Diversity and species overlap of *Botryosphaeriaceae* on some trees of *Anacardiaceae* in disturbed and undisturbed ecosystems in South Africa

by

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DECLARATION

I, Elelwani Ramabulana, declare that this dissertation submitted for the degree Doctor of Philosophy at the University of Venda is composed of my original work and contains no material previously published by another person except where due respect has been made in the text reference.

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ABSTRACT

The *Botryosphaeriaceae* are common and diverse on native and non-native plants. It has been established that the ability of *Botryosphaeriaceae* fungi to exist in healthy plant tissues as endophytes facilitates their global movement during the international trade of plants and plant products. It is important to understand whether fungi in the *Botryosphaeriaceae* have a restricted host range or a limited geographic distribution once introduced into new areas, and how ecosystem disturbances through human activities influence species diversity and distribution of fungi in the *Botryosphaeriaceae*. The current study explored *Botryosphaeriaceae* species diversity on native and non-native tree species of *Anacardiaceae* found in disturbed and undisturbed ecosystems, as well as species overlap of the *Botryosphaeriaceae* as endophytes and as pathogens in asymptomatic and symptomatic tissues of these trees. In addition, the genetic diversity and population structure of *N. parvum,* the dominant species occurring on asymptomatic mango fruit were assessed. These questions were addressed by sampling three tree species of *Anacardiaceae*, namely *Sclerocarya birrea* (marula), *Mangifera indica* (mango) and *Lannea Schweinfurthii* (false marula) in disturbed and undisturbed ecosystems in the Limpopo and Mpumalanga provinces, South Africa.

The ecosystem disturbance study identified 11 species of *Botryosphaeriaceae*, namely *Diplodia allocellula*, *Dothiorella brevicollis*, *Dothiorella dulcispinae*, *Dothiorella viticola*, *Lasiodiplodia crassispora*, *Lasiodiplodia exigua*, *Lasiodiplodia gonubiensis*, *Lasiodiplodia mahajangana*, *Neofusicoccum parvum*, *Oblongocollomyces ednahkunjekuae* and *Oblongocollomyces* sp. 1 occurring on native and non-native *Anacardiaceae* in disturbed and undisturbed ecosystems. Ten of the 11 species were identified on trees in undisturbed ecosystems, while three were identified on trees in disturbed ecosystems. *Lasiodiplodia crassispora* and *L. mahajangana* were generalists on trees in disturbed and undisturbed ecosystems. The results from this study also indicated the ability of some species to cross-infect native and non-native *Anacardiaceae* growing next to each other. For example, *N. parvum* isolates occurred on both *S. birrea* (a native species) and *M. indica* (a non-native species) trees adjacent to each other in disturbed ecosystems.

An investigation on species occurrence of the *Botryosphaeriaceae* as endophytes and as pathogens on the *Anacardiaceae* identified 12 species of *Botryosphaeriaceae*, *D. allocellula*, *Dothiorella brevicollis*, *Do. dulcispinae*, *Do. viticola*, *Lasiodiplodia crassispora*, *L. exigua*, *L. gonubiensis*, *L. mahajangana, L. pseudotheobromae, Neofusicoccum parvum,* *Oblongocollomyces ednahkunjekuae* and *Oblongocollomyces* sp. 1 in asymptomatic and symptomatic tissues. *Dothiorella dulcispinae, L. exigua, L. gonubiensis, Oblongocollomyces ednahkunjekuae* and *Oblongocollomyces* sp. 1 were exclusive to asymptomatic *Anacardiaceae*, while *D. allocellula*, *Do. brevicollis*, *Do. viticola*, *L. crassispora, L. mahajangana* and *N. parvum* occurred in asymptomatic and symptomatic *Anacardiaceae* indicating the ability to occupy healthy plant tissues as latent opportunistic pathogens on these trees.

The *N. parvum* population genetics data was assessed based on nine microsatellite markers. This study indicated that the *N. parvum* population consists of three distinct genetic clusters characterized by high levels of genetic diversity. The three clusters were not correlated to host or geographic location indicating the lack of barrier on the movement of the fungus between the hosts and locations sampled. The study highlights the potential of mango fruit to serve as a source of international spread of *Botryosphaeriaceae*, especially of *N. parvum*.

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LIST OF ABBREVIATIONS

AIC	Akaike Information Criterion
AMOVA	Analysis of Molecular Variance
BI	Bayesian Inference
BLAST	Basic Local Alignment Search Tool
BS	Bootstrap
BSR	Biological Species Recognition
B-TUB	Beta Tubulin
CAL	Calmodulin
CMW	Culture Mike Wingfield
CPHB	Centre of Excellence in Plant Health Biotechnology
DNA	Deoxyribonucleic Acid
DSI	Department of Science and Innovation
ESS	Effective Sampling Size
FABI	Forestry and Agricultural Biotechnology Institute
GCPSR	Genealogical Concordance Phylogenetic Species Recognition
GDP	Gross Domestic Product
ICN	International Code of Nomenclature for algae, fungi and plants
ITS	Internal Transcribed Spacer
MAFFT	Multiple Alignment using Fast Fourier Transform
MCMC	Markov Chain Monte Carlo
MEA	Malt Extract Agar
ML	Maximum Likelihood
MSR	Morphological Species Recognition
NCBI	National Center for Biotechnology Information
NRF	National Research Foundation
°C	Degree Celcius
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reactions
PP	Posterior Probabilities
PSR	Phylogenetic Species Recognition
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RPB2	RNA polymerase II Subunit
SNP	Single Nucleotide Polymorphism

Sp./spp.	Species
SSR	Simple Sequence Repeat
TEF-1α	Translation Elongation Factor
UV	Ultraviolet Light

CHAPTER 1: INTRODUCTION

Invasive, non-native plant pathogens are increasing, causing substantial damage to various tree species and challenging the sustainability of agriculture, forestry and natural forests (Burgess *et al.,* 2016; Ghelardini *et al.,* 2016). The global distribution of plant pathogens has been linked to the extensive international movement of living plants and their products, as well as the movement of people, shipping containers and vehicles (Santini *et al*., 2013; Burgess *et al*., 2016; Crous *et al*., 2016; Paap *et al*., 2022). Therefore, quarantine measures are put in place by government authorities to detect and prevent the introduction of non-native pathogens. Some pathogens, especially those occurring in asymptomatic plants as endophytes are, however, difficult to detect when visual inspections are made.

Emerging tree disease outbreaks are often the result of introduced invasive non-native pathogens or native pathogens due to changes in climatic conditions (Sturrock *et al*., 2011; Linnakoski *et al*., 2019; Burgess *et al*., 2022; Paap *et al*., 2022). It has been predicted that as the climate changes (causing extreme temperatures, changes in precipitation patterns and drought) pathogens will spread into new geographic areas, where they will come into contact with new potential hosts (Baker *et al.,* 2000; Etterson and Shaw, 2001). One such group of fungi, those belonging to *Botryosphaeriaceae*, are recognised as important potential pathogens in the era of global climate change (Desprez-Loustau *et al.,* 2006; Sturrock *et al*., 2011). Fungi in the *Botryosphaeriaceae* are widely distributed, can persist asymptomatically in healthy plant tissues and are capable of adapting and colonizing new hosts and become pathogenic when their hosts are under stress which may lead to the death of their hosts (Slippers and Wingfield, 2007; Galarneau *et al.,* 2019; Hrycan *et al.,* 2020). As a result, managing species of *Botryosphaeriaceae* poses significant challenges to plant pathologists globally (Anderson *et al*., 2004; Slippers and Wingfield, 2007; Burgess *et al*., 2019).

The taxonomy, systematics and biogeography of species in the *Botryosphaeriaceae* have been intensively studied in South Africa (Jami *et al*., 2017). However, the occurrence of these fungi on native trees was not considered until 2004 when the CPHB (Centre of Excellence in Plant Health Biotechnology, then the Centre of Excellence in Tree Health Biotechnology) was established with funding from the South African government through the DSI-NRF (Department of Science and Innovation of the National Research Foundation, then the Department of Science and Technology) to study the health of native trees. It has been established that *Botryosphaeriaceae* are more diverse on native hosts than on non-native tree species in South Africa (Jami *et al*., 2017). As our understanding of these fungi improves, more in-depth questions regarding the *Botryosphaeriaceae* arise. For instance, the influence of ecosystem disturbance through human activities on species diversity and distribution of *Botryosphaeriaceae* is poorly understood.

The introduction of plants into non-native areas and changes in land use or agricultural practices are some of the human activities that contribute to disturbance in an ecosystem. A study by Pavlic *et al.* (2015) showed that *Neofusicoccum* species distribution and genetic diversity on *Syzygium cordatum* is influenced by the level of disturbance in an ecosystem and that *N. parvum* was dominant in disturbed ecosystems, while *N. cordaticola*, *N. kwambonambiense* and *N. ribis* were common in undisturbed stands where *N. parvum* was absent. More recently, higher species diversity of *Botryosphaeriaceae* in disturbed ecosystems (vineyards) than in undisturbed ecosystems (forests) was reported (Laurent *et al.,* 2020). The results obtained from the two studies suggest that the level of disturbance, which may vary even between the same ecosystem type (land use in urban environments and management practices in vineyards), influences species diversity and distribution patterns of *Botryosphaeriaceae*.

Organisms with a cosmopolitan distribution and broad host range are often colonizers of disturbed ecosystems and invaders in new regions (Lee and Gelembiuk, 2008; Burgess *et al.,* 2022). Although the phenomenon of ecosystem disturbance and species distribution has not been widely reported for fungi in the *Botryosphaeriaceae*, Slippers and Wingfield (2007) reported that some prominent species are generalists regarding host association. In one of the studies presented in this dissertation (Chapter 3), I investigated whether some of the dominant species in the *Botryosphaeriaceae* might also act as generalists, colonizing trees of *Anacardiaceae* in human-disturbed and undisturbed ecosystems. To answer this question, samples were collected from trees in disturbed and undisturbed ecosystems in the Limpopo and Mpumalanga provinces. Disturbed ecosystems included developed sites, in close contact with human activities and intensively farmed, while undisturbed ecosystems included isolated and undeveloped protected parks.

The studies presented in this dissertation focused on the *Botryosphaeriaceae* from three tree species of *Anacardiaceae,* namely *Sclerocarya birrea* (marula)*, Mangifera indica* (mango) and *Lannea schweinfurthii* (false marula). These species were selected because they are important agricultural commodities used as raw material in the cosmetic industry, as traditional medicine by surrounding communities as well as a source of extra income in rural households (Mariod and Abdelwahab, 2012; Kalita, 2017; Maroyi, 2019). The distribution of the three tree species across disturbed and undisturbed ecosystems presents a model to study the influence of disturbance on the diversity and distribution of the *Botryosphaeriaceae* on these trees. The studies undertaken were organised as follows:

The current chapter (**Chapter 1**) is a preface of studies conducted and an outline of chapters presented in the dissertation. Chapter 1 explains the importance of undertaking the studies included in this dissertation. Repetitions in the Materials and Methods and Reference sections of some chapters were unavoidable due to the nature in which the research is presented in the different chapters.

A review of literature on the host range, distribution and population structure of *Botryosphaeriaceae* in South Africa is presented in **Chapter 2**. The review focuses on the taxonomy of the *Botryosphaeriaceae* and provides an overview of previous and current tools used to identify these fungi. Existing gaps regarding the knowledge of this important fungal group were identified from the literature. Furthermore, the literature was reviewed to guide the choice of the study locations, tree species sampled, sampling strategies and molecular tools used in this study.

Chapter 3 presents an investigation of the species diversity of endophytic *Botryosphaeriaceae* on selected tree species of *Anacardiaceae* in disturbed and undisturbed ecosystems. Asymptomatic branch samples were collected from native (*S. birrea* and *L. schweinfurthii*) and non-native (*M. indica*) trees in disturbed and undisturbed ecosystems. The general health status of individual trees sampled was assessed. Thereafter, the influence of ecosystem disturbance on diversity and distribution of *Botryosphaeriaceae* species was determined. This was done by comparing species identified on trees in disturbed and undisturbed ecosystems. Lastly, cross-infection by species of *Botryosphaeriaceae* was determined by investigating species overlap on indigenous and introduced tree species of *Anacardiaceae* growing next to each other in disturbed and undisturbed ecosystems. Isolates obtained from asymptomatic tissues were identified by partial sequencing of four gene regions including the internal transcribed spacer (ITS), translation elongation factor (*tef-1*α), β-tubulin (β-*tub*) and RNA polymerase II subunit (*rpb2*).

The occurrence of *Botryosphaeriaceae* species as endophytes and as pathogens often overlap (Slippers and Wingfield, 2007). For example, the well-known canker pathogen of *Pinus* trees, *Diplodia sapinea*, also occurs as an endophyte on branches, trunks and seed cones of these trees (Smith *et al.,* 1996; Burgess and Wingfield, 2002). It has been hypothesised that endophytic *Botryosphaeriaceae* are associated with general dieback of woody tree species (Jami *et al.,* 2013). Therefore, the objective of the research presented in **Chapter 4** was to test the hypothesis that endophytic species of *Botryosphaeriaceae* are the causal agents of dieback on tree species of *Anacardiaceae*. The objective was achieved by identifying species occurring on both asymptomatic and symptomatic tree species. Isolates obtained were characterized through phylogenetic inference of the ITS, *tef-1α, β-tub, rpb2,* as well as the combined sequence data.

In **Chapter 5**, the species and genetic diversity of endophytic *Botryosphaeriaceae* occurring on *M. indica* and *S. birrea* fruits were investigated. *Mangifera indica* (mango) is an economically important tropical fruit produced in at least 90 countries around the world (Ni *et al.,* 2012; Ismail *et al.,* 2012). In 2018/19, 6% of mango produced in South Africa was exported to the Middle East, Africa (Botswana and Ghana), Russia and the Netherlands (https://southafrica.co.za/mango-production.html). *Sclerocarya birrea* (marula) fruit contains four times as much vitamin C as common citrus, making it a prominent source of vitamin C in rural communities. The pulp of the fruit is processed into a commercial cream liqueur, while the oil from the nut is used in the cosmetic industry (Mutshinyalo and Tshisevhe, 2003; Mariod and Abdelwahab, 2012). Knowledge of the diversity of endophytic *Botryosphaeriaceae* occurring on fruits, especially on fruits that are exported, is important in understanding the pathway of these fungi in healthy plant material. The species diversity of endophytic *Botryosphaeriaceae* on *M. indica* and *S. birrea* fruits was determined using a multi-gene sequencing approach of the four loci used in chapters 3 and 4 of this dissertation. Thereafter, previously designed simple sequence repeat (SSR) markers were used to explore the genetic diversity and population structure of the most dominant species isolated from mango and marula fruits.

A general discussion of the results from the different research chapters is presented at the end of the dissertation.

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CHAPTER 2: LITERATURE REVIEW

Diversity, host range and population structure of *Botryosphaeriaceae* associated with woody tree species in South Africa

2.1. Introduction

Botryosphaeriaceae is a family of fungi known for their ecological and economic significance as plant pathogens (Slippers and Wingfield, 2007; Slippers *et al.,* 2017; Burgess *et al.,* 2019; Batista *et al.,* 2021). Fungi in the *Botryosphaeriaceae* have been isolated on more than 1000 plant hosts in temperate, tropical and subtropical environments, as well as on seagrasses in marine environments (Sakayaroj *et al.,* 2010; Lawrence *et al.,* 2017; Burgess *et al.,* 2019). A common feature of the biology of members of *Botryosphaeriaceae* is the ability to remain latent in healthy plant tissues without causing disease symptoms (Slippers and Wingfield, 2007; Sakalidis *et al.,* 2011a; Marsberg *et al.,* 2017; Slippers *et al.,* 2017). The endophytic nature of *Botryosphaeriaceae* species makes them ideal candidates for undetected long-distance dispersal with plant germplasm (Burgess *et al.,* 2019; Luo *et al.,* 2019).

The cosmopolitan distribution of some species of *Botryosphaeriaceae* suggests human-facilitated movement of species in the *Botryosphaeriaceae* with the international trade in plants and the inefficiency of current quarantine diagnostic techniques to detect species of *Botryosphaeriaceae* as latent endophytes (Burgess and Wingfield, 2002; Sakalidis *et al*., 2013; Crous *et al*., 2016; Marsberg *et al*., 2017; Burgess *et al*., 2017, 2018). Therefore, there is a need to develop effective techniques to accurately identify and detect species of *Botryosphaeriaceae* as endophytes in healthy plant tissues. Knowledge of the host ranges of *Botryosphaeriaceae*, especially on plants that are moved around the world for commercial purposes, is important if the impacts of these movements are to be understood and potentially managed (Wingfield *et al.,* 2001, 2015; Crous *et al.,* 2016). The purpose of this chapter is to provide an overview of the current state of knowledge about the taxonomy, diversity, host range, population structure and molecular techniques used for species identification of *Botryosphaeriaceae,* with a particular focus on South Africa.

2.2. Key genera in the *Botryosphaeriaceae*

The family *Botryosphaeriaceae* was first introduced with only three genera, namely *Botryosphaeria, Dibotryon* and *Phaebotryon* (Theissen and Sydow, 1918). The taxonomy of *Botryosphaeriaceae* has often been contentious due to most of the species having similar morphological characteristics that complicate species delineation (Zhang *et al.,* 2021). The availability of DNA sequencing methods has allowed taxonomists to determine phylogenetic relationships in fungi at various taxonomic levels, from species to orders (Zhang *et al.,* 2021). Schoch *et al.* (2006) constructed a multigene phylogeny and reported that species in the genera *Botryosphaeria* and *Guignardia* grouped into a distinct clade from all other orders and for that reason introduced the order *Botryosphaeriales* to accommodate the *Botryosphaeriaceae* family. *Botryosphaeriales* includes nine families with the *Botryosphaeriaceae* being the most diverse (Zhang *et al.,* 2021). The family *Botryosphaeriaceae* accommodates 23 genera, while the other eight families accommodate at least one or two genera each (Dissanayake *et al.,* 2016; Slippers *et al.,* 2017; Yang *et al.,* 2017; Zhang *et al.,* 2021). Several species in the *Botryosphaeriaceae* are currently recognised as species-complexes that comprise cryptic, hybrid species identified based on DNA sequence variation and phylogenetic placement (Pavlic *et al.,* 2009; Sakalidis *et al.,* 2011b; Cruywagen *et al.,* 2017).

Members of *Botryosphaeriaceae* occur in all climatic and geographical regions of the world except Antarctica (Slippers and Wingfield, 2007; Mehl *et al.,* 2017a; Marsberg *et al.,* 2017; Burgess *et al*., 2019). Species in the genera *Botryosphaeria*, *Diplodia*, *Lasiodiplodia* and *Neofusicoccum* have a broad host and geographic range (Marsberg *et al*., 2017; Burgess *et al.,* 2019). A review by Batista *et al.* (2021), based on information gathered from the National Center for Biotechnology Information (NCBI) database showed that *Lasiodiplodia theobromae* has the largest number of known hosts (666 of 749 hosts reported for the genus *Lasiodiplodia*), with the largest number of isolates in GenBank (1944), the largest number of country occurrences (97) and the largest number of host fungus interaction reports (365). For the other cosmopolitan species, some species can colonize a higher number of hosts than others. For example, *Botryosphaeria dothidea* has been recorded on 403 hosts in 66 countries, *Neofusicoccum parvum* on 223 hosts in 50 countries and *Diplodia sapinea* on 102 hosts in 62 countries (Batista *et al.,* 2021). These four species, *B. dothidea, D. sapinea, L. theobromae* and *N. parvum* have been recorded as the most common species on native and introduced hosts in South Africa (Figure 2.1) (Jami *et al.,* 2017). Species in other genera such as *Dothiorella*, *Eutiarosporella*, *Oblongocollomyces*, *Pseudofusicoccum*, *Saccharata* and *Sphaeropsis* are also known to occur in South Africa, but in this review we focus on the most common genera, namely *Botryosphaeria*, *Diplodia*, *Lasiodiplodia* and *Neofusicoccum.*

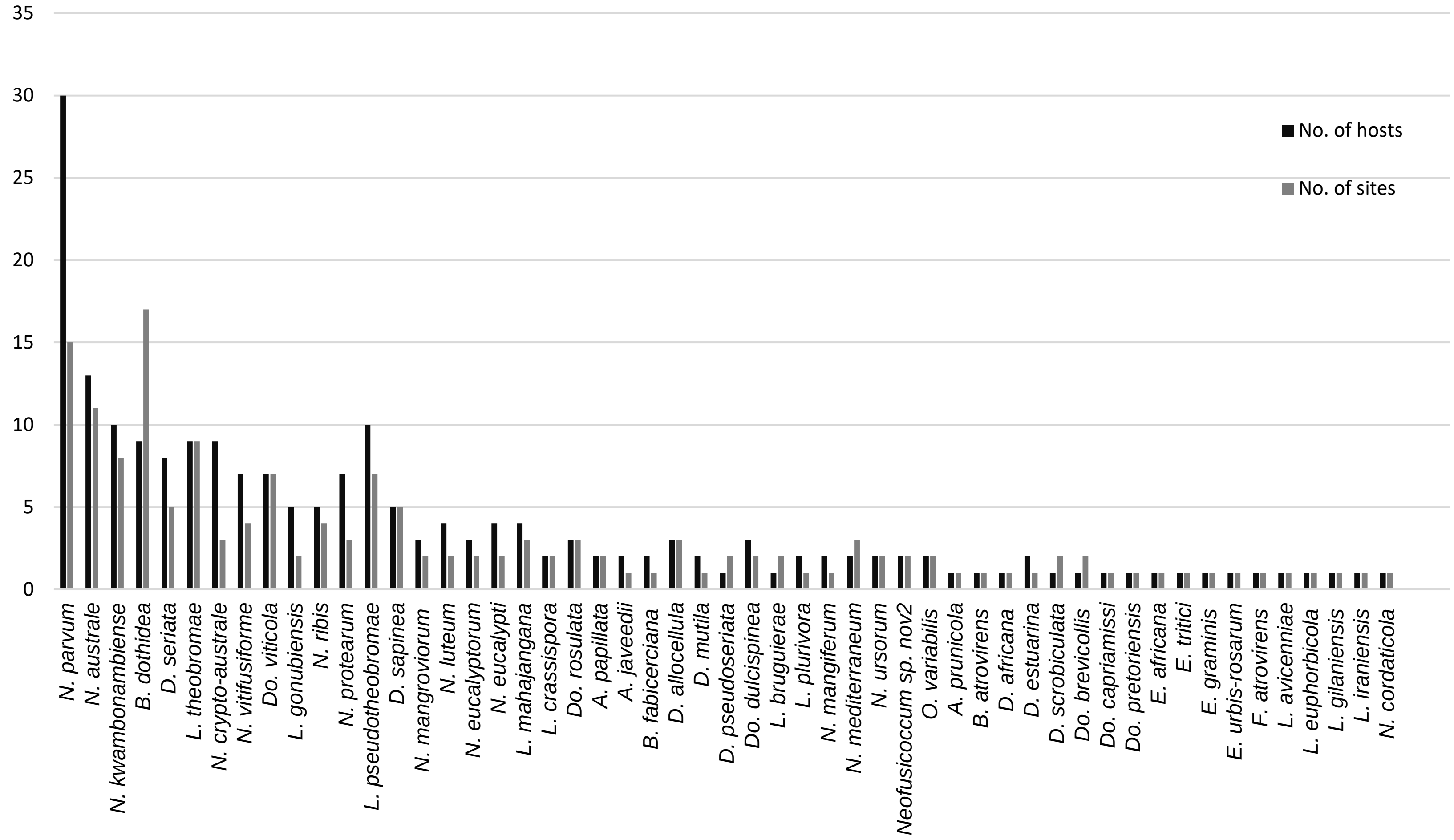

Figure 2.1. The number of hosts and sites infected by species of *Botryosphaeriaceae* in South Africa (Jami *et al.,* 2017).

2.2.1. *Botryosphaeria*

Botryosphaeria was described by Cesati and De Notaris (1863). At the time of description, Cesati and De Notaris (1863) listed twelve species under *Botryosphaeria* but did not designate a type species for the genus. These species were described based on morphology and host associations (Cesati and De Notaris, 1863). Theissen and Sydow (1915) designated *Botryosphaeria quercuum* as the type species for the genus. However, *B. quercuum* was not one of the twelve originally described species and was thus deemed unsuitable as a type species (Theissen and Sydow, 1915; Von Arx and Muller, 1954). Most species were synonymised with *B. dothidea* and *B. quercuum,* leading to a reduction of species from 183 to less than 10. Barr (1972) proposed *B. dothidea* as the type species. Slippers *et al.* (2004a) epitypified *B. dothidea* and confirmed it as a type species for the genus.

