.	KX244782	KX029225
D. seriata	*Citrus* sp	KX244783	-
D. seriata	Unknown	NR 111151	-
D. Africana	*Prunus* sp.	EF445344	-
D. Africana	*Prunus* sp.	NR 119635	-
D. allocellula	*Vachellia karroo*	KF270058	-
D. bulgarica	Unknown	KY039060	-
D. allocellula	Unknown	KU997376	-
D. alatafructa	*Pterocarpus angolensis*	-	FJ888446
D. bulgarica	Unknown	NR 111444	-
D. corticola	*Quercus* sp.	MG220435	-
D. corticola	*Quercus* sp.	NR 111152	-
D. intermedia	Unknown	NR 111445	-
D. pyri	Unknown	NR 152465	-
D. pyri	Unknown	KX464093	-
D. rosulata	Unknown	NR 111318	-
D. rosulata	*Prunus* sp.	AY210346	-
Lasiodiplodia theobromae	*Eucalyptus* spp.	-	HQ332210
Neofusicoccum sp	*Terminalia catappa*	-	FJ900655
N. kwambonambiense	Unknown	-	KY024656
Dothiorella longicollis	*Adansonia gibbosa*	NR 136999	-
Do. Omnivore	*Vitis* sp.	-	KY681038
Do. Omnivore	*Vitis* sp.	-	KY681037
Do. plurivora	Unknown	KX464126	-
Do. rosulata	*Vachellia mellifera*	NR 136991	-
Do. Sarmentorum	*Quercus agrifolia*	-	JQ512133
Do. viticola	Unknown	MG198185	-
Do. viticola	*Eriobotrya japonica*	KT240296	-
Do. viticola	Unknown	NR 111186	-
Do. viticola	Unknown	MH236148	-

| Do. viticola | *Prunus species* | EF445361 | - |

Appendix 1 (continued): Isolates used for phylogenetic analysis

Species	host	ITS	TEF
Do. ulmicola	Unknown	-	KR611910
Do. oblonga	Unknown	KF766163	-
Do. oblonga	*Vachellia* sp.	NR 137689	-
Do. dulcispinae	Unknown	NR 111702	-
Do. longicollis	Unknown	KF766162	-
Do. iberica	Unknown	NR 111165	KP828801
Do. acacicola	Unknown	NR 145255	-
Do. pretoriensis	*Vachellia karroo*	NR 111704	-
Do. dulcispinae	*Vachellia karroo*	JQ239401	-
Do. brevicollis	*Vachellia karroo*	JQ239404	-
Do. brevicollis	Unknown	NR 111703	-
Do. viticola	*Vitis* sp.	KP692191	-
Do. viticola	Unknown	KM103250	KC769865
Do. westrale	*Vitis* sp.	HM009376	-
Dothiorella sp.	*Eriobotrya japonica*	KT240299	-
Guignardia mangiferae	*Citrus maxima*	FJ538349	FJ538407
Diaporthe macintoshii	Unknown	KY420948	KJ197251
Diaporthe sp.	Unknown	KF675745	MH220825
D. anacardii	Unknown	NR 111841	-
D. anacardii	Unknown	KC343024	-
D. baccae	*Vitis* sp.	MG281013	MG281534
D. baccae	*Vitis* sp.	-	MG281533
D. foeniculina	Unknolwn	KP747693	-
D. foeniculina	*Citrus* sp.	MF774662	-
D. foeniculina	Unknown	NR 145303	-
D. foeniculina	*Citrus* sp.	MF774663	-
D. macintoshii	Unknown	NR 147539	-
D. neotheicola	Unknown	KC145914	-
D. parapterocarpi	Unknown	KJ869138	-
D. raonikavaporum	*Tectona grandis*	KU712450	-
D. raonikavaporum	Unknown	NR 111860	-
D. rhusicola	Unknown	MG828893	-
D. velutina	Unknown	NR 152470	-
D. velutina	Unknown	KX986792	-
Diaporthe sp.	*Coffea*	EU002922	-
D. vaccinia	*Cyanococcus*	KC488259	-
Diaporthe sp.	Unknown	JN153072	-
Diaporthe sp.	Unknown	JN153056	-
Diaporthe sp.	*Vitis labrusca*	KM362371	-
Diaporthe sp.	*Albizia adianthifolia*	KY369142	-
Diaporthe sp.	Unknown	KF128763	-
Cytospora acacia	Unknown	DQ243804	JX438560
C. austromontana	Unknown	LN808963	-
C. berkeleyi	*Populus tremuloides*	-	JX438562
C. brevispora	*Rhizophora mangle*	MF281195	-
C. cedri	*Populus tremuloides*	-	JX438575
C. diatrypelloidea	*Populus tremuloides*	-	JX438563
C. eucalyptina	*Eucalyptus*	AY347375	-
C. magnolia	*Platanus acerifolia*	KP881429	-
C. magnolia	*Populus tremuloides*	JX438623	JX438565
C. nitschkii	*Populus tremuloides*	-	JX438567
C. punicae	*Punica granatum*	-	KY131963
C. rhizophorae	Unknown	DQ996040	JX438609
C. rosarum	Unknown	EF447387	-
C. saccharl	*Populus tremuloides*	-	JX438569
C. tibouchinae	Unknown	KX228284	-
C. tibouchinae	Unknown	NR 154809	-