In culture, *Botryosphaeria* species are characterised by aerial mycelia with branched, septate and hyaline hyphae (Phillips *et al.,* 2005, 2013). The diversity in morphological characters used to describe *Botryosphaeria* species, together with the dual nomenclature approach gave preference to the name of the sexual state and led to the inclusion of more than 18 asexual genera within *Botryosphaeria,* including *Diplodia, Dothiorella, Fusicoccum, Lasiodiplodia* and others (Denman *et al.,* 2000; Phillips *et al.,* 2005; Luque *et al.,* 2005; Crous *et al.,* 2006). Crous *et al.* (2006) reported that *Botryosphaeria* included several phylogenetic lineages that corresponded to different asexual genera and separated these genera. Numerous species were described under *Botryosphaeria*, but they were later moved to their respective genera based on morphology and phylogenetic placement (Crous *et al.,* 2006; Phillips *et al.,* 2008). Thus, *Botryosphaeria* is now a relatively small genus with only 10 species (Zhang *et al.,* 2021).

The type species of *Botryosphaeria, B. dothidea* is a common pathogen on *Eucalyptus* species causing canker and dieback worldwide and has been well studied on this host (Smith *et al.,* 1994; Burgess *et al.,* 2005; Pérez *et al.,* 2008; Slippers *et al.,* 2009; Marsberg *et al.,* 2017). The fungus has a cosmopolitan distribution and occurs on monocotyledonous, dicotyledonous and gymnosperm hosts (Smith *et al.,* 1996; Slippers and Wingfield, 2007; Marsberg *et al.,* 2017). It occurs as a saprophyte, endophyte or pathogen on these plants. In South Africa, *B. dothidea* is known to occur on various hosts including *Vachellia, Eucalyptus* and *Podocarpus* species (Jami *et al.,* 2017). However, studies have indicated that *B. dothidea* is not widely distributed (Jami *et al.,* 2017). This may suggest that previous reports of *B. dothidea* referred not only to this fungus but to other species that were previously included in *Botryosphaeria* (Slippers *et al.,* 2004a).

2.2.2. *Diplodia*

Like many other genera of *Botryosphaeriaceae* for which the sexual state was not known, *Diplodia* was placed within *Botryosphaeria*. *Diplodia* was later separated from *Botryosphaeria* to accommodate the dark-spored anamorphs of *Botryosphaeria* (Crous *et al.,* 2006). Based on culture morphology, *Diplodia* species are characterised by aerial, septate and dark-brown mycelia (Denman *et al.,* 2000; Phillips *et al.,* 2013). Two types of conidial morphologies have been noted for *Diplodia* species. Type one has hyaline and aseptate conidia that slowly turn brown and become septate, while type two is characterised by conidia that become pigmented during the early stages of development and the conidia rarely become septate (Denman *et al.,* 2000; Phillips *et al.,* 2013). The genus consists of 26 species with a cosmopolitan distribution and a broad host range (Zhang *et al.,* 2021). The type species of the genus is *D. mutila* (Dissanayake *et al.,* 2016).

One of the best-studied species in *Diplodia* is *D. sapinea,* which is a well-known pathogen of pine trees and has been isolated from 33 pine species causing blight, dieback, canker and wood discoloration (Burgess *et al.,* 2004; Zlatković *et al.,* 2016). *Diplodia sapinea* was the first fungal pathogen found in South African pine plantations (Lundquist, 1987). Recent studies have reported an expansion on the host ranges of *D. sapinea* and it is known to infect other woody tree species in the genera *Abies, Cedrus, Chamaecyparis, Thuja, Cupressus, Juniperus* and *Sequoiadendron* on which it also causes extensive dieback and canker (Milijašević, 2003, 2009; Zlatković *et al.,* 2016). In South Africa, *D. sapinea* has been recorded on indigenous and introduced tree species including *Acacia mearnsii*, *Eucalyptus citriodora, Eucalyptus dorrigoensis, Eucalyptus microcorvs, Podocarpus longatus, Syzygium cordatum, Vachellia karroo and Podocarpus henkelii* (Jami *et al.,* 2017).

2.2.3. *Lasiodiplodia*

The genus *Lasiodiplodia* was introduced with the type species *Lasiodiplodia tubericola* (Ellis, 1894). Ellis (1894) did not provide morphological descriptions for either the genus or the species. Morphological descriptions were later provided by Clendenin (Clendenin, 1896). Based on morphological descriptions by Clendenin (1896), Griffin and Maublanc (1909) considered *Botryodiplodia theobromae* to be more suitably accommodated in *Lasiodiplodia.* Thus, *B. theobromae* was moved to *Lasiodiplodia* and referred to as *L. theobromae* and was subsequently designated as the type species of the genus (Griffin and Maublanc, 1909).

Several studies have shown that morphology alone is not reliable for the separation of species in *Lasiodiplodia* (Phillips *et al.,* 2013; Slippers *et al.,* 2014). Recent studies using DNA sequence data and phylogenetic inference have shown that *L. theobromae* represents a species complex (Pavlic *et al.,* 2004; Burgess *et al.,* 2006; Damm *et al.,* 2007; Alves *et al.,* 2008). Some species, including *L. mahajangana*, *L. iraniensis*, *L. pseudotheobromae*, *L. parva* and *L. viticola* that are closely related to *L. theobromae* were likely confused with this species in the past when using only morphologically discernible features (Alves *et al.,* 2008; Phillips *et al.,* 2013). Additionally, hybrids have been discovered in *Lasiodiplodia* adding to the complex picture of understanding the true identity of *L. theobromae* (Sakalidis *et al.,* 2011b; Cruywagen *et al.,* 2017). Cruywagen *et al.* (2017) further reported that due to the widespread distribution of *Lasiodiplodia* species, some of the recognised species may be hybrids. *Lasiodiplodia* currently includes 31 species (Dissanayake *et al.,* 2016; Yang *et al.,* 2017).

Lasiodiplodia theobromae is geographically distributed in tropical and subtropical regions (Yan *et al.,* 2013; Mehl *et al.,* 2017a). The fungus has been recorded on approximately 500 plant hosts as an endophyte and a pathogen causing various diseases such as dieback, root rot, fruit rots and leaf spots (Trakunyingcharoen *et al.,* 2014; Jami *et al.,* 2017; Mehl *et al.,* 2017a; Batista *et al.,* 2021). In rare cases, the fungus has been reported as an opportunistic human pathogen associated with keratomycosis and phaeohyphomycosis (Rebell and Forster, 1976; Summerbell *et al.,* 2004). *Lasiodiplodia theobromae* is common in tropical and sub-tropical climates in South Africa, with the fungus occurring mostly in the western and northern parts of the country. *Lasiodiplodia theobromae* has been recorded on eight hosts in South Africa, of which six are native and two are non-native (Jami *et al.,* 2017).

2.2.4. *Neofusicoccum*

Neofusicoccum was previously placed within *Botryosphaeria*. Crous *et al.* (2006) proposed *Neofusicoccum* as a new genus to accommodate species with *Fusicoccum*-like and *Dichomera*-like synanamorphs (Crous *et al.,* 2006). Before the separation of *Neofusicoccum* from *Botryosphaeria*, Slippers *et al*. (2004a) distinguished *B. dothidea*, *B. ribis* and *B. parva* from each other based on morphological features, thus *B. ribis* and *B. parva* were moved to *Neofusicoccum* and referred to as *N. parvum* and *N. ribis*. The two species are closely related and have often been treated as one species (Pavlic *et al.,* 2007; Sakalidis *et al.,* 2011c). It was later recognised in studies using DNA sequence data that *N. parvum* and *N. ribis* represent a species complex comprising at least 10 cryptic species (Pavlic *et al.,* 2009; Sakalidis *et al.,* 2011c, 2013). *Neofusicoccum* comprises 29 species described using DNA sequences and phylogenetic inference (Dissanayake *et al.,* 2016; Yang *et al.,* 2017). Not all delineated species have a Dichomera-like synanamorph as originally described for the genus (Dissanayake *et al.,* 2016; Yang *et al.,* 2017).

Neofusicoccum parvum is the most widespread species in the genus having been reported on 223 hosts in 50 countries (Sakalidis *et al.,* 2013; Batista *et al.,* 2021). In South Africa, the fungus has been recorded on 30 hosts (14 native and 16 non-native tree species) at 12 sites in the country (Jami *et al.,* 2017). *Neofusicoccum parvum* was reported as the most dominant species on native *S. cordatum* in South Africa (Pavlic *et al.,* 2007). The common occurrence of *N. parvum* on both native and introduced hosts and the intraspecific genetic variation in South African isolates, indicates the ease with which this invasive fungus spreads, infecting both indigenous and introduced hosts once established in the region (Pavlic *et al.,* 2007; Sakalidis *et al.,* 2013; Pavlic *et al.,* 2015).

2.3. Reproduction and dispersal of species in the *Botryosphaeriaceae*

The *Botryosphaeriaceae*, as with most ascomycota, reproduce both sexually and asexually. Sexual reproduction can either be heterothallic or homothallic (Bihon *et al.,* 2014; Nagel *et al.,* 2018). Heterothallic sexual reproduction involves the interaction of two individuals of the same species of opposite mating types for sexual reproduction to take place (Ni *et al.,* 2011; Nagel *et al.,* 2018; Du *et al.,* 2020). With homothallic sexual reproduction, one fungal individual can mate with any other individual of the same species, including itself (Ni *et al.,* 2011; Wilson *et al.,* 2015; Nagel *et al.,* 2018). Homothallism is beneficial when the probability of encountering a compatible mating partner is low (Ni *et al.,* 2011; Wilson *et al.,* 2015). A study focusing on the evolution of the mating types and mating strategies of *Botryosphaeriaceae* showed that heterothallism was the ancestral state for *Botryosphaeriaceae* and at least five transitions to homothallism have occurred (Nagel *et al.,* 2018).

Dispersal of fungi in the *Botryosphaeriaceae* occurs through spores, as infections in seeds or other plant parts and through other organisms such as insect vectors and humans (Slippers and Wingfield, 2007; Gramaje *et al.,* 2018; Wingfield *et al.,* 2025). The *Botryosphaeriaceae* produce a slimy mass of sexual and asexual spores from a central ostiole in the perithecium or pycnidium (Slippers and Wingfield, 2007). The production of spores is stimulated by the presence of moisture arising from fog, rainfall, dew or mist, and warmer temperatures (Pusey, 1989; Michailides *et al.,* 1991; Úrbez-Torres *et al.,* 2010). A study by van Niekerk *et al.* (2011) using spore traps indicated a strong relationship between rainfall and spore dispersal with more than 60% of spores trapped in rainy weather. The spores are dispersed by rainwater and wind, allowing for transmission between hosts (van Niekerk *et al.,* 2011; Úrbez-Torres *et al.,* 2010; Valencia *et al.,* 2015). Therefore, the inoculum is expected to be dispersed locally from a specific host to other hosts in the vicinity (Úrbez-Torres *et al.,* 2010). Stanosz *et al.,* (2005) indicated that nursery plants usually have low levels of *Botryosphaeriaceae* colonization unless exposed to infected mature trees in their proximity which serves as a source of inoculum.

Species of *Botryosphaeriaceae* have also been recovered from asymptomatic seeds suggesting transmission of *Botryosphaeriaceae* through seed infection could be possible (Cilliers *et al.,* 1993; Smith *et al.,* 1996; Burgess and Wingfield, 2002; Slippers and Wingfield, 2007; Bihon *et al.,* 2011). *Lasiodiplodia theobromae* and *D. sapinea* have been isolated from healthy *Pinus* seeds (Cilliers *et al.,* 1993; Smith *et al.,* 1996; Burgess and Wingfield, 2002) and *D. rosulata* from healthy *Podocarpus falcatus* and *Prunus africana* seeds (Gure *et al.,* 2005). These asymptomatic infections are thought to have facilitated the movement of *Botryosphaeriaceae* around the world during seed and seedling trade (Burgess and Wingfield, 2002; Burgess *et al.,* 2019; Marsberg *et al.,* 2017; Slippers *et al.,* 2017). However, vertical transmission of the fungus from the seed to the offspring does not happen, despite infections of the seed (Bihon *et al.,* 2011).

2.4. Mode of infection

Species of *Botryosphaeriaceae* infect plants through direct penetration of natural openings (Petrini and Fisher, 1988; Slippers and Wingfield, 2007; Pérez *et al.,* 2010; Úrbez-Torres and Gubler, 2011; Jurick *et al.,* 2013). Natural openings, including stomata on leaves, lenticels on fruit and inflorescences are often the entry sites, leading to endophytic colonization of healthy tissues. Endophytism appears to be common to most if not all, members of the *Botryosphaeriaceae* (Pillay *et al.,* 2013; Osorio *et al.,* 2017; Luo *et al.,* 2019). Endophytism was first reported by Petrini and Fisher (1988) who isolated *D. sapinea* from asymptomatic *Pinus sylvestris* stems. Fisher *et al.* (1993) later isolated *B. dothidea* from asymptomatic leaves and twigs of *Eucalyptus nitens.* Subsequent studies also confirmed the ability of species of *Botryosphaeriaceae* to remain latent in healthy plant tissues without showing disease symptoms (Slippers and Wingfield, 2007).

Another means by which species of *Botryosphaeriaceae* infect their hosts is through wounds (Michailides, 1991; Slippers and Wingfield, 2007; Úrbez-Torres and Gubler, 2011; Mehl *et al.,* 2013). Wounds may be a result of human activities such as pruning and harvesting, strong winds, hail or insect damage (Johnson, 1994; Menge and Ploetz, 2003; Mehl *et al.,* 2013). Physical wounds from strong winds have been reported as infection sites for fungal organisms (Cobb and Metz, 2017). An earlier study by Smith *et al.* (2002) reported that hail wounds on *P. patula* and *P. radiata* developed into cankers infected with *D. sapinea*. Insect damage has been reported to add additional stress and pressure on trees predisposing them to fungal infection (Cobb and Metz, 2017). Some members of *Botryosphaeriaceae* have been recovered from graft unions, suggesting that transmission could have been through contaminated grafting tools and stress resulting from graft incompatibility (Aroca *et al.,* 2006).

2.5. The role of climatic conditions on *Botryosphaeriaceae* and the diseases they cause

Members of the *Botryosphaeriaceae* are generally regarded as weak opportunistic pathogens that often cause disease when their hosts are subjected to unfavourable environmental conditions (Desprez-Loustau *et al.,* 2006; Piskur *et al.,* 2011; Zhang *et al.,* 2013; Mehl *et al.,* 2013). However, the threat associated with fungi belonging to *Botryosphaeriaceae* is increasing in the context of climate change (Desprez-Loustau *et al.,* 2007; Slippers and Wingfield, 2007). Various climatic conditions, such as moisture stress and extreme temperatures are conducive to disease development caused by species of *Botryosphaeriaceae* (Desprez-Loustau *et al.,* 2006*,* 2007; Slippers and Wingfield, 2007; van Niekerk *et al.,* 2011).

Moisture stress arising from either drought or high temperatures often leads to disease expression by species of *Botryosphaeriaceae*. Moisture stress results in the inability of the host to respond to infection and inhibits nutrient translocation, leading to nutrient deficiency (Arnold and Herre, 2003; Anderson *et al.,* 2004). Moisture stress increases host susceptibility to fungal colonization in general and especially to *Botryosphaeriaceae* (Old *et al.,* 1990; Madar *et al.,* 1995; Paoletti *et al.,* 2001). Pathogenicity tests on stressed and non-stressed dogwood stems indicated that the test fungus, *L. theobromae*, could be re-isolated from all the inoculated plants, but canker was only observed on moisture-stressed plants (Mullen *et al.,* 1991). Similarly, Hartill (1991) indicated that ring necrosis that develops on stressed avocado trees in dry conditions promotes susceptibility of the trees to infection by species of *Botryosphaeriaceae* causing post-harvest diseases.

Apart from causing moisture stress, high temperatures also induce spore germination, facilitating *Botryosphaeriaceae* infection (Ahimera *et al.,* 2003; Desprez-Loustau *et al.,* 2006). The higher average temperatures in tropical and subtropical regions typically range between 25 °C and 30 °C, which is ideal for spore germination of most species of *Botryosphaeriaceae* (Ahimera *et al.,* 2003). Several researchers predicted that warmer temperatures will increase disease outbreaks due to the positive effects they have on spore germination of pathogens (Coakley *et al.,* 1999; Davis and Shaw, 2001; Etterson and Shaw, 2001; Desprez-Loustau *et al.,* 2006).

Botryosphaeriaceae species can be dispersed over a wide range of temperatures. Some species in the family, including *B. dothidea*, *Diplodia seriata* and *L. theobromae* have been reported to sporulate at temperatures between 6 °C and 9 °C (Copes and Hendrix, 2004). Úrbez-Torres *et al.* (2010) also detected spore production during cold winter months at temperatures as low as 3 °C – 7 °C, demonstrating that spore production can occur at low temperatures. Disease progression also seems to be affected by low temperatures. Rayachhetry *et al*. (1996) reported increased canker length and sapwood discoloration caused by *B. ribis,* which is likely *N. ribis* or a related species, on *Melaleuca* species following exposure to low temperatures (6 °C).

2.6. *Botryosphaeriaceae* in South Africa

The *Botryosphaeriaceae* have been intensively studied in South Africa (reviewed in Jami *et al.,* 2017). Most studies on these fungi have been carried out in the Western Cape, KwaZulu-Natal, Gauteng and Mpumalanga provinces (Figure 2.2). Fifty-six species of *Botryosphaeriaceae* have been reported on indigenous and introduced hosts in South Africa (Table 2.1). Most species in the family are not host-specific, occurring on various hosts that may be related or unrelated to each other (Pavlic *et al.,* 2007; Pérez *et al.,* 2010; Pillay *et al.,* 2013; Jami *et al.,* 2014; Mehl *et al.,* 2017b). Although the ecological role of the large number of species discovered on these hosts in plantations, orchards and natural ecosystems in South Africa is still not well understood, it is clear that *Botryosphaeriaceae* are ubiquitous on all woody tree species sampled to date (Jami *et al.,* 2017; Burgess *et al.,* 2019; Li *et al.,* 2020).

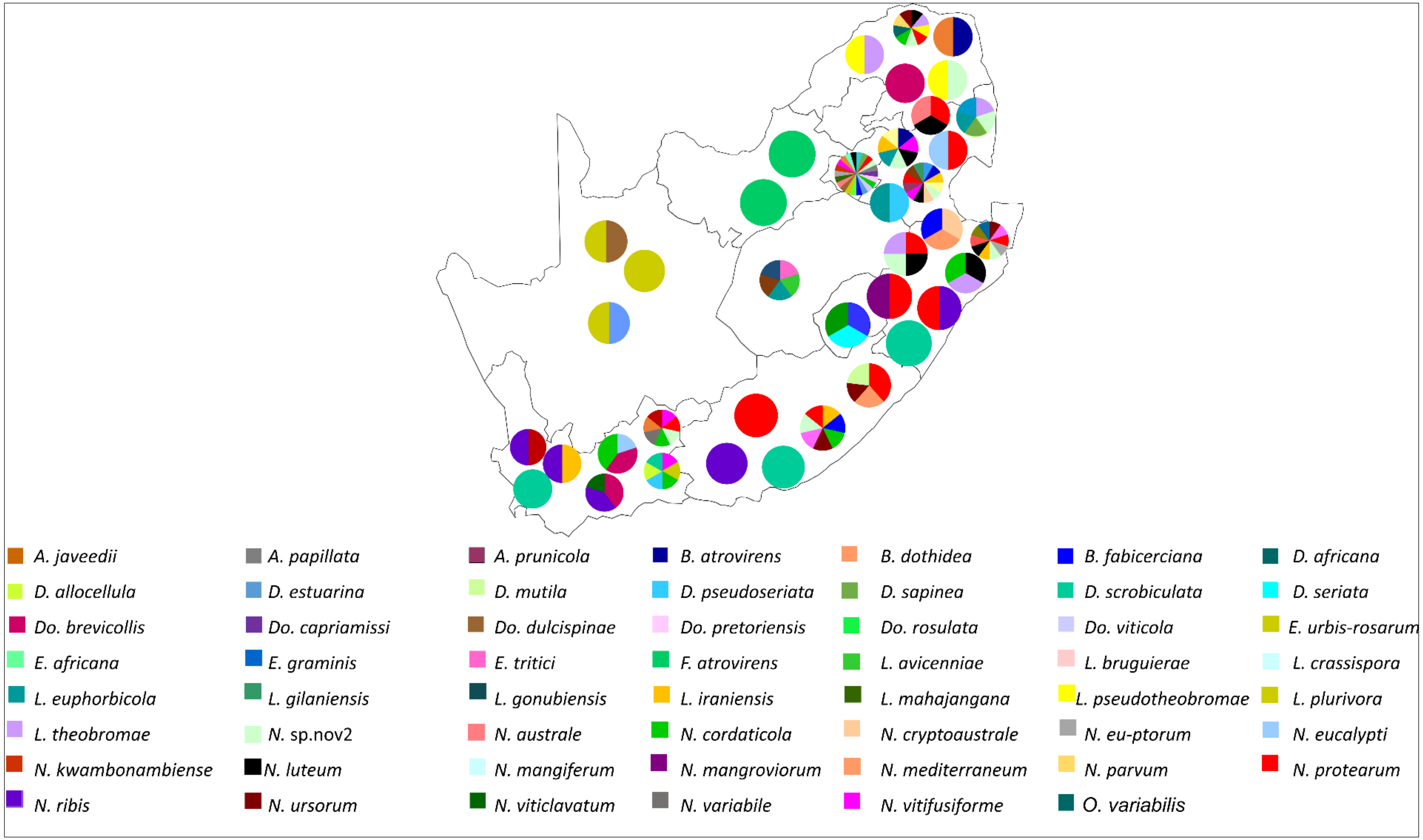

Figure 2.2. Species diversity and distribution of *Botryosphaeriaceae* across South Africa (Jami *et al.*, 2017).

Table 2.1. *Botryosphaeriaceae* species infecting native and non-native tree species in South Africa. Species names are as presented in the publications.

Species	**Host**	**Location**	**References**
Aplosporella javeedii	*Celtis africana*	Gauteng	Jami *et al.*, 2017
Aplosporella javeedii	*Sercia lancea*	Gauteng	Jami *et al.*, 2017
Aplosporella papillata	*Vachellia erioloba*	Northern Cape	Jami *et al.*, 2017
Aplosporella papillate	*Vachellia tortilis*	Northern Cape	Jami *et al.*, 2017
Aplosporella prunicola	*Prunus persica*	Limpopo	Jami *et al.*, 2017
Botryosphaeria atrovirens	*Pterocarpus angolensis*	Mpumalanga	Jami *et al.*, 2017
Botryosphaeria fabicerciana	*Sclerocarya birrea*	Mpumalanga	Jami *et al.*, 2017
Botryosphaeria fabicerciana	*Mangifera indica*	Mpumalanga	Jami *et al.*, 2017
Botryosphaeria dothidea	*Acacia mearnsii*	KwaZulu-Natal	Jami *et al.*, 2017
Botryosphaeria dothidea	*Eucalyptus citriodora*	Gauteng	Jami *et al.*, 2017
Botryosphaeria dothidea	*Eucalyptus dorrigoensis*	Gauteng	Jami *et al.*, 2017
Botryosphaeria dothidea	*Eucalyptus microcorvs*	Gauteng	Jami *et al.*, 2017
Botryosphaeria dothidea	*Eucalyptus* sp.	Gauteng	Jami *et al.*, 2017
Botryosphaeria dothidea	*Podocarpus elongates*	Gauteng	Jami *et al.*, 2017
Botryosphaeria dothidea	*Podocarpus henkelii*	Gauteng	Jami *et al.*, 2017
Botryosphaeria dothidea	*Syzygium cordatum*	Eastern Cape, KZN	Jami *et al.*, 2017
Botryosphaeria dothidea	*Vachellia karroo*	Gauteng	Jami *et al.*, 2017
Diplodia africana	*Prunus persica*	Western Cape	Jami *et al.*, 2017
Diplodia allocellula	*Sclerocarya birrea*	Mpumalanga	Jami *et al.*, 2017
Diplodia allocellula	*Vachellia karroo*	Gauteng	Jami *et al.*, 2017

Table 2.1. (Continued)

Species	**Host**	**Location**	**References**
Diplodia estuarina	*Avicennia marina*	KwaZulu-Natal, Eastern Cape	Jami *et al.*, 2017
Diplodia mutila	*Podocarpus henkelii*	Western Cape	Jami *et al.*, 2017
Diplodia mutila	*Prunus salicina*	Western Cape	Jami *et al.*, 2017
Diplodia pseudoseriata	*Pterocarpus angolensis*	Mpumalanga	Jami *et al.*, 2017
Diplodia pseudoseriata	*Vachellia karroo*	Gauteng	Jami *et al.*, 2017
Diplodia sapinea	*Pinus patula*	KwaZulu-Natal	Jami *et al.*, 2017
Diplodia sapinea	*Pinus radiata*	Eastern Cape, Western Cape	Jami *et al.*, 2017
Diplodia sapinea	*Cupressus lusitanica*	Mpumalanga	Jami *et al.*, 2017
Diplodia sapinea	*Protea repens*	KwaZulu-Natal	Jami *et al.*, 2017
Diplodia sapinea	*Prunus persica*	Western Cape	Jami *et al.*, 2017
Diplodia scrobiculata	*Pinus patula*	KwaZulu-Natal, Mpumalanga	Jami *et al.*, 2017
Diplodia seriata	*Malus* sp.	Western Cape	Jami *et al.*, 2017
Diplodia seriata	*Populas* sp.	Western Cape	Jami *et al.*, 2017
Diplodia seriata	*Protea magnifica*	Western Cape	Jami *et al.*, 2017
Diplodia seriata	*Prunus armeniaca*	Limpopo	Jami *et al.*, 2017
Diplodia seriata	*Prunus magnifica*	Western Cape	Jami *et al.*, 2017
Diplodia seriata	*Prunus persica*	Western Cape	Jami *et al.*, 2017
Diplodia seriata	*Prunus salicina*	Western Cape	Jami *et al.*, 2017
Diplodia seriata	*Pyrus communis*	Western Cape	Jami *et al.*, 2017
Diplodia seriata	*Vitis vinifera*	Western Cape	Jami *et al.*, 2017
Dothiorella brevicollis	*Vachellia karroo*	Gauteng	Jami *et al.*, 2017

Table 2.1. (Continued)

Species	Host	Location	References
Dothiorella capriamissi	*Vachellia erioloba*	Northern Cape	Jami *et al.*, 2017
Dothiorella dulcispinae	*Senegalia mellifera*	Gauteng	Jami *et al.*, 2017
Dothiorella dulcispinae	*Vachellia karroo*	Gauteng	Jami *et al.*, 2017
Dothiorella pretoriensis	*Vachellia karroo*	Gauteng	Jami *et al.*, 2017
Dothiorella rosulata	*Vachellia karroo*	Northern Cape	Jami *et al.*, 2017
Dothiorella rosulata	*Senegalia mellifera*	Gauteng, Northern Cape	Jami *et al.*, 2017
Dothiorella rosulata	*Vachellia tortilis*	Northern Cape	Jami *et al.*, 2017
Dothiorella viticola	*Celtis Africana*	Gauteng	Jami *et al.*, 2017
Dothiorella viticola	*Gymnosporia buxifolia*	Gauteng	Jami *et al.*, 2017
Dothiorella viticola	*Podocarpus henkelii*	Gauteng	Jami *et al.*, 2017
Dothiorella viticola	*Prunus persica*	Limpopo	Jami *et al.*, 2017
Dothiorella viticola	*Senegalia mellifera*	Gauteng	Jami *et al.*, 2017
Dothiorella viticola	*Vachellia karroo*	Gauteng	Jami *et al.*, 2017
Dothiorella viticola	*Vitis vinifera*	Western Cape	Jami *et al.*, 2017
Eutiarosporella africana	*Celtis Africana*	Gauteng	Jami *et al.*, 2017
Eutiarosporella graminis	*Nestlera* sp.	Free State	Jami *et al.*, 2017
Eutiarosporella tritici	*Triticum* sp.	Free State	Jami *et al.*, 2017
Eutiarosporella urbisrosarum	*Vachellia karroo*	Gauteng	Jami *et al.*, 2017
Fusicoccum atrovirens	*Pterocarpus angolensis*	Mpumalanga	Jami *et al.*, 2017
Lasiodiplodia avicenniae	*Avicennia marina*	KwaZulu-Natal	Jami *et al.*, 2017
Lasiodiplodia euphorbiicola	*Adansonia digitata*	KwaZulu-Natal	Jami *et al.*, 2017

Table 2.1. (Continued)

Species	Host	Location	References
Lasiodiplodia bruguierae	*Bruguiera gymnorrhiza*	KwaZulu-Natal	Jami *et al.*, 2017
Lasiodiplodia bruguierae	*Rhizophora mucronata*	KwaZulu-Natal	Jami *et al.*, 2017
Lasiodiplodia crassispora	*Pterocarpus angolensis*	Mpumalanga	Jami *et al.*, 2017
Lasiodiplodia crassispora	*Sclerocarya birrea*	Mpumalanga	Jami *et al.*, 2017
Lasiodiplodia crassispora	*Vitis vinifera*	Western Cape	Jami *et al.*, 2017
Lasiodiplodia gilanensis	*Podocarpus latifolius*	Western Cape	Jami *et al.*, 2017
Lasiodiplodia gonubiensis	*Bruguiera gymnorrhiza*	KwaZulu-Natal	Jami *et al.*, 2017
Lasiodiplodia gonubiensis	*Ceriops tagal*	KwaZulu-Natal	Jami *et al.*, 2017
Lasiodiplodia gonubiensis	*Rhizophora mucronate*	KwaZulu-Natal	Jami *et al.*, 2017
Lasiodiplodia gonubiensis	*Syzygium cordatum*	Eastern Cape	Jami *et al.*, 2017
Lasiodiplodia gonubiensis	*Sclerocarya birrea*	Mpumalanga	Jami *et al.*, 2017
Lasiodiplodia gonubiensis	*Vachellia karroo*	Western Cape	Jami *et al.*, 2017
Lasiodiplodia iraniensis	*Sclerocarya birrea*	Mpumalanga	Jami *et al.*, 2017
Lasiodiplodia mahajangana	*Euphorbia ingens*	Limpopo	Jami *et al.*, 2017
Lasiodiplodia mahajangana	*Mangifera indica*	Mpumalanga	Jami *et al.*, 2017
Lasiodiplodia mahajangana	*Sclerocarya birrea*	Mpumalanga	Jami *et al.*, 2017
Lasiodiplodia plurivora	*Vitis vinifera*	Western Cape	Jami *et al.*, 2017
Lasiodiplodia plurivora	*Prunus salicina*	Western Cape	Jami *et al.*, 2017
Lasiodiplodia pseudotheobtromae	*Mangifera indica*	Mpumalanga	Jami *et al.*, 2017
Lasiodiplodia pseudotheobtromae	*Pterocarpus angolensis*	Mpumalanga	Jami *et al.*, 2017
Lasiodiplodia pseudotheobtromae	*Sclerocarya birrea*	Mpumalanga	Jami *et al.*, 2017

Table 2.1. (Continued)

Species	Host	Location	References
Lasiodiplodia pseudotheobtromae	*Syzygium cordatum*	KwaZulu-Natal	Jami *et al.*, 2017
Lasiodiplodia pseudotheobtromae	*Terminalia catappa*	KwaZulu-Natal	Jami *et al.*, 2017
Lasiodiplodia pseudotheobtromae	*Vachellia karroo*	Limpopo	Jami *et al.*, 2017
Lasiodiplodia theobromae	*Barrringtonia racemose*	KwaZulu-Natal	Jami *et al.*, 2017
Lasiodiplodia theobromae	*Euphorbia ingens*	Limpopo	Jami *et al.*, 2017
Lasiodiplodia theobromae	*Mangifera indica*	Mpumalanga	Jami *et al.*, 2017
Lasiodiplodia theobromae	*Pterocarpus angolensis*	Mpumalanga	Jami *et al.*, 2017
Lasiodiplodia theobromae	*Sclerocarya birrea*	Mpumalanga	Jami *et al.*, 2017
Lasiodiplodia theobromae	*Syzygium cordatum*	Eastern Cape	Jami *et al.*, 2017
Lasiodiplodia theobromae	*Terminalia catappa*	KwaZulu-Natal	Jami *et al.*, 2017
Lasiodiplodia theobromae	*Vachellia karroo*	Gauteng	Jami *et al.*, 2017
Lasiodiplodia theobromae	*Vitis vinifera*	Western Cape	Jami *et al.*, 2017
Neofusicoccum australe	*Widdringtonia nodiflora*	Western Cape	Jami *et al.*, 2017
Neofusicoccum australe	*Eucalyptus grandis*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum australe	*Malus domestica*	Western Cape	Jami *et al.*, 2017
Neofusicoccum australe	*Mangifera indica*	Mpumalanga	Jami *et al.*, 2017
Neofusicoccum australe	*Prunus armeniaca*	Limpopo	Jami *et al.*, 2017
Neofusicoccum australe	*Prunus dulcis*	Western Cape	Jami *et al.*, 2017
Neofusicoccum australe	*Prunus persica*	Western Cape	Jami *et al.*, 2017
Neofusicoccum australe	*Prunus salicina*	Western Cape	Jami *et al.*, 2017
Neofusicoccum australe	*Pyrus communis*	Western Cape	Jami *et al.*, 2017

Table 2.1. (Continued)

Species	**Host**	**Location**	**References**
Neofusicoccum australe	*Syzygium cordatum*	Eastern Cape	Jami *et al.*, 2017
Neofusicoccum australe	*Vachellia karroo*	Gauteng	Jami *et al.*, 2017
Neofusicoccum australe	*Vitis vinifera*	Western Cape	Jami *et al.*, 2017
Neofusicoccum cordaticola	*Syzygium cordatum*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum crypto-australe	*Avicennia marina*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum crypto-australe	*Ceriops tagal*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum crypto-australe	*Eucalytus* sp.	Gauteng	Jami *et al.*, 2017
Neofusicoccum crypto-australe	*Podocarpus henkelii*	Gauteng	Jami *et al.*, 2017
Neofusicoccum crypto-australe	*Bruguiera gymnorrhiza*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum crypto-australe	*Podocarpus latifolius*	Western Cape	Jami *et al.*, 2017
Neofusicoccum crypto-australe	*Rhizophora mucronate*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum crypto-australe	*Syzygium cordatum*	Gauteng	Jami *et al.*, 2017
Neofusicoccum eucalypti	*Eucalyptus* sp.	Gauteng	Jami *et al.*, 2017
Neofusicoccum eucalypti	*Podocarpus henkelii*	Gauteng	Jami *et al.*, 2017
Neofusicoccum eucalypti	*Podocarpus latifolius*	Gauteng	Jami *et al.*, 2017
Neofusicoccum eucalyptorum	*Eucalyptus grandis*	Mpumalanga	Jami *et al.*, 2017
Neofusicoccum eucalyptorum	*Eucalyptus nitens*	Mpumalanga	Jami *et al.*, 2017
Neofusicoccum eucalyptorum	*Eucalyptus* sp.	Mpumalanga	Jami *et al.*, 2017
Neofusicoccum kwambonambiense	*Afrocarpus falcatus*	Mpumalanga	Jami *et al.*, 2017
Neofusicoccum kwambonambiense	*Bruguiera gymnorrhiza*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum kwambonambiense	*Celtis Africana*	Gauteng	Jami *et al.*, 2017

Table 2.1. (Continued)

Species	Host	Location	References
Neofusicoccum kwambonambiense	*Eucalyptus grandis*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum kwambonambiense	*Mangifera indica*	Mpumalanga	Jami *et al.*, 2017
Neofusicoccum kwambonambiense	*Podocarpus henkelii*	Mpumalanga	Jami *et al.*, 2017
Neofusicoccum kwambonambiense	*Rhizophora mucronate*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum kwambonambiense	*Syzygium cordatum*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum kwambonambiense	*Terminalia sericea*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum kwambonambiense	*Vachellia karroo*	Gauteng	Jami *et al.*, 2017
Neofusicoccum luteum	*Avicennia marina*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum luteum	*Bruguiera gymnorrhiza*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum luteum	*Rhizophora mucronate*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum luteum	*Syzygium cordatum*	Eastern Cape	Jami *et al.*, 2017
Neofusicoccum mangiferum	*Syzygium cordatum*	Eastern Cape	Jami *et al.*, 2017
Neofusicoccum mangiferum	*Tibouchina urvilleana*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum mangroviorum	*Avicennia marina*	KwaZulu-Natal, Eastern Cape	Jami *et al.*, 2017
Neofusicoccum mangroviorum	*Bruguiera gymnorrhiza*	KwaZulu-Natal, Eastern Cape	Jami *et al.*, 2017
Neofusicoccum mangroviorum	*Mimusops caffra*	Eastern Cape	Jami *et al.*, 2017
Neofusicoccum mangroviorum	*Rhizophora mucronate*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum mediterraneum	*Mangifera indica*	Mpumalanga	Jami *et al.*, 2017
Neofusicoccum mediterraneum	*Sclerocarya birrea*	Mpumalanga	Jami *et al.*, 2017
Neofusicoccum parvum	*Vitis vinifera*	Western Cape	Jami *et al.*, 2017
Neofusicoccum parvum	*Afrocarpus falcatus*	Mpumalanga	Jami *et al.*, 2017

Table 2.1. (Continued)

Species	Host	Location	References
Neofusicoccum parvum	*Avicennia marina*	KwaZulu-Natal, Eastern Cape	Jami *et al.*, 2017
Neofusicoccum parvum	*Lumnitzera racemose*	KwaZulu-Natal, Eastern Cape	Jami *et al.*, 2017
Neofusicoccum parvum	*Bruguiera gymnorrhiza*	KwaZulu-Natal, Eastern Cape	Jami *et al.*, 2017
Neofusicoccum parvum	*Eucalyptus dorrigoensis*	Gauteng	Jami *et al.*, 2017
Neofusicoccum parvum	*Eucalyptus grandis*	Mpumalanga	Jami *et al.*, 2017
Neofusicoccum parvum	*Eucalyptus microcorvs*	Gauteng	Jami *et al.*, 2017
Neofusicoccum parvum	*Eucalyptus nicholii*	Gauteng	Jami *et al.*, 2017
Neofusicoccum parvum	*Podocarpus henkelii*	Gauteng	Jami *et al.*, 2017
Neofusicoccum parvum	*Eucalyptus ovata*	Gauteng	Jami *et al.*, 2017
Neofusicoccum parvum	*Eucalyptus robusta*	Gauteng	Jami *et al.*, 2017
Neofusicoccum parvum	*Eucalyptus saligna*	Gauteng	Jami *et al.*, 2017
Neofusicoccum parvum	*Eucalupyus scoparia*	Gauteng	Jami *et al.*, 2017
Neofusicoccum parvum	*Eucalyptus smithii*	Mpumalanga	Jami *et al.*, 2017
Neofusicoccum parvum	*Eucalyptus* sp.	Gauteng	Jami *et al.*, 2017
Neofusicoccum parvum	*Eucalyptus tereticornis*	Gauteng	Jami *et al.*, 2017
Neofusicoccum parvum	*Gymnosporia buxifolia*	Gauteng	Jami *et al.*, 2017
Neofusicoccum parvum	*Heteropyxix natalensis*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum parvum	*Mangifera indica*	Mpumalanga	Jami *et al.*, 2017
Neofusicoccum parvum	*Rhizophora mucronate*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum parvum	*Schizolobium parahyba*	Gauteng	Jami *et al.*, 2017
Neofusicoccum parvum	*Sclerocarya birrea*	Mpumalanga	Jami *et al.*, 2017

Table 2.1. (Continued)

Species	Host	Location	References
Neofusicoccum parvum	*Sequoia gigantean*	Eastern Cape	Jami *et al.*, 2017
Neofusicoccum parvum	*Syzygium cordatum*	Eastern Cape	Jami *et al.*, 2017
Neofusicoccum parvum	*Terminalia catappa*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum parvum	*Syzygium guineense*	Eastern Cape	Jami *et al.*, 2017
Neofusicoccum parvum	*Terminalia sericea*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum parvum	*Tibouchina urvilleana*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum parvum	*Vachellia karroo*	Gauteng	Jami *et al.*, 2017
Neofusicoccum protearum	*Heteropyxix natalensis*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum protearum	*Leucadendron laureolum*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum protearum	*Protea cynaroides*	Western Cape	Jami *et al.*, 2017
Neofusicoccum protearum	*Protea eximia*	Western Cape	Jami *et al.*, 2017
Neofusicoccum protearum	*Protea magnifica*	Western Cape	Jami *et al.*, 2017
Neofusicoccum protearum	*Protea repens*	Western Cape	Jami *et al.*, 2017
Neofusicoccum ribis	*Bruguiera gymnorrhiza*	KwaZulu-Natal, Eastern Cape	Jami *et al.*, 2017
Neofusicoccum ribis	*Mangifera indica*	Mpumalanga	Jami *et al.*, 2017
Neofusicoccum ribis	*Avicennia marina*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum ribis	*Rhizophora mucronata*	KwaZulu-Natal	Jami *et al.*, 2017
Neofusicoccum ribis	*Sclerocarya birrea*	Mpumalanga	Jami *et al.*, 2017
Neofusicoccum ribis	*Syzygium cordatum*	Eastern Cape	Jami *et al.*, 2017
Neofusicoccum ursorum	*Podocarpus henkelii*	Gauteng	Jami *et al.*, 2017
Neofusicoccum ursorum	*Eucalyptus* sp.	Gauteng	Jami *et al.*, 2017

Table 2.1. (Continued)

Species	Host	Location	References
Neofusicoccum variabile sp. nov	*Mimisops caffra*	Eastern Cape	Jami *et al.*, 2017
Neofusicoccum viticlavatum	*Vitis vinifera*	Western Cape	Jami *et al.*, 2017
Neofusicoccum vitifursiforme	*Acacia mearnsii*	South Africa	Jami *et al.*, 2017
Neofusicoccum vitifursiforme	*Schizolobium parahyba*	Gauteng, Mpumalanga	Jami *et al.*, 2017
Neofusicoccum vitifursiforme	*Vachellia karroo*	Gauteng	Jami *et al.*, 2017
Neofusicoccum vitifursiforme	*Sclerocarya birrea*	Mpumalanga	Jami *et al.*, 2017
Neofusicoccum vitifursiforme	*Prunus persica*	Western Cape	Jami *et al.*, 2017
Neofusicoccum vitifursiforme	*Vitis vinifera*	Western Cape	Jami *et al.*, 2017
Neofusicoccum vitifursiforme	*Prunus salicina*	Western Cape	Jami *et al.*, 2017
Neofusicoccum sp. nov2	*Afrocarpus falcutus*	Gauteng	Jami *et al.*, 2017
Neofusicoccum sp. nov2	*Podocarpus henkelii*	Mpumalanga	Jami *et al.*, 2017
Oblongocollomyces variabilis	*Senegalia mellifera*	Northern Cape	Jami *et al.*, 2017
Oblongocollomyces variabilis	*Vachellia karroo*	Gauteng	Jami *et al.*, 2017

2.6.1. *Botryosphaeriaceae* on plantation trees in South Africa

The South African forestry industry relies heavily on plantations of exotic tree species including Australian *Acacia, Eucalyptus* and *Pinus* for pulp and timber (Albaugh *et al.,* 2013; Jami *et al.,* 2017). Exotic plantations were established in the country around the 1800s to meet the growing demands of timber that could not be supplied from indigenous forests (Albaugh *et al.,* 2013). More than 1.3 million hectares of the total land area in South Africa is planted with exotic plantation trees (Forestry South Africa, 2017). A forestry product report indicated that the contribution of plantation forests to South Africa's annual GDP was R38.4 billion in 2019 (Forestry South Africa, 2019).

Eucalyptus is the most widely planted hardwood genus worldwide (Albaugh *et al.,* 2013). In South Africa, about 660, 000 hectares are planted with *Eucalyptus* (https://forestry.co.za/industry-info/statistical-data/2019). *Eucalyptus* trees are often planted in areas with suboptimal growing conditions, making them more exposed to environmental stress and diseases (Albaugh *et al.,* 2013). One common group of fungi associated with diseases of *Eucalyptus* spp. worldwide is fungi belonging to the *Botryosphaeriaceae* (Pérez *et al.,* 2008; Maleme, 2009; Mohali *et al.,* 2009; Chen *et al.,* 2011; Iturritxa *et al.,* 2011). *Botryosphaeria dothidea*, *Lasiodiplodia theobromae*, *Neofusicoccum australe*, *N. kwambonambiense*, *N. eucalyptorum* and *N. parvum* are known to occur on *Eucalyptus* species in South Africa and on these hosts they are associated with various disease symptoms such as dieback, canker, blight and tree mortality (Smith *et al.,* 1994, 2001; Slippers *et al.,* 2004b; Burgess *et al.,* 2005; Jami *et al.,* 2017).

Another important forestry group of trees is the pines. In South Africa, *Pinus* plantations cover approximately 690, 000 hectares (https://forestry.co.za/industry-info/statistical-data/2019). The first commercial plantation of 30 hectares was established with *P. pinaster* at Genadendal, Western Cape in 1825 (Burgess and Wingfield, 2001). Early plantations were free of pests and diseases, but as the industry expanded, pests and diseases became a problem (Burgess and Wingfield, 2001). Among the many species of *Botryosphaeriaceae*, *D. sapinea* is one of the well-known pathogens of *Pinus* spp. Worldwide (Swart and Wingfield, 1991; Smith *et al.,* 1996; Burgess *et al.,* 2001). The endophytic nature of the fungus is thought to have facilitated its worldwide distribution during the international trade of seeds and planting stocks (Wingfield *et al.,* 2001; Burgess *et al.,* 2004; Bihon *et al.,* 2011).

Australian *Acacia* species are planted in South Africa for the production of paper, fuelwood, tannins and building materials (Gibson, 1975; Midgley and Turnbull, 2003). There are about 200, 000 hectares of Australian *Acacia* plantations in South Africa (Govender, 2007). *Acacia* *mearnsii* is the most commonly planted Australian *Acacia* species in South Africa, followed by A. *dealbata* and *A. decurrens* (Nyoka, 2003). Fungi in the *Botryosphaeriaceae* also infect *Acacia* species (Roux and Wingfield, 1997; Roux *et al.,* 1997). These include *Macrophomina phaseolina* causing root rot on *A. decurrens* (Gibson, 1975) and *B. dothidea, Aplosporella* spp., *Diplodia* spp. and *L. theobromae* causing stem canker, tip dieback and pith discoloration on *A. mearnsii* in the Eastern Cape, KwaZulu-natal, Mpumalanga and Western cape provinces (Gibson, 1975; Roux and Wingfield, 1997; Roux *et al.,* 1997).

2.6.2. *Botryosphaeriaceae* on fruit trees in South Africa

Diseases of economically important fruit trees such as *Mangifera indica* (mango)*, Vitis vinifera* (grape) and *Malus* spp. (apple) have been studied in South Africa (van Niekerk *et al.,* 2004; Damm *et al.,* 2007; Slippers *et al.,* 2007; Mehl *et al.,* 2017b). Approximately 80 and 90% production of grapes and apples in South Africa occurs in the Western Cape Province, while mango production occurs in the Limpopo and Mpumalanga provinces.

Mango is the most prevalent fruit tree in Limpopo and Mpumalanga. However, one of the most commonly reported limiting factors in mango production is diseases associated with members of the *Botryosphaeriaceae* (de Oliveira Costa *et al.,* 2010; Sakalidis *et al.,* 2011d; Ismail *et al.,* 2012; Trakunyingcharoen *et al.,* 2014). Trakunyingcharoen *et al.* (2014) identified *L. theobromae* and *N. parvum* for the first time in South Africa. Mehl *et al.* (2017b) discovered further diversity of *Botryosphaeriacea with species such as B. fabicerciana, L. mahajangana, L. pseudotheobromae, L. theobromae, N. austral, N. kwambonambiense, N. mediterraneum, N. parvum* and *N. umdonicala.* These species are reported on *M. indica* globally, with *N. parvum*, *N. mediterraneum* and *L. theobromae* being the abundant species (de Oliveira Costa *et al.,* 2010; Sakalidis *et al.,* 2011a; Ismail *et al.,* 2012; Trakunyingcharoen *et al.,* 2014).

Grapevine dieback commonly referred to as ‘‘*Botryosphaeria* dieback’’ is a common disease in vineyards globally (Billones-Baaijens and Savocchia, 2019; Rangel-Montoya *et al.,* 2021). At least 26 species of *Botryosphaeriaceae* belonging to the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Fusicoccum*, *Guignardia*, *Lasiodiplodia*, *Neofusicoccum* and *Phaeobotryosphaeria* are associated with dieback of grapevines worldwide (Gramaje *et al.,* 2018; Billones-Baaijens and Savocchia, 2019; Rangel-Montoya *et al.,* 2021). *Botrysphaeria dothidea*, *L. plurivora*, *L. theobromae*, *N. viticlavatum, N. vitifursiforme, N. australe* and *N. luteum* have been reported as pathogens of grapevines (Crous *et al.,* 2000; van Niekerk *et al.,* 2004).

In South Africa, stone and pome fruit trees are often planted next to vineyards. This allows *Botryosphaeriaceae* inoculum to move between vineyards and orchards. Species of

Botryosphaeriaceae have also been reported as pathogens of pome and stone fruit trees in South Africa (Damm *et al.,* 2007; Slippers *et al.,* 2007). *Botryosphaeria dothidea, D. africana*, *D. seriata*, *D. mutila*, *D. viticola*, *L. plurivora*, *L. theobromae*, *N. vitifusiforme*, *N. ribis*, *N. parvum* and *N. austral* infect apple, pear and plim in the Western Cape (Damm *et al.,* 2007; Slippers *et al.,* 2007). On these trees, *D. seriata* and *N. australe* are the most commonly reported species (Jami *et al.,* 2017).

2.6.3. *Botryosphaeriaceae* on native trees in South Africa

Knowledge about the diversity of fungi, including those in *Botryosphaeriaceae,* causing diseases on native trees in the country has only received attention in recent years. For example, studies conducted on native trees revealed diverse *Botryosphaeriaceae* in natural ecosystems (Pavlic *et al.,* 2007; Pillay *et al.,* 2013; Jami *et al.,* 2014, 2017; Mehl *et al.,* 2017b). A total of 27 species have been described as new taxa on these trees (Jami *et al.,* 2017). The results from these studies form a basis to intensify research focusing on fungal disease agents on native tree species.