Species	host	ITS	TEF
C. umbrina	*Populus tremuloides*	-	JX438606
C. viticola	*Vitis* sp.	KX256239	-
Cytospora sp.	Unknown	KU900329	-
Cytospora sp.	Unknown	KR093918	-
Cytospora sp.	Unknown	DQ996039	-
Fusarium decemcellulare	*Pinus* sp.	MF076589	KJ648617
F. equiseti	Unknown	NR 121457	KU939015
F. lateritium	Unknown	-	JF740854
F. verticillioides	*Zea mays*	KU204755	-
F. xylarioides	Unknown	KF889083	-
F.equiseti	*Sceletium tortuosum*	KY318493	-
F.lacertarum	Unknown	-	JF740828
Fusarium equiseti	*Chenopodium quinoa*	MF166765	-
Fusarium sp.	Unknown	DQ682580	-
Fusarium sp.	*Rhizophora mangle*	HQ023180	-
Neurospora sp.	Unknown	KX058050	-
A. alternata	Unknown	MF958649	KP009004
A. alternata	*Olea europaea*	MH716004	-
A. alternata	*Solanum lycopersicum*	MF693801	-
A. tenussima	*Phytolacca acinosa*	KX828180	-
Alternaria sp.	Unknown	MG209668	HQ413697
Alternaria sp.	*Cypripedium* species	MH730190	-
Didymella schachtii (=*Phoma schachtii*)	Unknown	FJ427066	-
Didymella sp.	*Eriobotrya japonica*	KY790596	-
Didymella sp.	*Vitis* sp.	KF128801	-
D. aliena	Unknown	GU237910	-
D. betae	Unknown	FJ426981	-
D. costarricencis	Unknown	GU237870	-
D. crystallifera	Unknown	KX342943	-
D. herbarum	Unknown	AY293803	-
D. neerlandica	Unknown	KT389535	-
D. saxea	Unknown	GU237860	-
D. unsulana	Unknown	GU237810	-
Neurospora sp.	Unknown	KX058050	-

Appendix 1 (continued): Isolates used for phylogenetic analysis

Species	host	GenBank accession no		
		ITS	BT	TEF
Diplodia africana	*Prunus* species	EF445343	KF766129	EF445382
D. alatafructa	*Pterocarpus angolensis*	FJ888460	NR_111416	FJ888444
D. allocellula	*Vachellia karroo*	JQ239937	JQ239379	JQ239384
D. corticola	*Quercus* species	AY259100	KX464789	AY573227
D. crataegicola	Unknown	KT290244	KT290246	KT290248
D. cupressi	*Cupressus* species.	DQ458893	DQ458861	DQ458878
D. estuarina	*Rhizophora* species	KP860831	KP860754	KP860676
D. fraxini	*Fraxinus* species	KF307700	MG015807	KF318747
D. galiicola	Unknown	KT290245	KT290247	KT290249
D. intermedia	Unknown	GQ923858	MG015814	GQ923826
D. mutila	*Quercus* species	AY259093	KU198426	AY573219
D. olivarum	*Olea* species	EU392302	HQ660079	EU392279
D. pseudoseriata	*Eucalyptus* species	EU080927	MG015820	EU863181
D. quercivora	*Quercus canariensis*	JX894205	MG015822	JX894229
D. rosacearum	*Eryobotria japonica*	KT956270	MG015823	KU378605
D. rosulata	*Olea* species	EU430265	EU673132	EU430267
D. sapinea	Unknown	DQ458895	KX464806	DQ458880
D. scrobiculata	*Pinus* species	AY253292	AY624258	AY624253
D. seriata	*Quercus* species	AY259094	KX464806	AY573220
D. tsugae	Unknown	DQ458888	DQ458855	DQ458873
Botryosphaeria agaves	Unknown	JX646791	JX646841	JX646856
B. auasmontanum	Unknown	KF766167	-	EU101348
B. corticis	*Olea europaea*	DQ299245	EU673107	EU017539
B. dothidea	Unknown	AY236949	AY236927	AY236898
B. fusispora	Unknown	JX646789	JX646839	JX646854
B. ramosum	*Adansonia*	EU144055	-	EU144070
N. algeriense	*Vitis* species	KJ657702	-	KJ657715
N. batangarum	*Terminalia catappa*	FJ900607	-	FJ900653
N. cordaticola	Unknown	EU821898	EU821838	EU821868
N. kwambonambiense	Unknown	EU821900	EU821840	EU821870
N. occulatum	Unknown	MH864743	EU339472	EU339509
N. brasiliense	*Mangifera indica*	JX513630	KC794031	JX513610
N. parvum	Unknown	AY236943	-	AY236888
N. umdonicola	Unknown	EU821904	EU821844	EU821874
Dothiorella acacicola	Unknown	KX228269	-	KX228376
Do. ulmicola	Unknown	KR611881	KR611909	KR611910
Do. tectonae	*Tectona grandis*	KM396899	-	KM409637
Do. brevicollis	*Vachellia karroo*	JQ239403	JQ239371	JQ239390
Do. dulcispinae	*Vachellia karroo*	JQ239400	JQ239373	JQ239387
Do. oblonga	*Vachellia mellifera*	EU101300	KX464862	EU101345
Do. yunnana	*Camellia* species	KX499643	-	KX499649
Do. yunnana	*Camellia* species	KX499644	-	KX499650
Do. mangifericola	Unknown	KC898221	-	KC898204
Do. rosulata	*Vachellia mellifera*	EU101290	KX464878	EU101335
Do. viticola	*Vitis* species	AY905554	EU673104	AY905559
Do. citricola	Unknown	EU673323	-	EU673290
Do. alpina	*Camellia* species	KX499645	-	KX499651
Do. casuarini	Unknown	DQ846773	DQ875340	DQ875331
Do. californica	*Laurus nobilis*	KX357188	KX357165	KX357211
Do. parva	Unknown	KC898234	-	KC898217
Do. iberica	Unknown	AY573202	EU673096	AY573222
Do. plurivora	Unknown	KC898225	KX464876	-

Appendix 2: Botryosphaeriaceae species used in phylogenetic analyses in this study

Appendix 2: (Continued) Botryosphaeriaceae species used in phylogenetic analyses in this study

Do. sarmentorum	Unknown	AY573212	KF575107	AY573235
Do. omnivora	*Corylus*	KP205497	KX464897	KP205470
Do. sempervirentis	Unknown	KC898236	-	KC898219
Do. symphoricarposicola	Unknown	KJ742378	-	KJ742381
Do. longicollis	*Adansonia* species	EU144054	KX464858	EU144069
Do. thailandica	Unknown	JX646796	JX646844	-
Do. iranica	Unknown	KC898231	KX464856	KC898214
Do. neclivorem	*Vitis* species	KJ573643	-	KJ573640
Do. uruguayensis	*Eucalyptus* species	EU080923	KX464886	EU863180
Do. vinea-gemmae	*Vitis* species	KJ573644	-	KJ573641
Do. moneti	*Eucalyptus gomphocephala*	EF591920	EF591954	EF591971
Do. santali	*Eucalyptus gomphocephala*	EF591924	EF591958	EF591975
Do. pretoriensis	*Vachellia karroo*	JQ239405	JQ239376	JQ239392
Do. capri-amissi	*Vachellia* species	EU101323	KX464850	EU101368
Macrophomina phaseolina	Unknown	KF951627	KF531806	KF952000
Phyllosticta citricarpa	*Quercus robur*	FJ824767	FJ824778	FJ538376