Studies have been conducted on native *Myrtaceae*, in particular S*. cordatum,* to identify fungi associated with this tree species (Pavlic *et al.,* 2007; Pillay *et al.,* 2013). *Syzygium cordatum* is widespread in the eastern and northeastern parts of South Africa and is commonly found close to *Eucalyptus* plantations. Being in the same family as *Eucalyptus, S. cordatum* provides a model to study species overlap of fungi in the *Botryosphaeriaceae* on native and introduced hosts*.* Pavlic *et al.* (2007) conducted a study throughout the distribution ranges of *S. cordatum* in South Africa and identified diverse species of *Botryosphaeriaceae,* including *B. dothidea, L. theobromae, L. gonubiensis, N. ribis*, *N. kwambonambiense*, *N. australe*, *N. mangiferae*, *N. parvum* and *N. luteum*. Some of the species identified on *S. cordatum*, including *N. australe*, *N. parvum* and *N. luteum* are known as pathogens of *Eucalyptus* across the world suggesting cross-infection of indigenous and introduced *Myrtaceae* by fungi in the *Botryosphaeriaceae* (Slippers *et al.,* 2004b; Burgess *et al.,* 2005; Mohali *et al.,* 2007; Pérez *et al.,* 2008).

Given the ability of *Botryosphaeriaceae* species to cross-infect native and introduced hosts, Pillay *et al.* (2013) considered species overlap of *Botryosphaeriaceae* between *Eucalyptus* and *S. cordatum*. *Neofusicoccum eucalyptorum, N. kwambonambiense* and *N. parvum* were found overlapping on the two hosts. Indeed, Pillay *et al.* (2013) recorded the first report of *N. eucalyptorum* on *S. cordatum* in South Africa. *Neofusicoccum eucalyptorum* was described on *E. grandis* and *E. nitens* (Smith *et al.,* 2001). *Neofusicoccum eucalyptorum* has been reported from other tree species of *Myrtaceae* including *Blepharocalyx salicifolius* and *Myreceugenia glaucescens.* Due to the widespread occurrence of *N. eucalyptorum* on

Eucalyptus species worldwide, Slippers *et al.* (2004b) assumed that the fungus may be endemic to Australia, but it can infect other members of the *Myrtaceae* and can easily move between indigenous and introduced *Myrtaceae* once introduced into new areas. Pérez *et al.* (2008) studied species diversity of the *Botryosphaeriaceae* on a native *Myrtaceae* spp. and *Eucalyptus* in Uruguay and found *B. dothidea*, *N. parvum* and *N. ribis* overlapping on native and non-native *Myrtaceae*.

Native *Vachellia* spp. (previously known as *Acacia*) have a significant ecological and economic impact in southern Africa. These trees are extensively used by local communities for firewood, stock feed, medical remedies and building materials (van der Walt, 2008). Studies on *Vachellia* species in different parts of the country have indicated a great diversity of the *Botryosphaeriaceae* species on these trees. *Vachellia karroo* hosts the highest diversity of *Botryosphaeriaceae* in South Africa, with 18 species, including *B. dothidea, Diplodia allocellula, D. pseudoseriata, Dothiorella brevicollis, Do. dulcispinae, Do. pretoriensis, Do. rosulata, Do. viticola, Eutiarosporella urbis-rosarum, L. pseudotheobromae, L. gonubiensis, L. theobromae, N. pretorium, N. parvum, N. australe, N. vitisifursiforme, N. kwambonambiense* and *Oblongocollomyces variabilis* identified on this host (Jami *et al.,* 2017). Of these, 13 species were described as new to this host (Jami *et al.,* 2017).

Other species identified on native hosts include *L. mahajangana* and *L. theobromae* on *E. ingens* (van der Linde *et al.,* 2011), *L. pseudotheobromae, L. theobromae* and *N. parvum* on *Terminalia catappa* (Begoude *et al.,* 2010), *D. viticola* and *N. parvum* on *Gymnosporia buxifolia, Aplosporella javeedii*, *Eutiarosporella africana* and *N. kwambonambiense* on *Celtis africana* and *A. javeedii* on *S. lancea* (Jami *et al.,* 2014)*, L. theobromae, L. pseudotheobromae, L. crassispora, Diplodia alatafructa* and *Fusicoccum atrovirens* on *P. angolensis* (Mehl *et al.,* 2011)*, B. dothidea*, *N. eucalypti* and *N. kwambonambiense* on *P. hankelii, P. elongates* and *P. latifolius*. (Ndove, 2015), *Botryosphaeria farbicerciana, D. allocellula, L. crassispora, L. gonubiensis, L. mahajangana, L. theobromae, N. mediterraneum, N. parvum, N. ribis* and *N. vitisifusiforme* on *Sclerocarya birrea* (Mehl *et al.,* 2017b), *D. estuarine*, *L. avicenniae*, *L. bruguierae, L. gonubiensis, L. theobromae, N. crypto-australe*, *N. luteum*, *N. mangroviorum*, *N. parvum, N. lumnitzerae, N. kwambonambiense* and *N. ribis* on mangrove species including *A. marina, B. gymnorrhiza, B. racemose, C. tagal, R. mucronata* and *L. racemosa* (Osorio *et al.,* 2017) and *N. variabile* and *N. mangroviorum* on *Mimusops caffra* (Jami *et al.,* 2018).

The broad host range of some species of *Botryosphaeriaceae* suggest that there is no barrier to disease transmission between native and non-native plant species or cultivated/urban and natural settings (Begoude *et al.,* 2012; marsberg *et al.,* 2016; Lauret *et al.,* 2020). The ability to infect different species may explain the invasive success of fungi in the *Botryosphaeriaceae*, enabling them to spread in heterogenous landscapes. A previous study by Fort *et al.* (2016) investigated fungal communities within vineyards and adjacent forest. The results from the study suggested that the major driver of differentiation between vineyards and forest patches was not dispersal, but rather selective pressures from host plants, microclimate and management practices. Biogeographic distribution of species in the *Botryosphaeriaceae* is still not well known. It is important to understand factors that shape *Botryosphaeriaceae* communities at a landscape level.

2.7. Molecular tools for species identification of *Botryosphaeriaceae*

The taxonomy of *Botryosphaeriaceae* has long been challenging because most species are cosmopolitan with a broad host range, as well as overlapping morphological characteristics between species (Crous *et al.,* 2006; Alves *et al.,* 2007; Phillips *et al.,* 2013; Dissanayake *et al.,* 2016; Cruywagen *et al.,* 2017; Yang *et al.,* 2017). The availability of DNA sequence data has significantly increased our understanding and ability to identify living organisms, including fungi belonging to *Botryosphaeriaceae* (Phillips *et al.,* 2013; Slippers *et al.,* 2017). The first DNA sequence data for this group were generated about 30 years ago (Jacobs and Rehner, 1988). To date, GenBank lists 28 genome assemblies for species of *Botryosphaeriaceae* (https://www.ncbi.nlm.nih.gov/genome, 2022).

Traditionally, fungi in the *Botryosphaeriaceae* were distinguished using morphological characteristics such as conidial size, colour, shape and septation, a criterion based on the Morphological Species Recognition (MSR) concept (Denman *et al.,* 2000; Phillips *et al.,* 2002; Crous *et al.,* 2006). The drawback of using MSR for this group of fungi is that *Botryosphaeriaceae* species are characterised by overlapping morphological features and MSR provides no measure of how much difference in morphological characters is significant in distinguishing organisms as separate species (Taylor *et al.,* 2000; Chethana *et al.,* 2016). Therefore, MSR alone is considered inadequate to define species and genera in the family *Botryosphaeriaceae* (Denman *et al.,* 2000; Phillips *et al.,* 2013; Slippers *et al.,* 2014).

It is assumed that species described based on morphological characters alone often include at least two or more cryptic species (Brasier, 1987; Taylor *et al.,* 2000). The *N. parvum* / *N. ribis* species complex thought to represent one species has been shown to represent more than 10 cryptic species (Pavlic *et al.,* 2009; Sakalidis *et al.,* 2011c; Slippers *et al.,* 2017). While some recent studies on the *Botryosphaeriaceae* claim to have applied a combination of morphological and molecular characters to identify species, a closer examination indicates that they rely on DNA sequence data to distinguish species and it appears that morphological characterisation is only added to comply with the regulations outlined by the International Code of Nomenclature for algae, fungi and plants (ICN) or expectations in the scientific community (Taylor *et al.,* 2000; Slippers *et al.,* 2014).

The Biological Species Recognition (BSR) concept defines species as groups of natural interbreeding populations that are reproductively separated from one another (Mayr, 1940; Taylor *et al.,* 2000). Thus, members of the same species can interbreed and produce fit-viable progeny (Mayr, 1940; Taylor *et al.,* 2000). However, there are reports that different fungal species can effectively cross and hybridise (Newcombe *et al.,* 2000; Joly *et al.,* 2006; De vos *et al.,* 2011; Cruywagen *et al.,* 2017). For example, Cruywagen *et al.* (2017), revealed the ability of *Lasiodiplodia* species to hybridise and form viable species. Hybridisation has also been reported in other fungal species, including *Cronartium*, *Fusarium* and *Melampsora* (Newcombe *et al.,* 2000; Joly *et al.,* 2006; De Vos *et al.,* 2011). Through backcrosses, hybrid species transfer novel genes to the parent population and eventually disappear from the population, while some hybrids become established in the environment (Brasier, 2001). The criterion of reproduction would define allopatric fungal populations that are genetically separated, but retain the interbreeding ancestral character as the same biological species (Taylor *et al.,* 2000).

The Phylogenetic Species Recognition (PSR) concept is currently the most widely used method of species identification for *Botryosphaeriaceae* (Phillips *et al.,* 2013; Slippers *et al.,* 2017; Cruywagen *et al.,* 2017). The PSR concept can effectively identify fungal species with similar morphological characteristics, but belonging to phylogenetically distinct lineages (Mayden, 1997; Taylor *et al.,* 2000; Stewart *et al.,* 2014). The phylogenetic analyses of DNA sequence data have elucidated the taxonomic confusion in the family, leading to the description of new genera, cryptic species and hybrids that could not be distinguished using morphological and biological characters (Alves *et al.,* 2008; Sakalidis *et al.,* 2011c; Cruywagen *et al.,* 2017). While in most cases the PSR approach provides confidence for the distinction of new genera, the approach has led to the reduction of some genera and species, such as *Dothiorella* and *Spencermartinsia* (Yang *et al.,* 2017). Also, PSR has some challenges, especially when only one genomic locus is used as some of the markers used in the systematics of species in the *Botryosphaeriaceae* (for example, the internal transcribed spacer and the beta-tubulin gene regions) have been thought to under estimate the magnitude of differentiation (Gazis *et al.,* 2011; Cruywagen *et al.,* 2017; Zhang *et al.,* 2021). Therefore, it is, recommended to consider at least two or three gene regions to identify species of *Botryosphaeriaceae* using PSR (Pavlic *et al.,* 2009; Liu *et al.,* 2012; Cruywagen *et al.,* 2017).

Phylogenetic relationships at family and genus level are described based on DNA sequence data from the large and small subunits, while the internal transcribed spacer (ITS) gene region is used to characterise intraspecific and interspecific relationships between species of *Botryosphaeriaceae* (Phillips *et al.,* 2013; Yang *et al.,* 2017). However, the ITS gene region alone is insufficient in distinguishing cryptic species of *Botryosphaeriaceae* (Slippers *et al.,* 2004a; Dissanayake *et al.,* 2016; Yang *et al.,* 2017). Pavlic *et al.* (2004, 2007) showed that the ITS gene region alone could not distinguish cryptic species in the *N. parvum* / *N. ribis* complex. Comparisons of the ITS sequence data with at least two gene regions including the translation elongation factor (*tef-1α*), RNA polymerase II (*rpb2*), a portion of the β-tubulin (*β-tub*) and to a lesser extent calmodulin (*cal*) have been used to provide sufficient phylogenetic signal in species separation of *Botryosphaeriaceae* (Phillips *et al.,* 2013; Cruywagen *et al.,* 2017; Slippers *et al.,* 2017; Yang *et al.,* 2017).

Multiple gene sequences allow for further development of the PSR into a combined phylogenetic species recognition analyses known as the GCPSR (genealogical concordance phylogenetic species recognition) criterion (Avise and Ball, 1990; Taylor *et al.,* 2000). The GCPSR criterion is based on the idea that recombination within lineage will create conflict between gene trees, with the transition from conflict to congruence representing the species limit (Taylor *et al.,* 2000). The GCPSR approach provided confidence in the delineation of *Botryosphaeriaceae* species (Phillips *et al.,* 2013; Dissanayake *et al.,* 2016; Yang *et al.,* 2017). Studies using the GCPSR criterion have revealed numerous cryptic and hybrid species in taxa that were previously treated as single species (Pavlic *et al.,* 2009; Phillips *et al.,* 2013; Cruywagen *et al.,* 2017; Yang *et al.,* 2017).

The first study to apply the GCPSR criterion on species of *Botryosphaeriaceae* was conducted by de Wet *et al.* (2003) using protein-coding genes to study relationships between *D. sapinea* morphotypes (A, B, C and I). The four morphotypes were described from *D. sapinea* based on morphological characteristics of the conidia (de Wet *et al.,* 2003). Phylogenetic genealogies generated from the study showed that isolates of the B morphotype represented a distinct species now known as *D. scrobiculata*, while the A and C morphotypes are part of the *D. sapinea* and the I morphotype represented *Botryosphaeria obtusa,* confirming previous results that the I morphotype represents a sexual state of *Diplodia seriata* (Burgess *et al.,* 2003).

GCPSR discovered cryptic and hybrid species in the *L. theobromae* and *N. parvum* / *N. ribis* species complex (Pavlic *et al.,* 2009; Phillips *et al.,* 2013; Cruywagen *et al.,* 2017). These species can only be identified successfully through careful analyses of multiple gene sequences (Phillips *et al.,* 2013; Cruywagen *et al.,* 2017). Two of the hybrid species identified by Cruywagen *et al.* (2017) were described as new, based on analyses of the ITS and *tef-1α* sequence data alone. This indicates that the interpretation of phylogenetic results from the two gene regions must be made with extreme caution and that in cases where species are thought to be new, analyses of at least one or two more robust gene regions must be considered (Cruywagen *et al.,* 2017).

2.8. Population genetics studies of *Botryosphaeriaceae*

Population genetics studies play an important role in understanding the evolutionary biology and genetic compositions of fungal plant pathogens (Bihon *et al.,* 2011, 2012; Pavlic *et al.,* 2015; Nagel *et al.,* 2020). Population genetics studies have been made easier by the use of highly polymorphic molecular markers (Burgess *et al.,* 2003; Begoude *et al.,* 2012; Pavlic *et al.,* 2015; Nagel *et al.,* 2020). DNA-based molecular markers are widely used in studies investigating gene flow, reproductive mode and population genetic structure of many fungal pathogens (Capote *et al.,* 2012). Popular genetic markers used in fungal population studies include random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), simple sequence repeats (SSRs), single nucleotide polymorphisms (SNPs) markers and genome sequences. SSRs are the most commonly used molecular markers in population genetics studies of the *Botryosphaeriaceae*.

Simple sequence repeat (SSR) markers, commonly known as microsatellites markers, are a class of co-dominant molecular markers that consist of short tandemly repeated nucleotide motifs rich in polymorphism (Mohali *et al.,* 2005; Richard *et al.,* 2008). Microsatellites are located in both coding and non-coding regions and they rapidly accumulate mutations over generations giving rise to genetic variability within species (Capote *et al.,* 2012; Nagel *et al.,* 2020). Due to the high mutation rate of microsatellites, they are among the most informative molecular markers in population genetics studies (Groppe *et al.,* 1995; Collevatti *et al.,* 2001; Zhou *et al.,* 2001; Nagel *et al.,* 2020).

Despite being effective population genetics tools, the development of microsatellite markers is expensive and time-consuming requiring sequencing and analyses of SSR-containing regions, as well as designing and testing primers. In a recent study, Nagel *et al.* (2020) designed cross-species transferable markers for *Neofusicoccum* and *Lasiodiplodia* species. These markers were mined from available genomes and are highly transferable between multiple species in a genus. Thus, they can be used to study populations of multiple species within the two genera, including those for which the genomes have not been sequenced yet. These cross-species transferable markers will significantly reduce the amount of work and costs for the development of species-specific markers (Nagel *et al.,* 2020).

Microsatellite markers have been widely applied in population genetics studies of *Botryosphaeriaceae* worldwide (Burgess *et al.,* 2004; Bihon *et al.,* 2012; Sarr *et al.,* 2014; Zlatković *et al.,* 2019). For example, microsatellite markers were used to assess population differentiation, gene and genotypic diversity of the globally distributed pathogen of pine trees, *D. sapinea* (Burgess *et al.,* 2004; Bihon *et al.,* 2012). Burgess *et al.* (2004) studied the genetic diversity of *D. sapinea* populations from America, Australia and South Africa, and observed higher genetic diversity in an introduced South African population than in the native American population. A previous study by Bihon *et al.* (2012) revealed high genetic diversity in the South African *D. sapinea* population when compared to other non-native populations in Argentina, Australia and Ethiopia. The results from previous studies suggest that the high genetic diversity in the South African population of *D. sapinea* might be due to multiple introductions of the fungus into the country through seeds and planting material (Burgess *et al.,* 2004; Bihon *et al.,* 2012).

Single nucleotide polymorphisms (SNPs) are also used in population genetics studies. They are the most abundant form of variation in DNA sequences amongst individuals in a population (Guichoux *et al.,* 2011; DeFaveri *et al.,* 2013). Most studies using SNPs markers have focused on model organisms and relatively little is known regarding the distribution and patterns of SNPs in non-model organisms, including *Botryosphaeriaceae.* Another limitation of using SNPs is that it requires a large number of isolates for the identification of fixed single nucleotide polymorphisms and whole genomes of many individuals within a species. The increasing availability of complete genome sequences for the *Botryosphaeriaceae*, coupled with the ability to genotype thousands of single-nucleotide polymorphisms, makes SNPs a less desirable tool for future population genetics studies (Guichoux *et al.,* 2011; DeFaveri *et al.,* 2013).

Population genomics is viewed as an extension of population genetics that alters and expands the types of questions that can be addressed (Grünwald *et al.,* 2016). Recent technological advances in both high-throughput sequencing and computational tools have made it possible to sequence and analyze whole genomes of many plant pathogens including the *Botryosphaeriaceae* (Marsberg *et al.,* 2017; Wang *et al.,* 2018; Nagel *et al.,* 2020; Yu *et al.,* 2022). Although genomes of some species of *Botryosphaeriaceae* have not been analysed, studies using available genome sequences are beginning to bring insight into the interaction of species of *Botryosphaeriaceae* with their hosts.

Genome analyses have elucidated the biology of *Botryosphaeriaceae* (Bihon *et al.,* 2014; van der Nest *et al.,* 2014; Lopes *et al.,* 2017; Nagel *et al.,* 2018). Nagel *et al.* (2018) assessed the mating strategies of various species of *Botryosphaeriaceae* by comparing the size, position and distance between the mating genes and reported that *B. dothidea*, which is known to have a sexual state is homothallic, while *D. sapinea* has an active heterothallic reproductive system, although a sexual state has not been encountered for this fungus. *Lasiodiplodia gonubiensis* was shown to be homothallic (Nagel *et al.,* 2018). It is clear from these studies that genomes provide a powerful tool to study the biology of species in the *Botryosphaeriaceae*. Our understanding of these fungi will no doubt be transformed as more whole genomes for closely related cryptic and hybrid species become available.

2.9. Conclusions

This review highlights the broad host range and cosmopolitan distribution of the *Botryosphaeriaceae* in South Africa. These fungi occur on both native and introduced tree species in plantations, orchards and natural ecosystems. Much of the local diversity of *Botryosphaeriaceae* occurs on native hosts rather than non-native hosts. The number of species identified and described as new will continue to increase as new hosts and areas are sampled. While we understand that the endophytic nature of these fungi allows them to be moved around the world because they cannot be detected during quarantine inspection, a genomic study revealed that these fungi have a unique interaction with their hosts and do not trigger host defense responses, deceiving the host into allowing them to infect, but later become pathogenic when the host is subjected to stress. As our understanding of these fungi increases and more tools become available to identify them, it is also time for quarantine routines to use robust tools to identify these organisms in their latent phase.

Advances in DNA-based phylogenetic inference of multiple loci have made it possible for researchers to revise the taxonomy of *Botryosphaeriaceae*, recognize species complexes and identify hybrids that could not be identified based on biological and morphological characters Taxonomic resolution of the *Botryosphaeriaceae* has significantly influenced our understanding of this important group of fungi. The use of microsatellite markers to characterise the population dynamics of species in the *Botryosphaeriaceae* has yielded significant insights into the genetic diversity of these fungi. Population genetics studies that analysed multi-locus genotypes of species in the *Botryosphaeriaceae* have indicated that some populations are clonal, while others are outcrossing even though sexual structures are not known. Molecular markers have also become useful in confirming the separation of cryptic species by supporting the lack of gene flow between distinct species that were separated using DNA sequences. The development of cross-species microsatellite markers will reduce both the amount of time and the costs required for the development of species-specific markers, allowing population genetics studies for species whose genome sequences may not be available for some time.

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CHAPTER 3

Diversity of endophytes in the *Botryosphaeriaceae* differs on *Anacardiaceae* in disturbed and undisturbed ecosystems in South Africa

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Abstract

Botryosphaeriaceae represent a diverse family of fungi with a cosmopolitan distribution and a wide host range. We studied species diversity and overlap of *Botryosphaeriaceae* on selected tree species of *Anacardiaceae* in disturbed (farming or forestry) and undisturbed (isolated and/or protected) ecosystems in the Limpopo and Mpumalanga provinces, South Africa. The disturbed sites were at Tshikundamalema and Tshipise in Limpopo and the undisturbed sites at Nwanedi and the Mapungubwe National Park in Limpopo and the Kruger National Park in Mpumalanga. Asymptomatic branches were collected from *Mangifera indica*, *Sclerocarya birrea* and *Lannea schweinfurthii* trees in 2017 and 2018. Eleven species, including *Dothiorella dulcispinae, Dothiorella viticola, Dothiorella brevicollis, Diplodia allocellula, Lasiodiplodia exigua, Lasiodiplodia crassispora, Lasiodiplodia gonubiensis, Lasiodiplodia mahajangana, Neofusicoccum parvum,* a new species described as *Oblongocollomyces ednahkunjekuae* and an un described species referred to as *Oblongocollomyces* sp. 1 were identified using a multi-gene sequencing approach. Ten of the 11 species were identified in undisturbed ecosystems (eight species being unique), while only three species were identified in disturbed ecosystems (one species being unique). Two species were generalists on trees in disturbed and undisturbed ecosystems. *Lasiodiplodia mahajangana* was the most dominant species as it occurred on the three tree species of *Anacardiaceae.* Isolates of *N. parvum* occurred on both *S. birrea* (a native species) and *M. indica* (a non-native species) trees that occurred adjacent to each other in disturbed ecosystems, confirming the ability of this invasive pathogen to cross-infect native and non-native hosts and its abundance in human-disturbed environments. The findings from this study confirm the lack of host specificity for most species of *Botryosphaeriaceae*. The results also indicate that disturbance through human activities such as clear-cutting, selective cutting and land-use changes, negatively influences the diversity of fungal species in the *Botryosphaeriaceae*.

Keywords: endophyte, fungal tree pathogen, tree health, invasive pathogen

3.1. Introduction

Fungi in the *Botryosphaeriaceae* infect a wide range of monocotyledonous, dicotyledonous and gymnospermous hosts (Jami *et al.,* 2017). Species of *Botryosphaeriaceae* are common on a variety of plants in South Africa, including commercial fruit trees (van Niekerk *et al.,* 2004; Damm *et al.,* 2007; Slippers *et al.,* 2007), plantation trees (Smith *et al.,* 1996; Slippers *et al.,* 2009) and native trees (Pavlic *et al.,* 2007; van der Linde *et al.,* 2011; Jami *et al.,* 2014). The ability of species in the *Botryosphaeriaceae* to infect multiple hosts facilitates their spread and establishment in new areas, increasing their threat as potential pathogens of woody trees globally (Slippers and Wingfield, 2007; Mehl *et al.,* 2013; Burgess *et al.,* 2019). Once introduced into a new environment, members of the *Botryosphaeriaceae* infect related and unrelated hosts (Slippers and Wingfield, 2007; Pillay *et al.,* 2013; Jami *et al.,* 2014).

In South Africa, *Botryosphaeriaceae* species have been found overlapping on related tree species of *Anacardiaceae* and *Myrtaceae* (Pavlic *et al.,* 2007; Pillay *et al.,* 2013; Mehl *et al.,* 2017a) and unrelated hosts in the *Cannabaceae, Celastraceae* and *Fabaceae* (Jami *et al.,* 2014). Several species of *Botryosphaeriaceae*, including *Botryosphaeria dothidea, Lasiodiplodia theobromae, L. mahajangana, L. pseudotheobromae, Neofusicoccom kwambonambiense, N. vitifusiforme, N. parvum* and *N. luteum* occur on indigenous and introduced hosts such as *Eucalyptus, Mangifera indica, Malus*, *Pinus*, *Prunus, Vachellia karroo, Sclerocarya birrea*, *Vitis vinifera* and *Syzygium cordatum* (Jami *et al.,* 2017).

It is likely that some prominent species that are commonly recorded on trees in agricultural and forestry ecosystems are also invaders of trees in natural stands. This is expected for species such as *B. dothidea*, *D. sapinea*, *L. theobromae* and *N. parvum* that have been reported on various hosts globally (Bihon *et al.,* 2011; Yan *et al.,* 2013; Sakalidis *et al.,* 2013; Marsberg *et al.,* 2017; Mehl *et al.,* 2017b; Burgess *et al.,* 2019). The broad host distribution of these species also indicates their ability to grow under varying climatic conditions (Yan *et al.,* 2013; Jami *et al.,* 2017; Burgess *et al.,* 2019).

Some species, however, appear to have a narrow host range and are known from a single host or region (Jami *et al.,* 2017). Species that are not commonly reported in South Africa include *Botryosphaeria atrovirens, Diplodia africana, D. scrobiculata, Dothiorella brevicollis, Do. capri-amissi, Do. pretoriensis, Eutiarosporella urbis-rosarum, E. africana, E. graminis, E. tritici, Lasiodiplodia avicenniae, L. bruguierae, L. euphorbicola* and *Neofusicoccum viticlavatum* (Jami *et al.,* 2017). Their limited host record might not necessarily reflect the inability to infect other plants. Therefore, it is not clear whether the narrow host range that has been observed for some species can be attributed to host specificity or the sampling strategies that were followed during sample collection.

To understand the diversity of *Botryosphaeriaceae* in a region, these fungi must be isolated from a variety of woody plants growing across diverse ecosystems. While the *Botryosphaeriaceae* have been intensively studied in South Africa, they have not been widely investigated in undisturbed ecosystems. This is because most of the work on *Botryosphaeriaceae* has focused on commercially important trees in human-disturbed ecosystems such as plantations, orchards and cities (Smith *et al.,* 1996; van Niekerk *et al.,* 2004; Slippers *et al.,* 2007). Where studies have been conducted on native trees in South Africa, they have revealed a great diversity of these fungi on various native hosts such as *Vachellia* spp., *Euphorbia ingens, Pterocarpus angolensis*, *S. birrea*, mangrove species and *S. cordatum* (Jami *et al.,* 2017).

Fungi in the *Botryosphaeriaceae* have been identified on trees of *Anacardiaceae* in South Africa. Currently, 11 species are known to occur on *S. birrea* (Mehl *et al.,* 2017a), 10 on *M. indica* (Trakunyingcharoen *et al.,* 2014; Mehl *et al.,* 2017a) and one on *Searsia lancea* (Jami *et al.,* 2014). The family *Anacardiaceae* includes more than 800 species worldwide and several genera in the family including *Anacardium*, *Pistacia*, *Mangifera* and *Sclerocarya* are economically important (Christenhusz and Byng, 2016). *Anacardium occidentale* and *S. birrea* are highly prized for their use in traditional medicine in rural parts of Africa (Kudi *et al.,* 1999; Shackleton *et al.,* 2002).

In this study, we contribute to a larger body of work considering the diversity of the *Botryosphaeriaceae* on indigenous and introduced trees in South Africa, and the role of agricultural and forestry-related disturbance in the distribution of species. Specifically, the study determined species diversity and overlap of *Botryosphaeriaceae* on three species, including native (*Sclerocarya birrea* and *Lannea schweinfurthii*) and non-native (*Mangifera indica*) *Anacardiaceae* in disturbed (agricultural and developed areas) and undisturbed (isolated and undeveloped or protected areas) ecosystems.

3.2. Materials and Methods

3.2.1. Sampling sites, tree health assessment and sample collection

Asymptomatic branches (20 – 30 cm long and 2 – 5 mm thick) were collected from three tree hosts, namely *S. birrea, M. indica* and *L. schweinfurthii*, in five locations that included disturbed (developed and intensively farmed) and undisturbed or low disturbance (isolated and undeveloped, and/or protected parks) sites.

The disturbed sites were Tshikundamalema (Latitude 22° 40′17.87 S, Longitude 30° 41′27.29 E) and Tshipise (Latitude 22° 36′15.59 S, Longitude 30° 09′59.08 E), which are approximately 90 km apart. The land is used for agricultural purposes for the cultivation of maize, groundnuts, vegetables, as well as for livestock production. Sample collections in Tshikundamalema were made from 49 *S. birrea* and 54 *M. indica* trees that were approximately 25 m from each other. In Tshipise, samples were collected from 30 *M. indica* and 20 *S. birrea* trees.

The first undisturbed or low disturbance site was Nwanedi (Latitude 22° 32′02.40 S, Longitude 30° 40′12.00 E). Nwanedi is situated between Tshikundamalema and Tshipise. It is a remote area with little to no human activity. The area sampled had *L. schweinfurthii* trees only. Samples were collected from 30 randomly selected trees that were approximately 25 m from each other.

The second undisturbed or low disturbance site was in the Mapungubwe National Park (Latitude 22° 13′19.37 S, Longitude 29° 20′50.17 E). Samples were collected from 54 *S. birrea* trees at the eastern and western sides of the park. Of these, 12 trees were in a residential yard inside the park and watered twice a week. Samples from these trees were recorded for comparison of fungal species occurring on *S. birrea* trees in the same environment, but with different moisture levels.

The third undisturbed or low disturbance site was in the Kruger National Park (Latitude 24° 59′47.21 S, Longitude 31° 35′30.79 E). Samples were collected from 68 *S. birrea* and 99 *L. schweinfurthii* trees around the Skukuza research camp. While the camp itself has human activity, the surrounding area is natural and not affected by human activities. To get a good representation of the area, samples were collected from trees in 11 different sections in the east, north, south and west of Skukuza. From each section, a minimum of 10 to a maximum of 30 trees were sampled. Five sections had *S. birrea* and *L. schweinfurthii* trees growing close to each other, three sections comprised *S. birrea* trees only and the other three sections comprised *L. schweinfurthii* trees.

In addition to collecting asymptomatic branches, the trees from which we sampled were scored for general health on a scale of 0 – 4; where 0 = healthy, 1 = <25% dieback branches, 2 = 25 – 50% dieback branches, 3 = >50% dieback branches and 4 = appearing to be dying or nearly dead, but still with some asymptomatic shoots. Samples were collected and transported to the laboratory for fungal isolations. Data from tree health assessment was analysed statistically using the Chi-square (χ^2) test to assess the goodness of fit between health status, tree species and site using SPSS v.24 [IBM, Armonk, New York, USA].

3.2.2. Fungal isolations

Samples were surface disinfected by immersing ~ 10 cm of the branch in 10% hydrogen peroxide for 2 min, followed by rinsing two times in sterile distilled water for 1 min. Subsequently, 12 discs (0.5 cm) were cut from each branch and plated onto 2% MEA (2% malt extract and 1.5% agar, Biolab, Midrand, South Africa) with Streptomycin to inhibit the growth of bacteria. Primary isolates were incubated at 25 °C and monitored regularly. Single hyphal tips from developing colonies resembling species of *Botryosphaeriaceae* were transferred onto clean 2 % MEA to purify cultures.

Isolates were grouped based on culture morphology and colour characteristics using the colour chart by Rayner (1970). Isolates were grouped into 11 cultural morphogroups, from which 2 to 10 isolates representing the different tree species and sites were selected. In total, 78 isolates were selected for preliminary ITS identification. Of these, 52 isolates were obtained from *S. birrea* trees sampled at Tshikundamalema, Tshipise, Mapungubwe and Kruger National Park, 16 from *L. schweinfurthii* trees at Nwanedi and Kruger National Park and 10 from *M. indica* trees at Tshipise and Tshikundamalema. Isolates used in this study (Table 3.1) are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Table 3.1. Representative fungal isolates obtained from tree species of *Anacardiaceae* in this study and used in the phylogenetic analyses.

Isolate No.	**Identity**	**Host**	**Site**	**GenBank accession numbers**			
				ITS	***tef-1α***	***β-tub***	***rpb2***
CMW54349	*Diplodia allocellula*	*Lannea schweinfurthii*	Nwanedi	OL441853	OL441909	OL441965	OM585579
CMW54351	*D. allocellula*	*Sclerocarya birrea*	Kruger	OL441855	OL441911	OL441967	OM585581
CMW54353	*D. allocellula*	*Lannea schweinfurthii*	Kruger	OL441857	OL441913	OL441969	OM585583
CMW57629	*Dothiorella brevicollis*	*Lannea schweinfurthii*	Nwanedi	OL441858	OL441914	OL441970	-
CMW57630	*Do. brevicollis*	*Sclerocarya birrea*	Mapungubwe	OL441859	OL441915	OL441971	-
CMW57462	*Do. dulcispinae*	*Sclerocarya birrea*	Kruger	OL441861	OL441917	OL441973	-
CMW57466	*Do. dulcispinae*	*Sclerocarya birrea*	Mapungubwe	OL441862	OL441918	OL441974	-
CMW57463	*Do. viticola*	*Sclerocarya birrea*	Mapungubwe	OL441863	OL441919	OL441975	-
CMW57464	*Do. viticola*	*Sclerocarya birrea*	Mapungubwe	OL441864	OL441920	OL441976	-
CMW54318	*Lasiodiplodia crassispora*	*Lannea schweinfurthii*	Kruger	OL441866	OL441922	OL441978	OL442021

Table 3.1. (Continued)

Isolate No.	Identity	Host	Site	GenBank accession numbers			
				ITS	*tef-1α*	*β-tub*	*rpb2*
CMW54319	*L. crassispora*	*Sclerocarya birrea*	Tshikundamalema	OL441867	OL441923	OL441979	OL442022
CMW54320	*L. crassispora*	*Sclerocarya birrea*	Mapungubwe	OL441868	OL441924	OL441980	OL442023
CMW54321	*L. crassispora*	*Mangifera indica*	Tshipise	OL441869	OL441925	OL441981	OL442024
CMW57579	*L. crassispora*	*Sclerocarya birrea*	Kruger	OL441870	OL441926	OL441982	OL442025
CMW54314	*L. gonubiensis*	*Sclerocarya birrea*	Kruger	OL441873	OL441929	OL441985	OL442028
CMW54315	*L. gonubiensis*	*Sclerocarya birrea*	Kruger	OL441874	OL441930	OL441986	OL442029
CMW54312	*L. exigua*	*Sclerocarya birrea*	Mapungubwe	OL441875	OL441931	OL441987	OL442030
CMW54313	*L. exigua*	*Sclerocarya birrea*	Mapungubwe	OL441876	OL441932	OL441988	OL442031
CMW54326	*L. mahajangana*	*Mangifera indica*	Tshikundamalema	OL441877	OL441933	OL441989	OL442032
CMW54329	*L. mahajangana*	*Lannea schweinfurthii*	Nwanedi	OL441878	OL441934	OL441990	OL442033

Table 3.1. (Continued)

Isolate No.	Identity	Host	Site	GenBank accession numbers			
				ITS	***tef-1α***	***β-tub***	***rpb2***
CMW54331	*L. mahajangana*	*Sclerocarya birrea*	Mapungubwe	OL441880	OL441936	OL441992	OL442035
CMW54334	*L. mahajangana*	*Sclerocarya birrea*	Kruger	OL441881	OL441937	OL441993	OL442036
CMW54357	*Neofusicoccum parvum*	*Sclerocarya birrea*	Tshipise	OL441892	OL441948	OL442004	OL442047
CMW54359	*N. parvum*	*Mangifera indica*	Tshipise	OL441893	OL441949	OL442005	OL442048
CMW54360	*N. parvum*	*Mangifera indica*	Tshikundamalema	OL441894	OL441950	OL442006	OL442049
CMW57467	*Oblongocollomyces ednahkunjekuae*	*Sclerocarya birrea*	Mapungubwe	OL441905	OL441961	OL442017	OL442060
CMW57572	*Oblongocollomyces ednahkunjekuae*	*Sclerocarya birrea*	Mapungubwe	OL441906	OL441962	OL442018	OL442061
CMW57465	*Oblongocollomyces* sp. 1	*Sclerocarya birrea*	Mapungubwe	OL441907	OL441963	OL442019	OL442062
CMW57573	*Oblongocollomyces* sp. 1	*Sclerocarya birrea*	Mapungubwe	OL441908	OL441964	OL442020	OL442063

3.2.3. DNA extraction, amplification and sequencing

Representative isolates selected for preliminary identification were transferred onto clean 2% MEA and incubated at 25 °C for 7 days, from which mycelia were scrapped and powdered. DNA was extracted following the protocol published by Möller *et al.* (1992). The extracted DNA was stained with GelRed® (Biotium, Haward, California, USA) and visualized under UV light after electrophoresis on 1% agarose gel. DNA concentrations were determined using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and adjusted to a working concentration of 50 ηg µL^{-}1 using sterile water.

Four gene regions that are commonly used in the systematics of *Botryosphaeriaceae* including the internal transcribed spacer (ITS), translation elongation factor (*tef-1α*), β-tubulin (*β-tub*) and the RNA polymerase II subunit (*rpb2*) were amplified*.* The ITS region, which includes the ITS-1 spacer, 5.8S gene and ITS-2 spacer, was amplified and sequenced for all the 78 isolates selected as representatives for the different morphological groups using primer pairs ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.,* 1990). A subset of 29 isolates was selected based on the ITS phylogeny and analysed using other gene regions for phylogenetic confirmation of species identity. For these isolates, regions of the translation elongation factor (*tef-1α*), using primer pairs tef1-728F (5'-CATCGAGAAGTTCGAGAAGG-3') and tef1-986R (5'-TACTTGAAGGAACCCTTACC-3') (Carbone and Kohn, 1999)*,* RNA polymerase II subunit (rpb2) using primer pairs rpb2-LasF (5'-GGTAGCGACGTCACTCCT-3') and rpb2-LasR (5'-GCGCAAATACCCAGAATCAT-3') (Cruywagen et al., 2017) and rpb2bot6F (5'-GGTAGCGACGTCACTCCC-3') and rpb2bot7R (5'-GGATGGATCTCGCAATGCG-3') (Sakalidis *et al.,* 2011), as well as the β-tubulin gene using primer pairs Bt2a (5'-GGTAACCAAATCGGTGCTCGTTTC-3') and Bt2b (5'-ACCCTCAGTGTAHTGACCCTTGGC-3') (Glass and Donaldson, 1995), were amplified and sequenced.

PCR reactions consisted of ~ 40 to 50 ηg genomic DNA, 0.2 µM of each primer, 0.5 u MyTaq® DNA polymerase (Bioline, London, UK), 5 µL MyTaq® PCR reaction buffer (10 mM Tris-HCL [pH 8.3], 3.0 mM $MgCl_{2,}$ 50 mM KCl, Roche Diagnostics, Mannheim, Germany) and PCR grade water at a final volume of 25 µL. PCR cycling conditions were initial denaturation for 2 min at 94 °C, followed by 30 cycles for 30 sec at 94 °C, annealing for 30 sec at 54 °C (ITS and *rpb2*) and 56 °C (*tef-1α* and *β-tub*), extension for 1 min at 72 °C and final extension for 7 min at 72 °C. Amplicons were separated by gel electrophoresis on 1% agarose gel stained with GelRed® and visualised under UV light. Exosap (Mixture of Exonuclease I and FastAP Alkaline Phosphatase) (Thermo Fisher Scientific Inc. Waltham, MA, USA) was used to purify amplicons following the manufacturer’s specifications.

Purified PCR fragments were sequenced in both directions using the same primer pairs utilised in PCR reactions. Sequencing reactions were conducted using the ABI Prism® Big Dye™ Sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing of the amplicons was done at the sequencing facility of the University of Pretoria. Consensus sequences were built from the forward and reverse sequence reads using CLC Main Workbench v.7.9 (QIAGEN, Aarhus, Denmark).

3.2.4. DNA sequence and phylogenetic analyses

Nucleotide sequences generated in this study were subjected to BLASTn searches against sequences on Gen Bank (http://www.ncbi.nlm.gov/genbank) (accessed on 25 February 2019) for preliminary identification. To confirm species identity, phylogenetic analyses were performed individually for each locus dataset. Additional reference sequences showing similarity to query sequences in BLASTn searches were retrieved from GenBank. Sequences were aligned using an online interface of MAFFT v.7 server (Katoh *et al.,* 2019). Alignments were verified and adjusted manually where necessary using BioEdit Sequence Alignment Editor v.7.2.5 (Hall, 1999). Nucleotide models were determined with jModelTest v0.1.1 (Posada, 2008) to select the model that optimally fits the data. The models HKY + I + G, HKY + G, GTR + G and GTR + G were applied to the ITS, *tef-1α*, *β-tub* and *rpb2* datasets, respectively. Maximum likelihood (ML) phylogenetic analyses were performed for the four datasets using RAxML v.8 (Stamatakis, 2014). To determine the robustness of the trees, bootstrap analyses were done using 1000 replicates.

Bayesian inference (BI) of phylogenetic trees was done using MrBayes v.3.2.6 (Ronquist *et al.,* 2012). Four simultaneous Markov chains were run for 3000000 generations and trees were sampled every 100th generation. The first 7500 trees representing the burn-in phase of the analyses were discarded and the remaining 22500 trees were used for calculating posterior probabilities (PP) based on a majority rule consensus tree. Effective sampling size (ESS) values were assessed using Tracer v.1.7.1 (http://tree.bio.ed.ac.uk/software/tracer/) (accessed on 23 February 2021). Both the ML and BI phylogenetic trees were rooted to sequences of *Melanops tulasnei* (*Botryosphaeriales*; *Melanopsaceae*) as the outgroup taxa.

3.3. Results

3.3.1. Sample collection and tree health assessment

Asymptomatic branches were collected from 191 *S. birrea*, 129 *L. schweinfurthii* and 84 *M. indica* trees in disturbed (Tshikundamalema and Tshipise) and undisturbed (Nwanedi, Mapungubwe and Kruger National Parks) ecosystems of the Limpopo and Mpumalanga provinces.

Sclerocarya birrea trees were the most damaged, followed by *L. schweinfurthii* and *M. indica* (Table 3.2). Results obtained from the χ^2 test indicated a statistically significant correlation between health status and tree species ($P = 0.00$). Physical damage caused by elephants was evident on *S. birrea* and *L. schweinfurthii* trees at the two National Park sites (Figure 3.1).

Table 3.2. Health status of the three tree species of *Anacardiaceae* assessed in this study.

Health status	***S. birrea***	***M. indica***	***L. schweinfurthii***	**Total**
0 = healthy	6	0	0	6
1 = <25% dieback branches	117	17	81	215
2 = 25–50% dieback branches	52	55	34	141
3 = >50% dieback branches	12	12	9	33
4 = appearing to be dying or nearly dead	4	0	5	9
Total	191	84	129	404

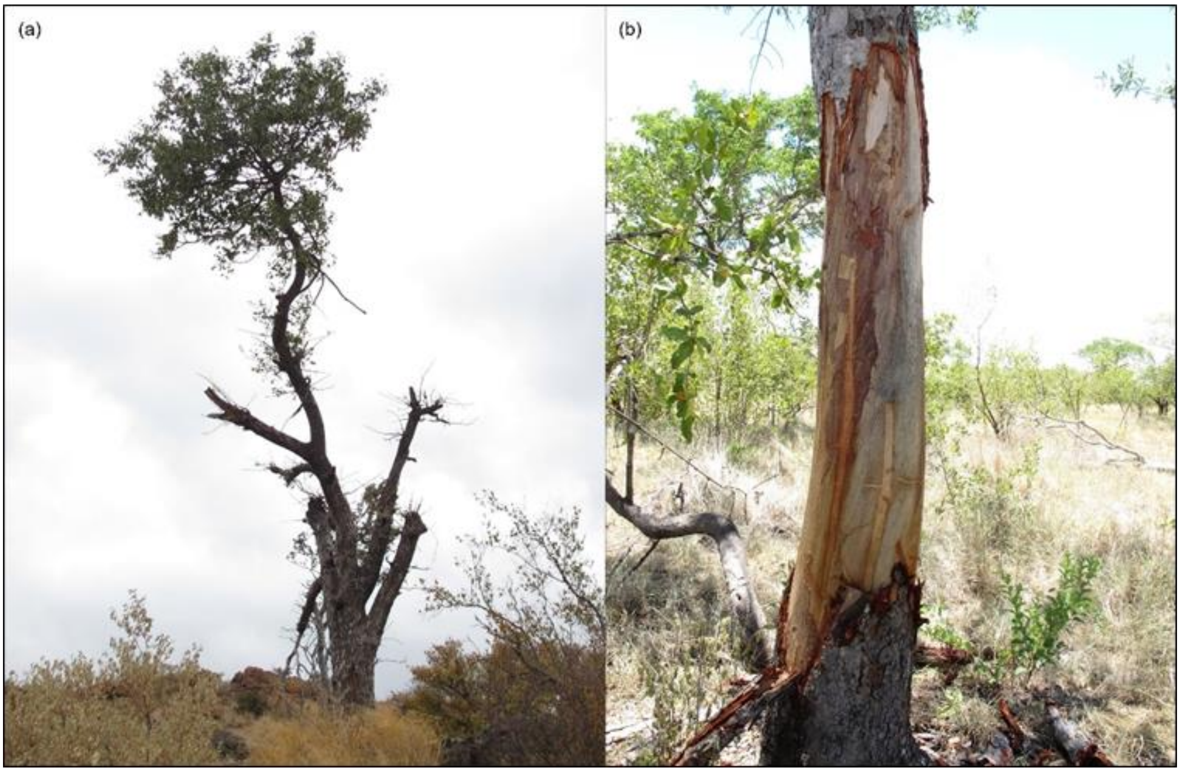

Figure 3.1. Trees with dieback and damage by elephants at the two National Parks; (a) Damaged *Sclerocarya birrea* tree at Mapungubwe National Park; (b) Bark stripped *Lannea schweinfurthii* tree at Kruger National Park.

Of the 404 trees surveyed and sampled, most trees at Kruger National Park were infected, followed by Tshikundamalema, Mapungubwe National Park and Tshipise. Nwanedi had the least number of infected trees (Table 3.3).

Table 3.3. Health status of the *Anacardiaceae* at the five sites sampled in this study.

Site	**Healthy**	**<25% dieback branches**	**25–50% dieback branches**	**>50% dieback branches**	**Appearing to be dying or nearly dead**	**Total**
Tshikundamalema	0	51	42	10	0	103
Tshipise	0	24	24	2	0	50
Nwanedi	0	28	2	0	0	30
Mapungubwe	6	11	26	7	4	54
Kruger	0	101	47	14	5	167

3.3.2. Fungal isolation and preliminary identification

A total of 404 *Botryosphaeriaceae*-like isolates that actively grew on MEA (based on culture morphology and colour characteristics) were obtained from the three hosts; 192 from *S. birrea*, 112 from *L. Schweinfurthii* and 100 from *M. indica*. Eleven culture morphogroups were made from these isolates.

3.3.3. Phylogenetic analyses and confirmation of species identification

From the 11 cultural morphogroups, 78 isolates were selected for preliminary ITS identification and selection of related reference sequences from GenBank. From these, 29 isolates were selected to represent the species diversity represented in the ITS sequences, and three additional gene regions were sequenced.

The datasets for the ITS, *tef-1α* and *β-tub* gene regions included 29 isolates obtained in this study and 62 reference isolates obtained from GenBank for ITS, 60 for *tef-1α* and 54 for *β-tub* (Table 3.4). The *rpb2* dataset included 23 sequences generated in this study. This was because amplicons for the *rpb2* gene region could not be obtained for *Dothiorella* isolates even after using two primer sets.

The topologies of the phylogenetic trees generated from ML and BI analyses of the four datasets were similar in the separation of clades representing genera of *Botryosphaeriaceae*. However, some clades of interest did not receive bootstrap (BS) and posterior probability (PP) support. Some phylogenies were incongruent on the placement of isolates obtained in this study. For example, the *Lasiodiplodia* sub-clade accommodating *L. citricola*, *L. magnolia*, *L. pseudotheobromae* and *L. vaccinii* on the ITS phylogeny (Figure 3.2). Most isolates on the *tef-1α* phylogeny did not form monophyletic clades with species of *Botryosphaeriaceae* (Figure 3.3). Some rearrangements were also observed on the backbone of individual gene trees, e.g., the position of the clade accommodating isolates corresponding to *L. crassispora* on the *β-tub* phylogeny (Figure 3.4). The most variable locus was *rpb2* which could distinguish between most species of *Botryosphaeriaceae* (Figure 3.5). Five main clades corresponding to *Diplodia*, *Dothiorella, Lasiodiplodia, Neofusicoccum* and *Oblongocollomyces*/*Sphaeropsis* were identified.

Table 3.4. Reference sequences obtained from GenBank and used for phylogenetic analyses in this study (species names are as indicated in the GenBank record).

Species	**Country**	**Strain number**	**GenBank accession numbers**			
			ITS	***tef-1α***	***rpb2***	***β-tub***
Diplodia allocellula	South Africa	CBS 130408	JQ239397	JQ239384	None	JQ239378
D. allocellula	South Africa	CMW 36469	NR111701	JQ239385	None	JQ239379
D. eriobotryicola	Spain	CBS 140851	NR152462	KT240193	None	MG015806
D. sapinea	Netherlands	CBS 393.84	DQ458895	DQ458880	None	DQ458863
D. sapinea	South Africa	CBS 109726	KX464094	KX464568	KX463956	KX464800
D. sapinea	Australia	CBS 189.37	KX464099	KX464573	KX463957	KX464808
Dothiorella brevicollis	South Africa	CMW 36464	JQ239404	JQ239391	None	JQ239372
Do. Brevicollis	South Africa	CBS 130411	NR111703	JQ239390	None	JQ239371
Do. Dulcispinae	South Africa	CMW 36461	JQ239401	JQ239388	None	JQ239374
Do. Dulcispinae	Namibia	CMW 36460	NR111702	JQ239387	None	JQ239373
Do. Longicollis	Australia	CBS 122068	NR136999	EU144069	KX463972	KF766246
Do. Longicollis	Australia	CMW 26165	EU144053	EU144068	None	None
Do. Oblonga	South Africa	CBS 121765	EU101301	EU101345	None	KX464862
Do. Oblonga	South Africa	CBS 121766	NR137689	EU101346	None	KX464863
Do. Plurivora	Iran	CBS 124724	KC898225	KC898208	None	KX464874
Do. Plurivora	USA, California	CBS 120999	KX464125	KX464617	None	KX464870
Do. Pretoriensis	South Africa	CBS 130404	JQ239405	JQ239392	None	JQ239376
Do. Pretoriensis	South Africa	CMW 36481	JQ239406	JQ239393	None	JQ239377
Do. Viticola	South Africa	CMW 37928	JX283730	JX283741	None	JX283717

Table 3.4. (Continued)

Species	**Country**	**Strain number**	**GenBank accession numbers**			
			ITS	***tef-1α***	***rpb2***	***β-tub***
Do. Viticola	South Africa	CMW 37933	JX283735	JX283743	None	JX283719
Do. Viticola	Spain	CBS 117009	MH863011	AY905559	DQ677985	EU673104
Do. Viticola	South Africa	STE-U5048	AY343373	AY343336	EF204479	None
L. americana	USA, Arizona	CERC 1962	KP217060	KP217068	None	KP217076
L. americana	USA, Arizona	CERC 1961	KP217059	KP217067	None	KP217075
Lasiodiplodia chonburiensis	Thailand	MFLUCC 16-0376	MH275066	MH412773	None	MH412742
L. citricola	Iran	IRNKB3	MN634040	MN633994	None	None
L. citricola	Iran	CBS 124707	GU945354	GU945340	KP872455	KU887505
L. citricola	Iran	CBS 124706	GU945353	GU945339	KP872456	KU887504
L. crassispora	Venezuela	CMW 13488	DQ103552	DQ103559	KP872458	KU887507
L. crassispora	Australia	CBS 118741	NR111194	EU673303	KP872457	KU887506
L. exigua	Tunisia	CBS 137785	KJ638317	KJ638336	KU696355	KU887509
L. exigua	Tunisia	BL184	KJ638318	KJ638337	None	None
L. gonubiensis	South Africa	CBS 115812	DQ458892	DQ103566	KP872464	KU887512
L. gonubiensis	South Africa	CBS 116355	AY639594	DQ103567	KP872465	KU887513
L. lignicola	Thailand	MFLUCC 11-0435	JX646797	KP872375	KP872470	JX646845
L. lignicola	India	SUF161	MT081525	None	None	None
L. magnoliae	China	MFLUCC 18-0948	MK499387	MK568537	None	MK521587
L. mahajangana	Madagascar	CMW 27801	FJ900595	FJ900641	KP872471	FJ900630
L. mahajangana	Madagascar	CMW 27818	FJ900596	FJ900642	KU696366	FJ900631

Table 3.4. (Continued)

Species	**Country**	**Strain number**	**GenBank accession numbers**			
			ITS	***tef-1α***	***rpb2***	***β-tub***
L. margaritacea	Australia	CBS 122519	NR136998	EU144065	KP872473	KX464903
L. pandanicola	Thailand	MFLUCC 16-0265	MH275068	MH412774	None	None
L. pandanicola	China	GBLZ16BO-008	MN540679	None	None	MN539183
L. pseudotheobromae	Costa Rica	CBS 116459	EF622077	EF622057	KU696376	EU673111
L. pseudotheobromae	Zaire	CBS 374.54	KX464139	KX464633	None	KX464906
L. pseudotheobromae	China	ZYP151106-14	KX499902	KX499940	KX499977	None
L. pyriformis	Namibia	CBS 121771	EU101308	EU101353	KU696379	KU887528
L. pyriformis	Namibia	CMW 25416	EU101309	EU101354	None	None
L. pyriformis	Namibia	CBS 121770	NR136993	EU101352	KP872483	KU887527
L. theobromae	Papua New Guinea	CBS 164.96	AY640255	AY640258	KU696383	KU887532
L. theobromae	Unknown	CBS 111530	EF622074	EF622054	KU696382	None
L. vaccinii	China, Beijing	CGMCC 3.19022	MH330318	MH330327	MH330321	MH330324
L. vaccinii	China, Beijing	CGMCC3.19256	MK157139	MK157166	MK157148	MK157157
Neofusicoccum parvum	South Africa	CMW 41213	KP860849	KP860693	KU587896	KP860771
N. parvum	New Zealand	CBS 138823	NR119487	AY236888	EU821963	AY236917
N. ribis	USA, New York	CMW 7772	AY236935	AY236877	EU863170	AY236906
N. ribis	USA, New York	CMW 7773	AY236936	AY236878	EU863169	AY236907
N. umdonicola	South Africa	CMW 14058	EU821904	EU821874	EU821934	EU821844
N. umdonicola	South Africa	CBS 123646	EU821905	EU821875	EU821935	KF766145
Oblongocollomyces variabilis	Namibia / RSA	CMW 36482	JX283726	JX283738	None	JX283714

Table 3.4. (Continued)

Species	**Country**	**Strain number**	**GenBank accession numbers**			
			ITS	***tef-1α***	***rpb2***	***β-tub***
O. variabilis	Namibia	CBS 121774	NR136994	EU101357	KX464053	JX283715
Sphaeropsis porosa	South Africa	CBS 110496	NR119492	AY343340	KX464076	EU673130
Melanops tulasnei	Germany	CBS 116805	FJ824769	None	None	FJ824780
M. tulasnei	Germany	CBS 116806	FJ824770	FJ824775	KX463998	FJ824781

3.3.3.1. ITS phylogeny

The phylogeny obtained from ML and BI analyses of the ITS sequence dataset grouped isolates obtained in this study into five clades representing five genera of *Botryosphaeriaceae* (Figure 3.2). The five clades represented *Lasiodiplodia* (clade I), *Diplodia* (clade II), *Oblongocollomyces*/*Sphaeropsis* (clade III), *Dothiorella* (clade IV) and *Neofusicoccum* (clade V). In the *Lasiodiplodia* clade, four isolates (CMW54326, CMW54329, CMW54331 and CMW54334) could not be identified. These isolates clustered with *L. citricola*, *L. magnoliae, L. pseudotheobromae* and *L. vaccinii*. Isolates CMW54312 and CMW54313 grouped with *L. americana* and *L. exigua*. Two isolates, CMW54314 and CMW54315 formed a monophyletic clade with *L. gonubiensis*. Isolates CMW54318, CMW54319, CMW54320, CMW54321 and CMW57579 formed a polytomy with *L. crassispora,* separate from *L. pyriformis*.

The *Diplodia* clade included *D. allocellula*, *D. eriobotryicola* and *D. sapinea* sequences. Three isolates obtained in this study (CMW54349, CMW54351 and CMW54353) formed a polytomy with *D. allocellula,* separate from *D. eriobotrycola.* Clade III included four isolates (CMW57573, CMW57465, CMW57572 and CMW57467) that formed a distinct clade from previously described species of *Botryosphaeriaceae*. The four isolates formed two sub-clades indicating that they possibly represent two closely related but distinct species. Isolates CMW57572 and CMW57467 were subsequently described as *Oblongocollomyces ednahkunjekuae,* while isolates CMW57573 and CMW57465 could not be assigned to a species based on ITS sequence data and are referred to as *Oblongocollomyces* sp. 1. Clade IV included *Dothiorella* sequences. Two isolates, CMW57463 and CMW57464 grouped with *Do. plurivora* and *Do. viticola*. Isolates CMW57629 and CMW57630 formed a polytomy within the genus based on the ITS sequence data. Isolates CMW57462 and CMW57466 grouped with *Do. dulcispinae* and *Do. oblonga*. The *Neofusicoccum* clade included *N. parvum, N. ribis* and *N. umdonicola* sequences, with isolates obtained in this study (CMW54357, CMW54359 and CMW54360) corresponding to *N. parvum*.

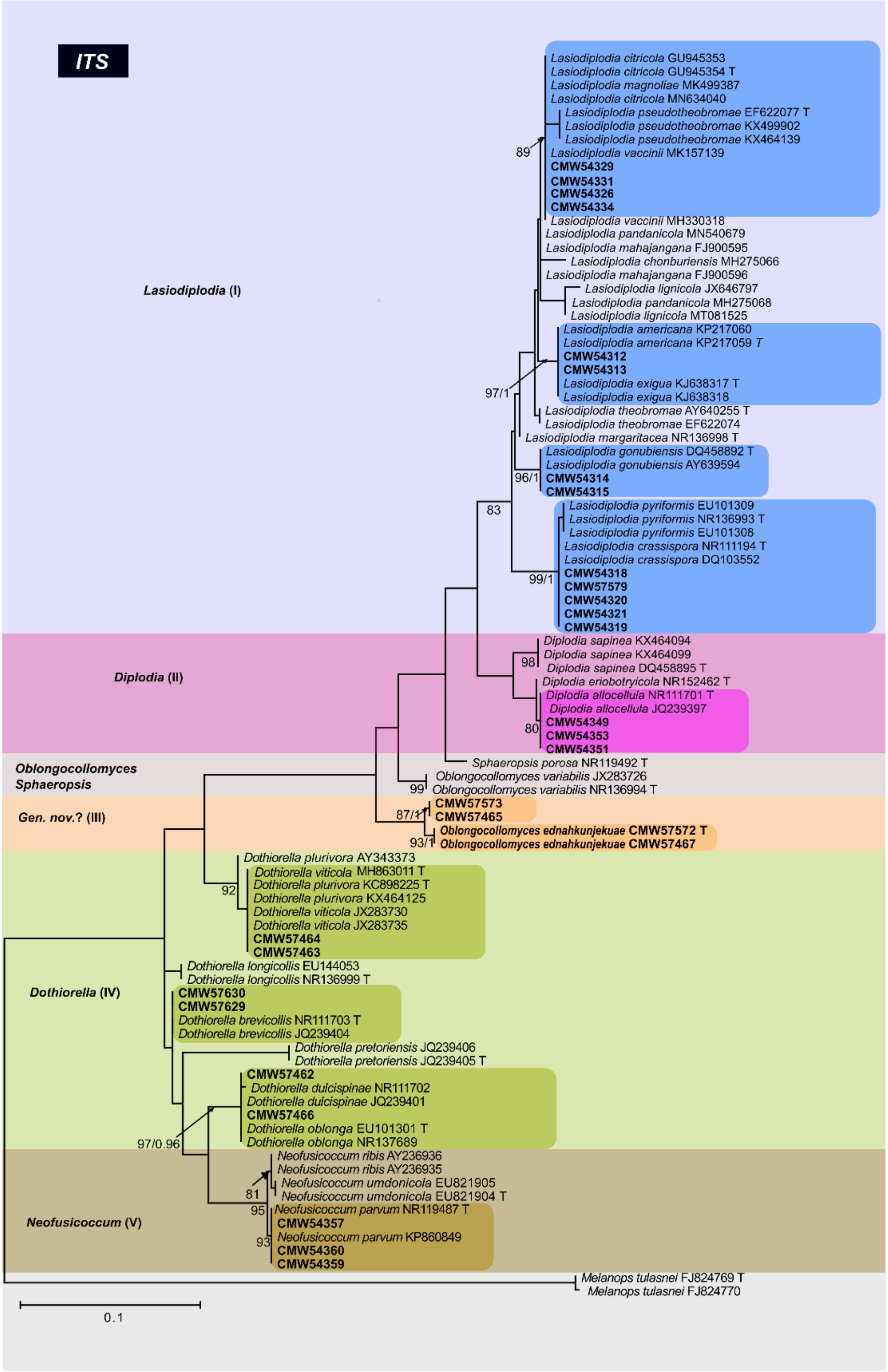

Figure 3.2. Phylogenetic tree generated from maximum likelihood and bayesian inference analyses of the ITS sequence data. Bootstrap support values > 70 % and PP values > 0.95 are shown at the nodes. Sequences in **bold** were generated in this study. The tree was rooted to *Melanops tulasnei.*

3.3.3.2. *Tef-1α* phylogeny

The *tef-1α* phylogeny separated sequences from the isolates obtained in this study into five clades. These clades represented *Lasiodiplodia* (clade I), *Diplodia* (clade II), *Oblongocollomyces/Sphaeropsis* (clade III), *Neofusicoccum* (clade IV) and *Dothiorella* (clade V) (Figure 3.3). The *Lasiodiplodia* clade included isolates (CMW54326, CMW54329, CMW54331 and CMW54334) that grouped with *L. mahajangana*. Two isolates, CMW54312 and CMW54313 formed a polytomy with *L. exigua*. Isolates CMW54318, CMW54319, CMW54320, CMW54321 and CMW57579 grouped with *L. crassispora.* Isolates CMW54314 and CMW54315 grouped with *L. gonubiensis*. The *Diplodia* clade included *D. allocellula*, *D. eriobotryicola* and *D. sapinea* sequences, with isolates obtained in this study (CMW54349, CMW54351 and CMW54353) corresponding to *D. allocellula*.

Clade III included four isolates, with isolates CMW57573 and CMW57465 forming a sister clade with *O. variabilis,* and *Oblongocollomyces ednahkunjekuae* forming a sister clade with both *O. variabilis* and *Oblongocollomyces* sp. 1. The *Neofusicoccum* clade included isolates (CMW54357, CMW54359 and CMW54360) that formed a monophyletic clade with *N. parvum.* Clade V included *Dothiorella* sequences*.* Isolates CMW57463 and CMW57464 grouped with *Do. viticola.* Isolates CMW57462 and CMW57466 formed a polytomy with *Do. oblonga*, separate from *Do. dulcispinae*. Isolates CMW57629 and CMW57630 formed a polytomy with *Do. brevicollis,* separate from *Do. longicollis*.

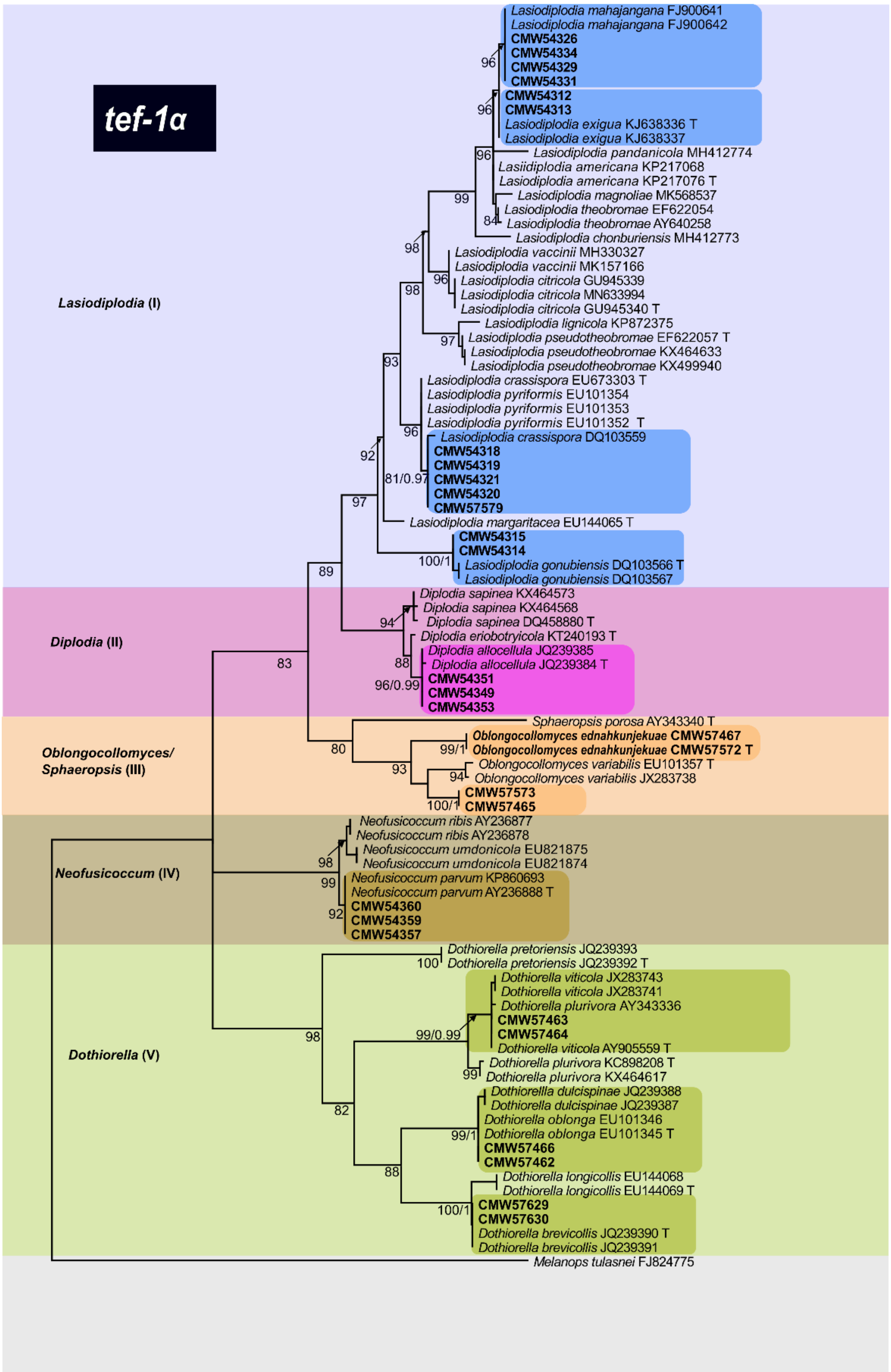

Figure 3.3. Phylogenetic tree generated from maximum likelihood and Bayesian inference analyses of the *tef-1α data* including species of *Botryosphaeriaceae*. Bootstrap values > 70% and posterior probabilities values > 0.95 are shown at the nodes. Sequences obtained in this study are indicated in **bold**. The tree was rooted to *Melanops tulasnei.*

3.3.3.3. *β-tub* phylogeny

The phylogeny emerging from ML and BI analyses of the *β-tub* dataset separated isolates obtained in this study into clades corresponding to five genera of *Botryosphaeriaceae* including *Dothiorella* (clade I), *Neofusicoccum* (clade II), *Sphaeropsis* (clade III), *Lasiodiplodia* (IV) and *Diplodia* (V) (Figure 3.4). The *β-tub* phylogeny varied from the other gene trees in the placement of some isolates obtained in this study. For example, the *Lasiodiplodia* clade including isolates corresponding to *L. crassispora* formed a separate clade from other *Lasiodiplodia* species. Isolates CMW57463 and CMW57464 grouped with *Do. plurivora* and *Do. viticola*. Isolates CMW57629 and CMW57630 grouped with *Do. brevicollis.* Isolates CMW57462 and CMW57466 grouped with *Do. dulcispinae* and *Do. oblonga*. The *Neofusicoccum* clade included *N. parvum, N. ribis* and *N. umdonicola* sequences. Isolates obtained in this study (CMW54357, CMW54359 and CMW54360) grouped with *N. parvum*.

The four isolates (CMW57467, CMW57572, CMW57465 and CMW57573) that could not be assigned to a species on the ITS and *tef-1α* phylogeny, grouped with *Sphaeropsis porosa* on the *β-tub* phylogeny. Two of these isolates (CMW57467 and CMW57572) formed a sister clade with *S. porosa*, while the other two isolates (CMW57465 and CMW57573) formed a sister clade with both *S. porosa* and the other closely related isolates (CMW57467 and CMW57572). The *Lasiodiplodia* clade included five isolates (CMW54318, CMW54319, CMW54320, CMW54321 and CMW57579) that grouped with *L. crassispora* and *L. pyriformis*. Clade V included *Diplodia* sequences. The *Diplodia* clade included *D. allocellula*, *D. eriobotryicola* and *D. sapinea* sequences. Isolates CMW54349, CMW54351 and CMW54353 grouped with *D. allocellula* and *D. eriobotryicola*. Isolates CMW54312 and CMW54313 together with *L. exigua* formed a polytomy within the *Lasiodiplodia* genus based on the *β-tub* sequence data. Isolates CMW54326, CMW54329, CMW54331 and CMW54334 grouped with *L. mahajangana*. Isolates CMW54314 and CMW54315 formed a monophyletic clade with *L.*

gonubiensis.

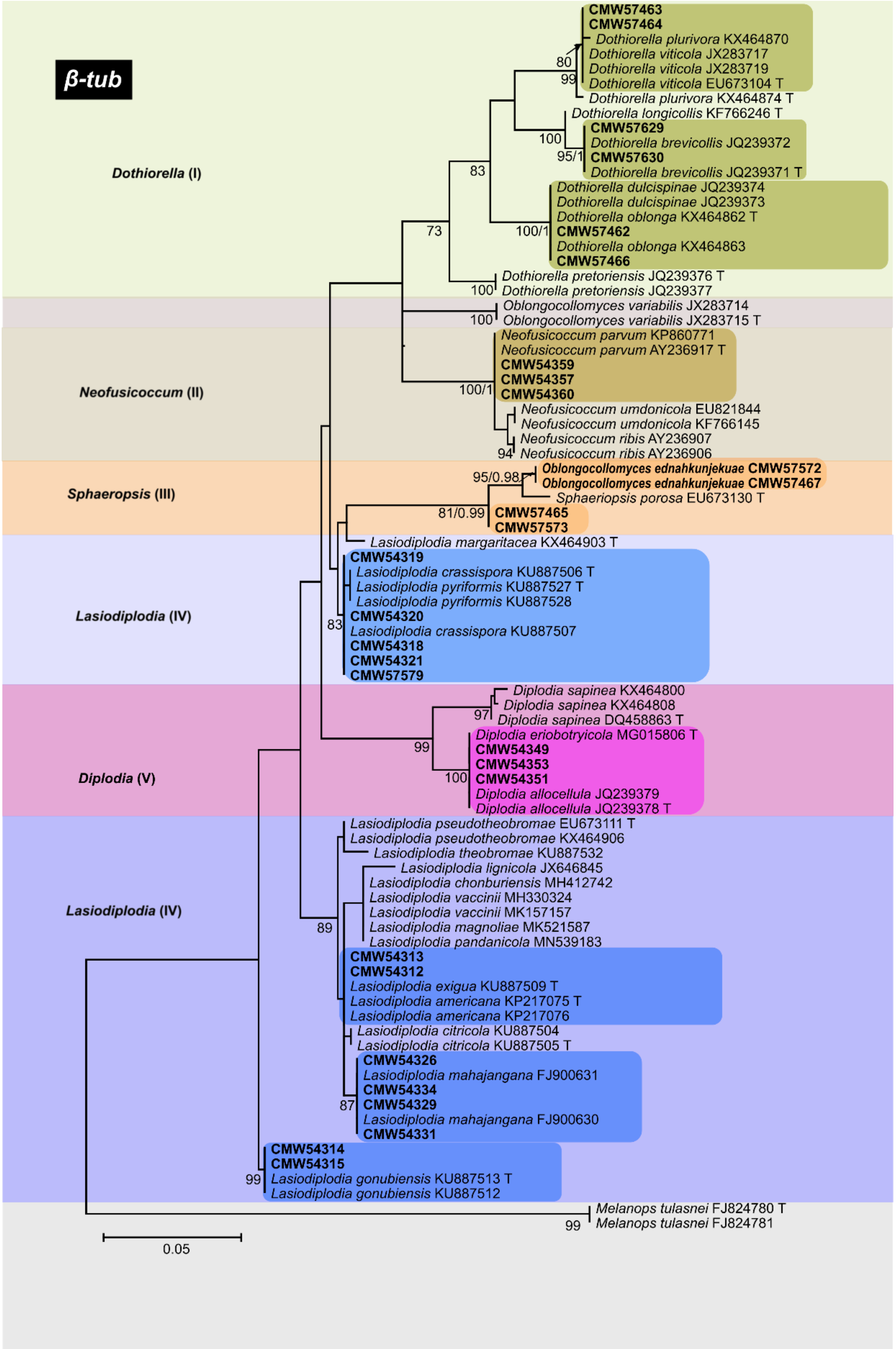

Figure 3.4. Phylogenetic tree obtained from ML and BI analyses of the *β-tub* dataset. Bootstrap values (> 70%) and PP values (> 0.95) appear at the nodes. Sequences generated in the current study are in **bold**. The tree was rooted to isolates of *Melanops tulasnei.*

3.3.3.4. *Rpb2* phylogeny

The *rpb2* phylogeny separated isolates obtained in this study into four clades. These clades represented *Lasiodiplodia* (clade I), *Diplodia* (clade II), *Oblongocollomyces*/*Sphaeropsis* (clade III) and *Neofusicoccum* (clade IV) (Figure 3.5). Four isolates in the *Lasiodiplodia* clade (CMW54326, CMW54329, CMW54331 and CMW54334) grouped with *L. mahajangana*. Two isolates, CMW54312 and CMW54313 grouped with *L. exigua.* Isolates CMW54314 and CMW54315 formed a monophyletic clade with *L. gonubiensis*. Isolates CMW54318, CMW54319, CMW54320, CMW54321 and CMW57579 formed a polytomy with *L. crassispora*, separate from *L. pyriformis*. The *Diplodia* clade included *D. sapinea* sequences only because reference sequences for other *Diplodia* species for the *rpb2* gene region are not available on public databases. Three isolates (CMW54349, CMW54351 and CMW54353) identified as *D. allocellula* on the ITS, *tef-1α* and *β-tub* phylogenetic trees formed a sister clade with *D. sapinea* indicating that they reside in the same genus.

Clade III included *O. variabilis* and *S. porosa* sequences. *Oblongocollomyces* sp. 1 formed a sister clade with *O. variabilis* and *Oblongocollomyces ednahkunjekuae* formed a sister clade with both *O. variabilis* and *Oblongocollomyces* sp. 1. The *Dothiorella* clade did not include any isolates obtained in this study because amplicons for *Dothiorella* isolates could not be obtained despite efforts to sequence two different primer sets. The *Neofusicoccum* clade included *N. parvum, N. ribis* and *N. umdonicola* sequences. Three isolates obtained in this study (CMW54357, CMW54359 and CMW54360) grouped with *N. parvum*.

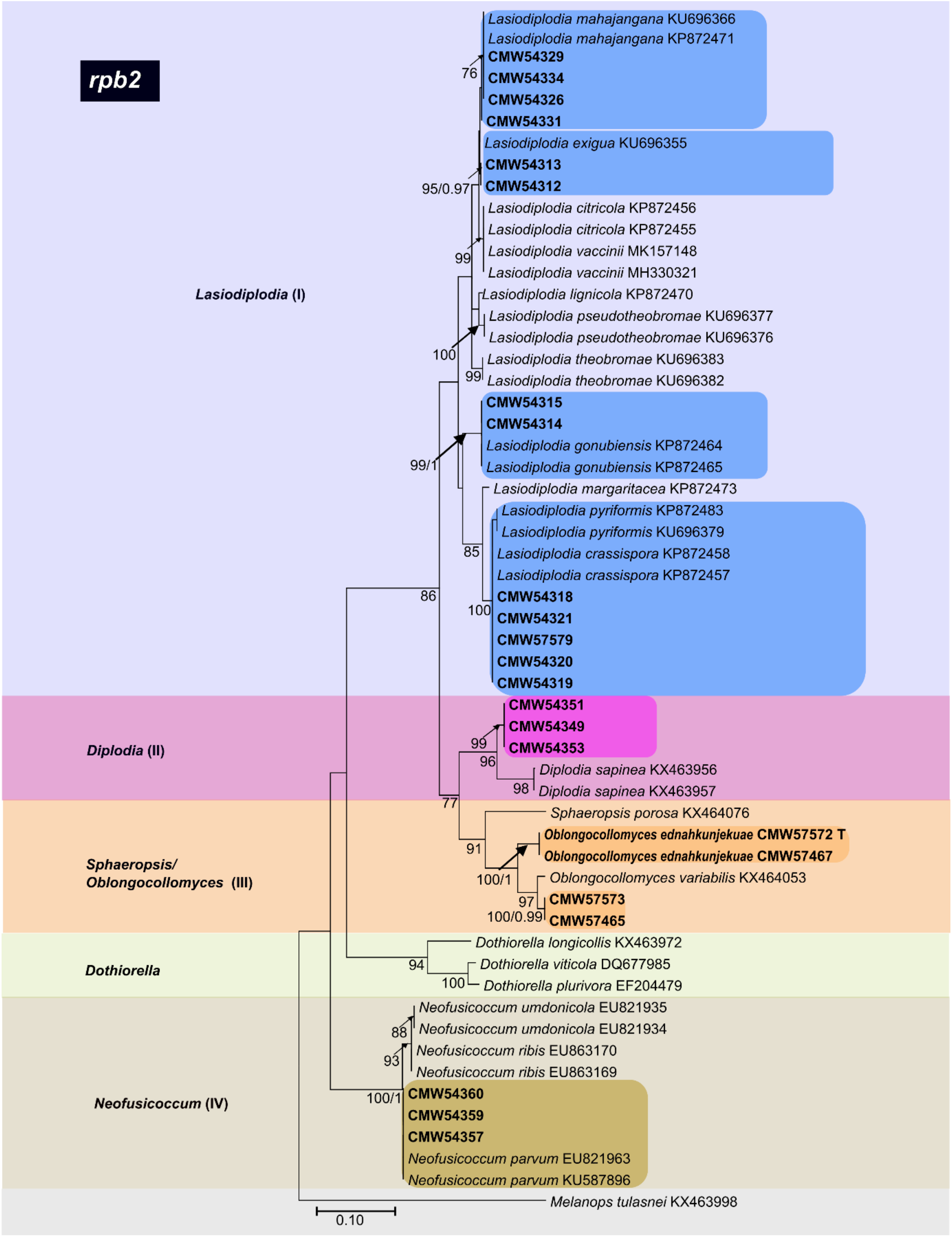

Figure 3.5. Phylogenetic tree generated from ML and BI analyses of the *rpb2* sequence data showing the relationship between species of *Botryosphaeriaceae.* Bootstrap values (> 70%) and PP values (> 0.95) appear at the nodes. Sequences generated in the current study are in **bold.** *Melanops tulasnei* sequences are rooted to the trees as outgroup taxa.

Taxonomy

The following are descriptions of new species based on morphology:

Oblongocollomyces ednahkunjekuae Slippers, Ramabulana & M.P.A. Coetzee, **sp. nov**. MycoBank MB 853978 (Figure. 3.6). Etymology: Named after Prof. Ednah Kunjeku, who has played an instrumental role in training postgraduate students at the University of Venda and enabling their sampling in unique habitats in the northern parts of South Africa.

Asexual morph: Conidiomata pycnidial, globose to subglobose, rarely with a short neck, superficial, immersed or semiimmersed, scattered on the medium, rarely in clusters; wall of 3–6 layers of brown textura angularis. Paraphyses intermingled among conidiogenous cells, hyaline, smooth, flexuous, aseptate, rarely branched, with obtuse apices. Conidiophores reduced to conidiogenous cells. Conidiogenous cells hyaline, holoblastic, cylindrical to ampulliform, proliferating at the same level to form periclinical thickenings or rarely proliferating percurrently. Conidia hyaline to honey coloured to brown, 0–1-septate, smooth, hilum truncate, moderately thick-walled, ellipsoidal to obtuse; 18.8–25.5 × 11–14 µm (av. 22.1 × 12.5 µm) in size.

Growth rate: The optimal growth rate was on PDA at 30 °C (6.3 cm/d), followed by 35 °C (5.3 cm/d) and 25 °C (5 cm/d). Growth rapidly decreased below these temperatures to 2.3 cm/d at 20 °C, 8 mm/d at 15 °C and less than 1 mm/d at 10 °C. Typus: South Africa, Mapungubwe National Park, on *Sclerocarya birrea*, 2018, E. Ramabulana (holotype PRU(M) 4586, preserved as dried culture; culture ex-holotype CMW 57572). Additional material examined: South Africa, Mapungubwe National Park, on *Sclerocarya birrea*, 2018, E. Ramabulana (culture CMW 57467).

Notes: Sporulation was induced on VMM using the methods described in Oostlander *et al.* 2023. The dried, sporulating culture serves as holotype.

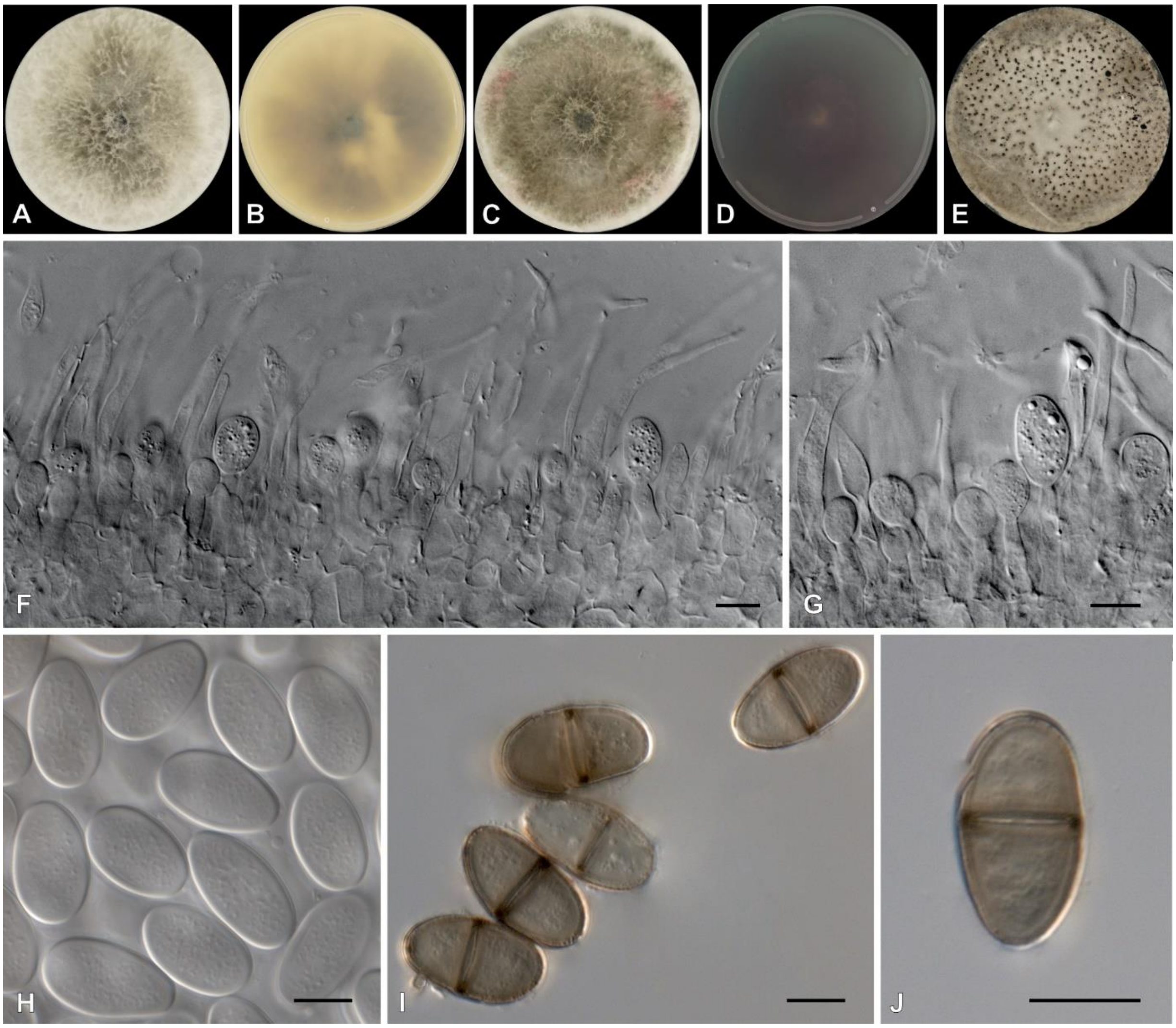

Figure 3.6. *Oblongocollomyces ednahkunjekuae*. A. Top view of a colony on PDA at 25 °C after 5 d. B. Bottom view of a colony on PDA at 25 °C after 5 d. C. Top view of a colony on PDA at 35 °C after 4 d. D. Bottom view of a colony on PDA at 35 °C after 4 d. E. Sporulating culture on VMM after 21 d. F, G. Conidiogenous cells, developing conidia and paraphyses. H. Immature conidia. I. Mature conidia. J. Mature conidium, showing the thickened cell wall and septum. Scale bars = 10 µm.

3.4. Species diversity distribution

The phylogenies obtained from ML and BI analyses of the four loci distinguished isolates obtained in this study as *D. allocellula, Do. plurivora*/*Do. viticola*, *Do. dulcispinae*/*Do. oblonga*, *Do. brevicollis*, *L. americana*/*L. exigua, L. crassispora*/*L. pyriformis*, *L. gonubiensis*, *L. citricola*/*L. magnoliae*/*L. pseudotheobromae*/*L. vaccinii*, *L. mahajangana*, *N. parvum*, *Oblongocollomyces ednahkunjekuae* and *Oblongocollomyces* sp. 1.

Isolates CMW54312 and CMW54313 that grouped with *L. americana* and *L. exigua* are treated as *L. exigua* based on a previous study that synonymised *L. americana* with *L. exigua* (Rodríguez-Gálvez *et al.,* 2017). Four isolates (CMW54326, CMW54329, CMW54331 and CMW54334) that grouped with *L. citricola*, *L. magnoliae*, *L. pseudotheobromae* and *L. vaccinii* on the ITS phylogeny grouped with *L. mahajangana* on the *tef-1α*, *β-tub* and *rpb2* phylogenies. Therefore, these isolates are treated as *L. mahajangana*. Isolates CMW54318, CMW54319, CMW54320, CMW54321 and CMW57579 that grouped with *L. crassispora* and *L. pyriformis* are treated as *L. crassispora* as they formed unresolved groups and based on an earlier study that reduced *L. pyriformis* to synonymy with *L. crassispora* (Zhang *et al.,* 2021). Similarly, isolates CMW57462 and CMW57466 that grouped with *Do. dulcispinae* and *Do. oblonga* are treated as *Do. dulcispinae* as they formed unresolved groups and based on a study that reduced *Do. oblonga* to synonymy With *Do. dulcispinae* (Zhang *et al.,* 2021). Isolates CMW57463 and CMW57464 that grouped with *Do. plurivora* and *Do. viticola* are treated as *Do. viticola* based on analyses of the *tef-1α* sequence data.

Eleven species of *Botryosphaeriaceae* including *D. allocellula*, *Do. brevicollis*, *Do. dulcispinae*, *Do. viticola*, *L. crassispora*, *L. exigua, L. gonubiensis*, *L. mahajangana*, *N. parvum*, *Oblongocollomyces ednahkunjekuae* and *Oblongocollomyces* sp. 1 were identified as endophytes on tree species of *Anacardiaceae* in this study. Identities of the 11 species were assigned to all 404 *Botryosphaeriaceae* isolates based on the combined results of the morphogroup identification and phylogenetic analyses of the ITS, *tef-1α*, *β-tub* and *rpb2* sequence datasets (Table 3.5).

Table 3.5. Number of *Botryosphaeriaceae* isolates identified based on morphological and phylogenetic grouping.

Site	**Host**	**Identity**	**No. isolate**
Tshikundamalema	*Sclerocarya birrea*	*Lasiodiplodia crassispora*	10
		Lasiodiplodia mahajangana	7
		Neofusicoccum parvum	4
Tshikundamalema	*Mangifera indica*	*Lasiodiplodia crassispora*	28
		Lasiodiplodia mahajangana	26
		Neofusicoccum parvum	4
Tshipise	*Sclerocarya birrea*	*Lasiodiplodia mahajangana*	25
		Neofusicoccum parvum	4
Tshipise	*Mangifera indica*	*Lasiodiplodia crassispora*	3
		Lasiodiplodia mahajangana	5
		Neofusicoccum parvum	34
Kruger	*Lannea schweinfurthii*	*Diplodia allocellula*	27
		Lasiodiplodia crassispora	27
		Lasiodiplodia mahajangana	6
Kruger	*Sclerocarya birrea*	*Diplodia allocellula*	25
		Dothiorella dulcispinae	6
		Lasiodiplodia crassispora	9
		Lasiodiplodia gonubiensis	2
		Lasiodiplodia mahajangana	7
Nwanedi	*Lannea schweinfurthii*	*Dothiorella brevicollis*	34
		Lasiodiplodia mahajangana	20
Mapungubwe	*Sclerocarya birrea*	*Dothiorella brevicollis*	20
		Dothiorella dulcispinae	2
		Dothiorella viticola	6
		Lasiodiplodia crassispora	16
		L. exigua	3
		Lasiodiplodia mahajangana	40
		O. ednahkunjekuae	2
		Oblongocollomyces sp. 1	2

The number of *Botryosphaeriaceae* species identified on the three tree species of *Anacardiaceae* varied across the different sampling sites in disturbed and undisturbed ecosystems (Figure 3.7). The highest species diversity was observed on *S. birrea* in Mapungubwe National Park with eight species identified.

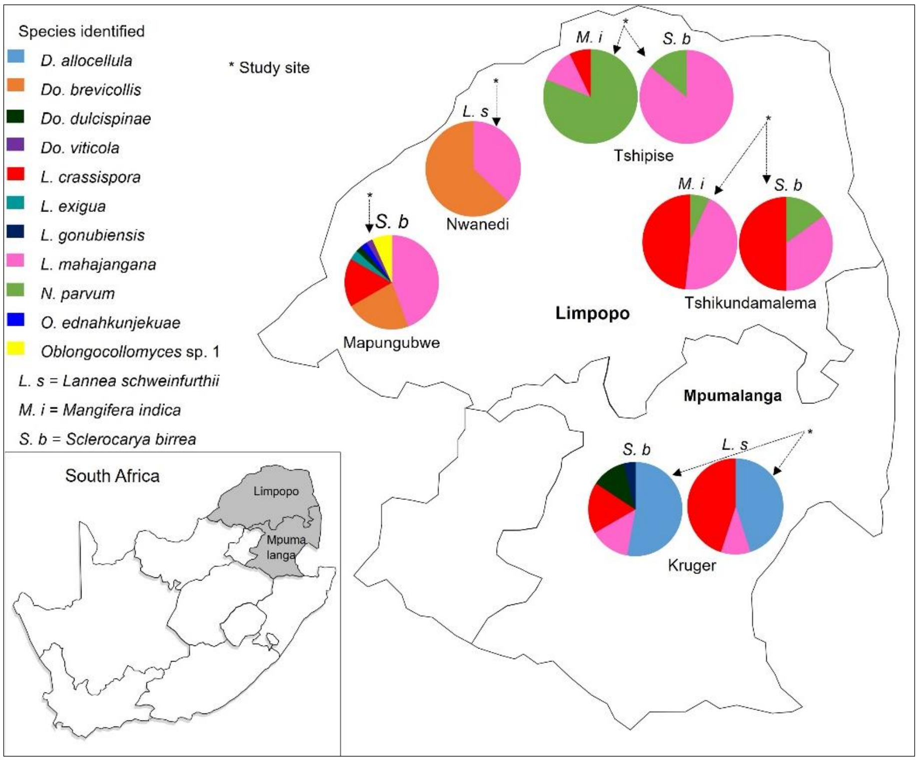

Figure 3.7. Species diversity and distribution of the *Botryosphaeriaceae* on *Sclerocarya birrea*, *Mangifera indica* and *Lannea schweinfurthii* trees in Mapungubwe, Nwanedi, Tshipise, Tshikundamalema and Kruger National Park. Insert: Map of South Africa with the two provinces in which the sampling sites reside shown in grey.

There was variation in *Botryosphaeriaceae* species diversity and distribution on trees of *Anacardiaceae* in disturbed and undisturbed ecosystems (Figure 3.8). Eight species were unique to trees in undisturbed ecosystems, two occurred as generalists on trees in disturbed and undisturbed ecosystems and only one species was unique to trees in disturbed ecosystems.

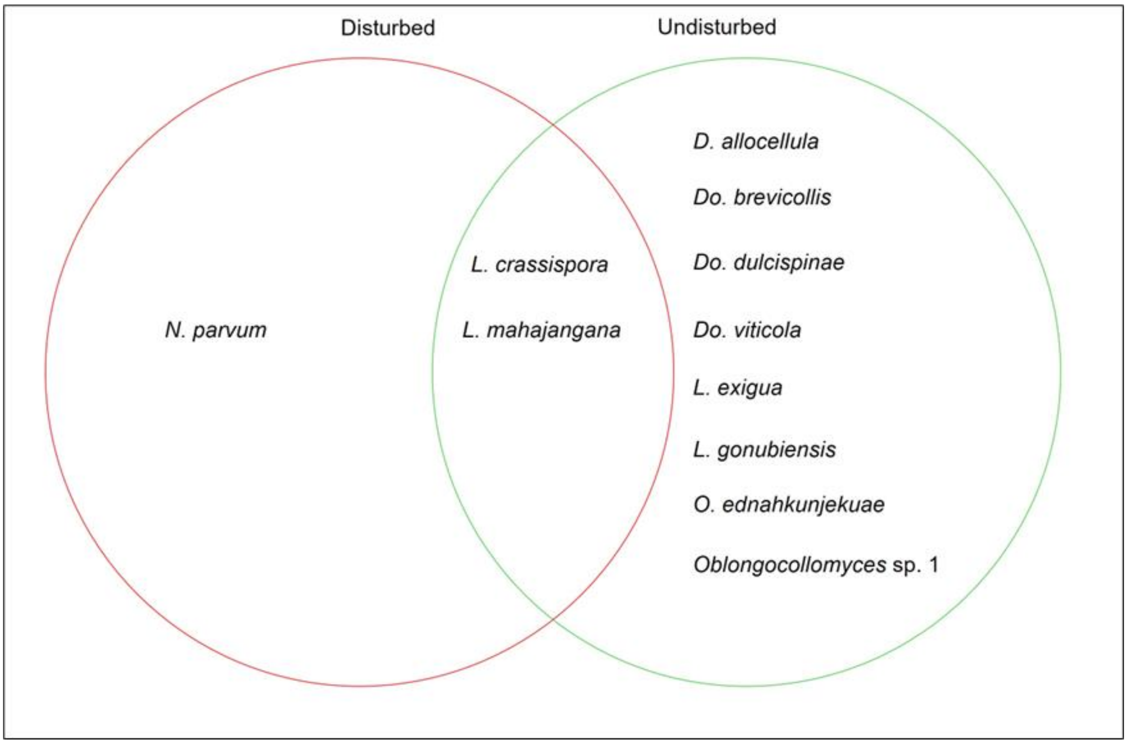

Figure 3.8. Venn diagram indicating *Botryosphaeriaceae* species diversity and distribution on three tree species of *Anacardiaceae* in disturbed (including *S. birrea* and *M. indica* at Tshikundamalema and Tshipise) and undisturbed (including *S. birrea* and *L. schweinfurthii* at Nwanedi, Mapungubwe and Kruger National Park) ecosystems.

Some species of *Botryosphaeriaceae* displayed the ability to infect both native and non-native *Anacardiaceae* (Figure 3.9). *Lasiodiplodia crassispora* and *L. mahajangana* overlapped on all three tree species of the *Anacardiaceae*. *Neofusicoccum parvum* overlapped between *S. birrea* (native) and *M. indica* (non-native) where they were growing next to each other at the two disturbed ecosystems. *Diplodia allocellula* and *Do. brevicollis* overlapped between native *Anacardiaceae* (*S. birrea* and *L. schweinfurthii*) in undisturbed ecosystems. Some species were only found on *S. birrea*. These included *Do. dulcispinae, Do. viticola, L. exigua*, *L. gonubiensis, Oblongocollomyces ednahkunjekuae* and *Oblongocollomyces* sp. 1.

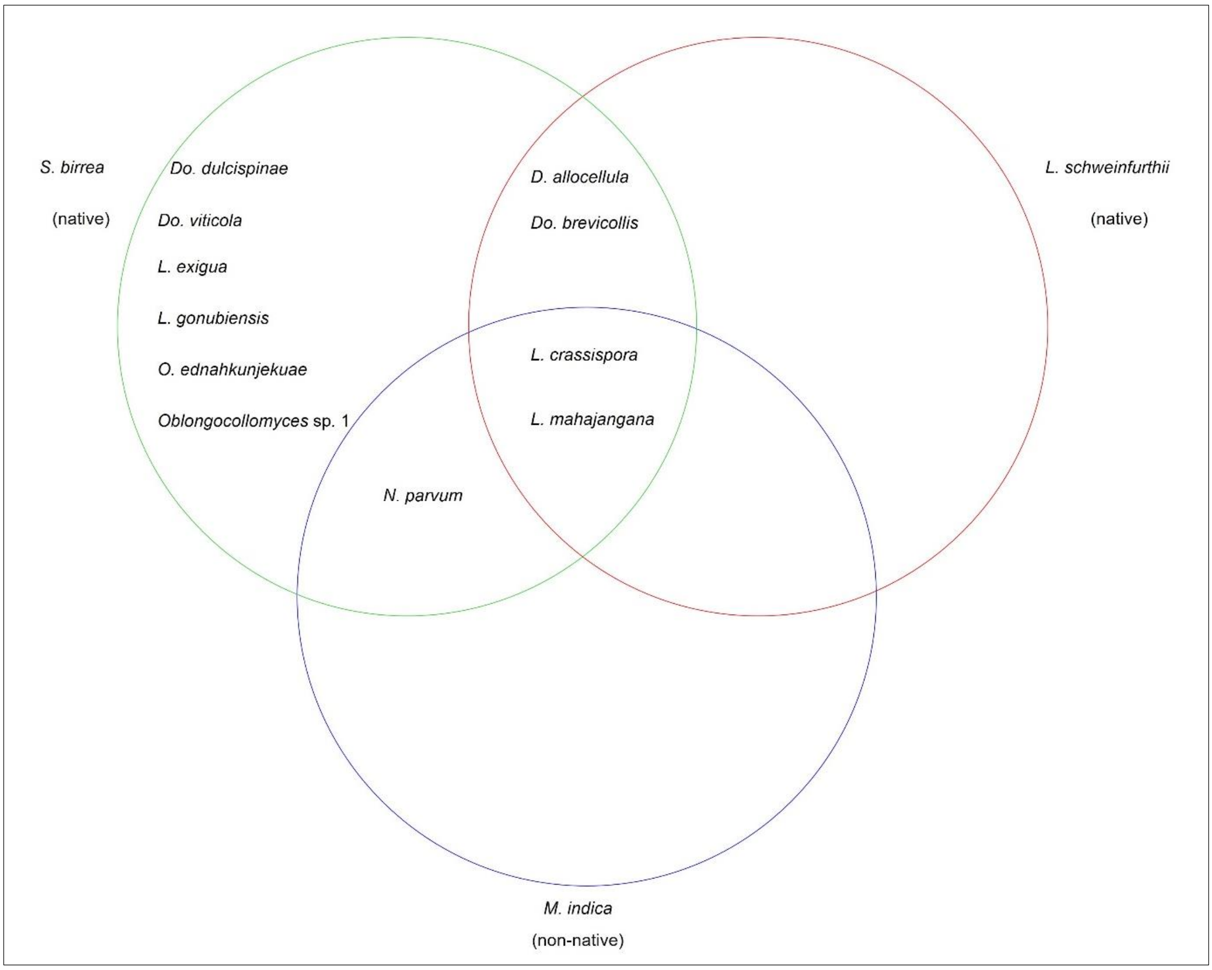

Figure 3.9. Venn diagram showing species overlap of *Botryosphaeriaceae* on *Sclerocarya birrea, Lannea schweinfurthii* and *Mangifera indica.*

3.5. Discussion

In this study, we report the presence of 11 species of *Botryosphaeriaceae*, namely *D. allocellula*, *Do. Brevicollis*, *Do. dulcispinae*, *Do. viticola*, *L. crassispora*, *L. exigua*, *L. gonubiensis*, *L. mahajangana, N. parvum, Oblongocollomyces ednahkunjekuae* and *Oblongocollomyces* sp. 1 on three tree species of *Anacardiaceae* in the north-eastern parts of South Africa. The majority of these species (10) occurred on trees in undisturbed (isolated and/or protected) ecosystems, compared to only three species in disturbed (agriculture and forestry) ecosystems. Three species overlapped on both native and non-native species. Some species identified in this study are reported for the first time in South Africa or on specific hosts.

The high species diversity recorded on trees in undisturbed ecosystems is consistent with findings that biodiversity in natural ecosystems increases fungal diversity compared to ecosystems frequently disturbed by human activities such as clear-cutting, selective cutting and land-use changes (Opik *et al.,* 2006). *Diplodia allocellula*, *Do. brevicollis*, *Do. dulcispinae*, *Do. viticola*, *L. exigua*, *L. gonubiensis, Oblongocollomyces ednahkunjekuae* and *Oblongocollomyces* sp. 1 were identified on trees in undisturbed ecosystems only. The influence of human activity on *Botryosphaeriaceae* species composition was previously reported by Pavlic *et al.* (2015). In that study, *N. parvum* was predominant on *S. cordatum* in disturbed stands and absent in undisturbed stands where other *Neofusicoccum* species dominated. Our results indicate that activities between the two ecosystems (including deforestation in disturbed ecosystems, as opposed to rich plant diversity in undisturbed ecosystems) influence *Botryosphaeriaceae* species composition. The absence of *N. parvum* on trees in undisturbed ecosystems despite its known presence in the region suggests that undisturbed ecosystems are more resilient to invasion by some invasive *Botryosphaeriaceae*. This hypothesis is worth testing, as this would imply a potential influence of conserved areas on the spread of invasive species of *Botryosphaeriaceae*. However, such areas might also serve as a reservoir of new species that might infect agricultural and forestry tree species.

Lasiodiplodia crassispora and *L. mahajangana* were common among the three tree species in both ecosystems. Organisms with a broad niche are often prominent colonizers of disturbed environments and also successful invaders in new regions (Lee and Gelembiuk, 2008; Pavlic *et al.,* 2015). The presence of *L. crassispora* and *L. mahajangana* in both ecosystems was not surprising from the perspective that *Lasiodiplodia* species occur predominantly in tropical and subtropical regions, such as those sampled in this study (Cruywagen *et al.,* 2017; Mehl *et al.,* 2017a). Also, the two species have been reported in the region before and on tree species of *Anacardiaceae* (Mehl *et al.,* 2017a). *Lasiodiplodia crassispora* has been recorded on *P. angolensis* and *S. birrea* in Mpumalanga and Limpopo, respectively (Mehl *et al.,* 2011, 2017a), while *L. mahajangana* is known to occur on *Adansonia digitata, E. ingens* and *S. birrea* in Limpopo and Mpumalanga (Cruywagen *et al.,* 2017; Mehl *et al.,* 2017a).

Neofusicoccum parvum was the only species unique to trees in disturbed, agricultural ecosystems. The fungus was also the most dominant species overlapping between *S. birrea* (native) and *M. indica* (non-native) trees in disturbed ecosystems. These results indicate the ease with which this important global pathogen spreads between indigenous and introduced hosts in human-disturbed ecosystems (Pillay *et al.,* 2013; Pavlic *et al.,* 2015; Mehl *et al.,* 2017a). The abundance and distribution of *Neofusicoccum* species, and *N. parvum* in particular, on *Syzygium cordatum,* are linked to host composition and ecosystem disturbance through human activities (Pavlic *et al.,* 2015). Our results provide further support for this hypothesis.

Our study is not the first to investigate species overlap of these fungi on indigenous and non-native *Anacardiaceae* in South Africa. Mehl *et al.* (2017a) also reported an overlap of *N.*

parvum on *M. indica* and *S. birrea* in Hoedspruit, Mpumalanga Province. The absence of *N. parvum* on *S. birrea* trees in undisturbed areas suggests that the fungus is not indigenous to native hosts, but it is spreading from a non-native (*M. indica*) to a native (*S. birrea*) host in disturbed ecosystems, rather than *vice versa.*

This study represents the first record of *L. exigua* in South Africa. *Lasiodiplodia exigua* was described from *Retama raetam* in Tunisia and *Pistacia vera* in the United States (Linaldeddu *et al.,* 2015). The fungus has also been reported to cause canker, dieback, discolouration and streaks on grapevine wood in Turkey and Mexico (Akgül *et al.,* 2019; Montoya *et al.,* 2021). In the current study, *L. exigua* was isolated from *S. birrea* at Mapungubwe National Park. Curiously, this species was identified on a native host in an isolated low disturbance site only, even though it is known from other hosts globally. This demonstrates the ability of this fungus to spread between continents and hosts. Our results indicate that other species that are thought to not occur in the country might exist on unsampled hosts in conserved areas.

Dothiorella brevicollis, Do. dulcispinae, Do. viticola, O. ednahkunjekuae and *Oblongocollomyces* sp. 1 on *S. birrea,* and *L. crassispora* on *M. indica* are the first reports on these hosts, albeit that these hosts were sampled before for the *Botryosphaeriaceae* in South Africa. *Dothiorella brevicollis* and *Do. dulcispinae* appear to have a limited geographic distribution and narrow host range in South Africa, in contrast to *Do. viticola* which appears to have a much wider host and geographic distribution. *Dothiorella brevicollis* and *Do. dulcispinae* were both described from *V. karroo* in South Africa and they have not been reported on any other host elsewhere (Jami *et al.,* 2012). Therefore, *S. birrea* is the second host for the two species. *Dothiorella viticola* was first described as a saprophyte from declining *V. vinifera* in Spain (Luque *et al.,* 2005). In South Africa, the fungus is known to occur on *Celtis africana*, *Gymnosporia buxifolia, Prunus persica*, *Podocarpus henkelii*, *Senegalia mellifera, V. karroo* and *V. vinifera* (Jami *et al.,* 2017). *Lasiodiplodia crassispora* is known to occur on *M. indica* in Brazil (Marques *et al.,* 2013). On native hosts, *L. crassispora* has been reported on *P. angolensis* (Mehl *et al.,* 2011) and *S. birrea* (Mehl *et al.,* 2017a) in Mpumalanga. These species are not necessarily new introductions, but their discovery might have been influenced by the more extensive (geographic and host) sampling in this study compared to previous efforts.

The six species, *Do. brevicollis*, *Do. dulcispinae, Do. viticola, L. exigua, Oblongocollomyces ednahkunjekuae* and *Oblongocollomyces* sp. 1 recorded on *S. birrea* for the first time in this study increases the number of *Botryosphaeriaceae* species known on this host to 17. Some species known to occur on *S. birrea* in South Africa were not identified in this study. These include *B. fabicerciana, L. iraniensis, N. mediterraneum, N. umdonicola* and *N. vitifusiforme*

(Mehl *et al.,* 2017a). Except for *N. umdonicola* and *N. vitifusiforme,* the other three species that were not isolated in this study are possibly alien and they were recently identified in South Africa (Mehl *et al.,* 2017a). Timing and location of sampling likely influence these outcomes of species diversity on this host and more *Botryosphaeriaceae* can be expected in previously unsampled areas.

The three fungal species identified on *M. indica*, namely *L. crassispora*, *L. mahajangana* and *N. parvum* are well-known pathogens of this host worldwide (Ismail *et al.,* 2012; Ni *et al.,* 2012; Marques *et al.,* 2013; Trakunyingcharoen *et al.,* 2014). These species are associated with mango diseases in countries such as Brazil (Marques *et al.,* 2013), Egypt (Ismail *et al.,* 2012), Iran (Abdollahzadeh *et al.,* 2010) and Thailand (Trakunyingcharoen *et al.,* 2014). Only two of the identified species, *N. parvum* and *L. mahajangana* have been isolated on mangoes in South Africa (Trakunyingcharoen *et al.,* 2014; Mehl *et al.,* 2017a). This is the first study to report the presence of *L. crassispora* on mango in South Africa, which increases the number of *Botryosphaeriaceae* species recorded on mangoes in South Africa to 11.

This is the first study to consider the presence of *Botryosphaeriaceae* on *L. schweinfurthii* in South Africa. Four species, including *D. allocellula*, *Do. brevicollis*, *L. crassispora* and *L. mahajangana* were identified on this host in undisturbed ecosystems. *Diplodia allocellula* has been reported on *V. karroo* in Gauteng (Jami *et al.,* 2012) and *S. birrea* in Mpumalanga (Mehl *et al.,* 2017a). *Dothiorella brevicollis* has only been reported on *V. karroo* and was isolated on *S. birrea* in this study, making *L. schweinfurthii* a third host for the fungus. *Lasiodiplodia mahajangana* is known to occur on *Adansonia digitata* (Cruywagen *et al.,* 2017), *E. ingens* (van der Linde *et al.,* 2011), *M. indica* and *S. birrea* in Limpopo and Mpumalanga (Mehl *et al.,* 2017a), while *L. crassispora* is known to occur on *S. birrea* and *Pterocarpus angolensis* in Mpumalanga and Kwa-Zulu Natal (Mehl *et al.,* 2011, 2017a). The presence of these fungi on *L. schweinfurthii* supports the assumption that *Botryosphaeriaceae* species occur on virtually all woody tree species and many species remain undiscovered on unsampled native hosts in unexplored areas (Jami *et al.,* 2014).

A new species, *O. ednahkunjekuae* was described in this study. The genus *Oblongocollomyces* was introduced by Yang *et al*. (2017) to accommodate an anomalous “sphaeropsis/diplodia-like” species, originally named *Sphaeropsis variabilis* (Slippers *et al.,* 2014), that was distinct from other *Botryosphaeriaceae* genera based on phylogeny and morphology. *Oblongocollomyces variabilis* was isolated from *Vachellia erioloba* (= *Acacia erioloba*; *Fabaceae*) in Namibia (Slippers *et al.,* 2014). The only other species described in the genus is *O. zhivanae* from north Queensland, Australia, interestingly also from a *Vachellia* species (*V. farnesiana*) (Limbongan *et al.,* 2023). *Oblongocollomyces ednahkunjekuae* was isolated in this study from a different plant family (*S. birrea*; *Anacardiaceae*) in the Mapungubwe National Park in the north of South Africa. These sites from which all three species were isolated are all on a similar latitude below 20th parallel south and are in hot, dry and fairly remote areas. It would appear that this genus is adapted to such conditions. This was also evident in the optimal growth rate of the *O. ednahkunjekuae* at 30 and 35 °C, compared to many other *Botryosphaeriaceae* that typically grow fastest at 20 to 25 °C.

Results obtained from analyses of data on tree health assessment show that dieback is common on tree species of *Anacardiaceae* in the northeastern parts of the Limpopo and Mpumalanga provinces. Various species of *Botryosphaeriaceae* have been reported to cause dieback on various trees globally (Iturritxa *et al.,* 2011; van der Linde *et al.,* 2011; Ismail *et al.,* 2012), but none of the species identified in this study could be consistently linked to these symptoms. This study will, however, lay a foundation for future studies to investigate *Botryosphaeriaceae* species associated with dieback on these trees.

3.6. Conclusions

Members of *Botryosphaeriaceae* are diverse on native and non-native *Anacardiaceae* in disturbed and undisturbed ecosystems. It is evident from this study that land use and disturbance through human activities influence species diversity and distribution of *Botryosphaeriaceae*. The higher species diversity of *Botryosphaeriaceae* and the discovery of potential new species in undisturbed ecosystems emphasise the need to consider different habitats and hosts that may influence patterns of diversity and distribution of these fungi. These results indicate the ease with which invasive pathogens such as *N. parvum* introduced with non-native hosts can spread and infect native hosts once they are established in the region.

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Supplementary Materials

Figure S1: Genealogical concordance phylogenetic species recognition (GCPSR) based on analyses of the ITS, *tef-1α, β-tub* and *rpb2* sequence data. Bootstrap values above 70% and PP values above 0.95 are shown at the nodes. Isolates in **bold** were obtained in this study. The trees are rooted to isolates of *Melanops tulasnei* (CBS116805, CBS116806).

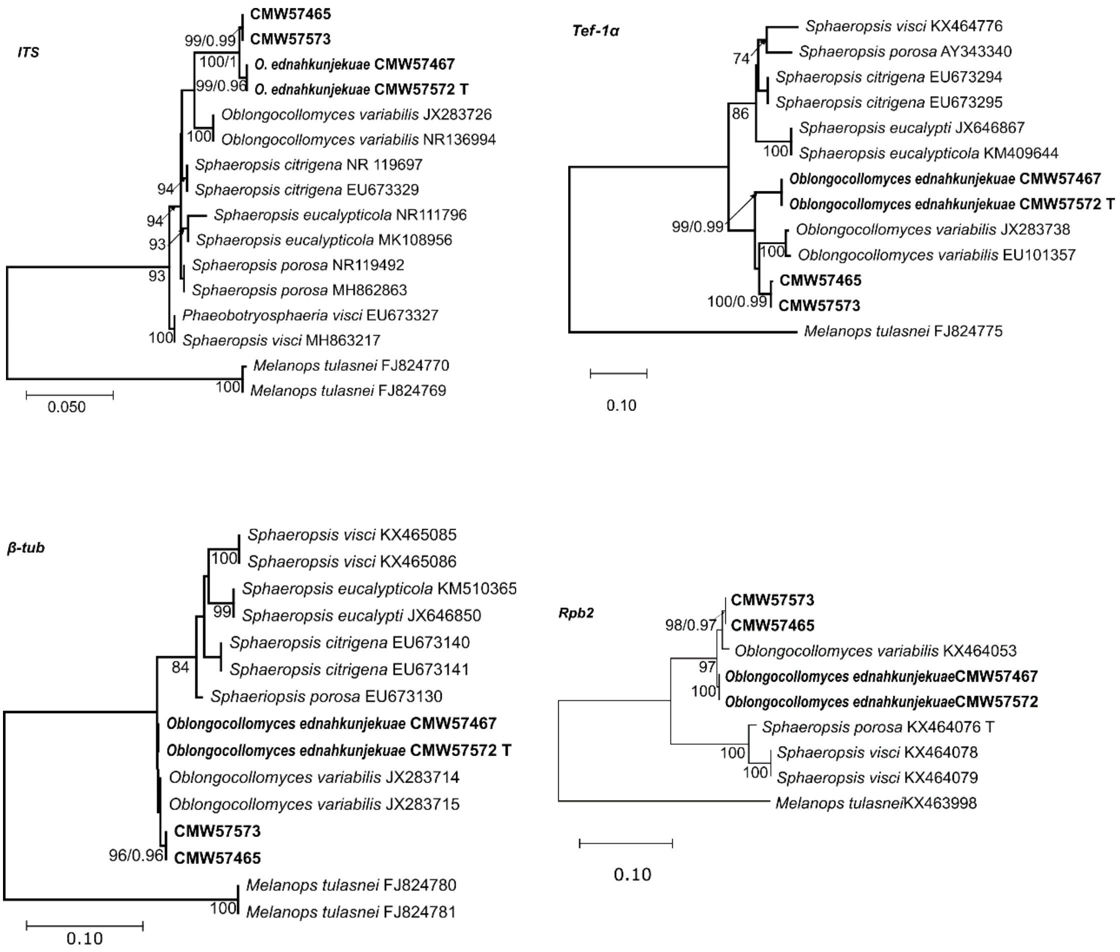

Figure S1: Genealogical concordance phylogenetic species recognition (GCPSR) based on analyses of the ITS, *tef-1α, β-tub* and *rpb2* sequence data*.* Bootstrap values above 70% and PP values above 0.95 are shown at the nodes. Sequences in **bold** were generated in this study. The trees are rooted to *Melanops tulasnei.*

CHAPTER 4

Species of *Botryosphaeriaceae* occurring on asymptomatic and symptomatic tissues of *Anacardiaceae* in South Africa

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Abstract

Members of the *Botryosphaeriaceae* are well-known endophytes and stress-related pathogens. This study focused on the overlap between *Botryosphaeriaceae* associated with three tree species in the *Anacardiaceae*, namely *Sclerocarya birrea*, *Mangifera indica* and *Lannea schweinfurthii*. Isolates were made from both asymptomatic and symptomatic branches from the three tree species. Samples were collected from disturbed (Tshikundamalema and Tshipise in Limpopo) and undisturbed (Nwanedi and the Mapungubwe National Park in Limpopo and the Kruger National Park in Mpumalanga) ecosystems. DNA sequences were produced for selected isolates from regions of the translation elongation factor (*tef-1α*), β-tubulin (*β-tub*), internal transcribed spacer (ITS) and RNA polymerase II subunit genes and included in phylogenetic analyses. Twelve species, *Dothiorella dulcispinae, Dothiorella viticola, Dothiorella brevicollis, Diplodia allocellula, Lasiodiplodia exigua, Lasiodiplodia crassispora, L. gonubiensis, L. mahajangana, L. pseudotheobromae, Neofusicoccum parvum,* a previously unknown species described as *Oblongocollomyces ednahkunjekuae* sp. Nov and *Oblongocollomyces* sp. 1, were identified from the asymptomatic and symptomatic branch samples. Species diversity and dominance of the *Botryosphaeriaceae* occurring in both asymptomatic and symptomatic branches of the same tree species varied across the different sampling sites in disturbed and undisturbed ecosystems. Six species, *D. allocellula, Do. brevicollis*, *Do. viticola*, *L. crassispora*, *L. mahajangana* and *N. parvum,* were identified from both asymptomatic and symptomatic branches of the three tree species of *Anacardiaceae*. *Lasiodiplodia pseudotheobromae* was only isolated from symptomatic samples but is also known as an endophyte from previous studies. These results demonstrate the ability of the *Botryosphaeriaceae* to occupy healthy plant tissues as potential pathogens on these trees.

Keywords: *Anacardiaceae,* asymptomatic, symptomatic, *Botryosphaeriaceae*, potential pathogens, species overlap

4.1. Introduction

Species of *Botryosphaeriaceae* have been implicated in the decline of numerous woody tree species globally (Golzar and Burgess, 2011; Panzavolta *et al.,* 2017; Soltaninejad *et al.,* 2017; Mahamedi *et al.,* 2020). Species of *Botryosphaeriaceae* occur as endophytes and as pathogens on various hosts (Yang *et al.,* 2017; Batista *et al.,* 2021). In the endophytic phase, the *Botryosphaeriaceae* can remain latent in healthy plant tissues without causing disease symptoms. However, they can cause disease on physically stressed trees caused by injury or harsh environmental conditions (Slippers and Wingfield, 2007; Mayorquin *et al.,* 2012). Some species, such as *Diplodia sapinea* on *Pinus* and *Botryosphaeria dothidea* on *Eucalyptus* spp., have been reported to cause disease without the host being under obvious stress (Slippers and Wingfield, 2007; Mohali *et al.,* 2007). Disease symptoms associated with infection by fungi in the *Botryosphaeriaceae* manifest as dieback, canker, gummosis and fruit rots, among others (Mehl *et al.,* 2013; Adesemoye *et al.,* 2014; Netto *et al.,* 2014; Valencia *et al.,* 2019).

Dieback is one of the most commonly reported symptoms associated with infection by fungi in the *Botryosphaeriaceae* (Mehl *et al.,* 2013; Lawrence *et al.,* 2017; Machado *et al.,* 2019). Infection occurs directly through natural openings, as well as through wounds (Slippers and Wingfield, 2007; Mehl *et al.,* 2013; Burgess *et al.,* 2016). Dieback caused by species of *Botryosphaeriaceae* is characterised by dry branches, giving the tree an appearance of fire scorch. Infection usually begins at the tip of the branches where the fungus colonises the soft tissues before moving downwards into the main stem (Iturritxa *et al.,* 2011; Zlatković *et al.,* 2016; Alvindia *et al.,* 2017). The disease has been linked to various species in the family, such as *B. dothidea*, *N. parvum* and *L. theobromae* on *Vitis vinifera* (Qiu *et al*., 2011; Úrbez-Torres *et al.,* 2011; Yan *et al.,* 2013; Stempien *et al.,* 2017), *Fusicoccum aesculi*, *L. crassispora*, *L. pseudotheobromae*, *L. iraniensis, L. mahajangana*, *L. theobromae* and *N. parvum* on *Mangifera indica* (de Oliveira Costa *et al.,* 2010; Ismail *et al.,* 2012; Marques *et al.,* 2013; Trakunyingcharoen *et al.,* 2014), *L. mahajangana* and *L. theobromae* on *Euphorbia ingens* (van der Linde *et al.,* 2011) and *N. protearum* on *Protea* species (Denman *et al.,* 2003; Jami *et al.,* 2017).

Some species in the family are known to occur on both asymptomatic and symptomatic tissues on numerous hosts worldwide (Mohali *et al.,* 2007; Mehl *et al.,* 2013). Examples include *Do. viticola* on *Vachellia karroo* and *Diplodia alatafructa* on *Pterocarpus angolensis* in South Africa (Mehl *et al.,* 2011; Jami *et al.,* 2013)*, Lasiodiplodia margaritaceae* on *Adansonia gregorii* in Australia (Pavlic *et al.,* 2008; Sakalidis *et al.,* 2011a) (Pavlic *et al.,* 2008; Sakalidis *et al.,* 2011a), *Botryosphaeria mamane* on *Eucalyptus* spp. and *Vachellia mangium* in Venezuela (Mohali *et al.,* 2007). Because of this overlap in their occurrence in asymptomatic and symptomatic tissue, species of *Botryosphaeriaceae* are recognised as opportunistic pathogens with a latent endophytic phase, that can easily spread in healthy plant tissues when plants or plant parts are transported (Slippers and Wingfield, 2007; Mohali *et al.,* 2007; Jami *et al.,* 2013).

It was hypothesised that endophytic *Botryosphaeriaceae* are associated with dieback symptoms that have been observed on woody tree species in South Africa (Jami *et al.,* 2013). Testing this hypothesis is hampered by the fact that most studies do not sample from both healthy and diseased tissue. There are no detailed studies that have been conducted to identify species of *Botryosphaeriaceae* occurring on both asymptomatic and symptomatic tissues of tree species of the *Anacardiaceae* at a particular point in time. In the present study, species overlap of the *Botryosphaeriaceae* occurring on asymptomatic and symptomatic branches of three *Anacardiaceae* species, namely *Sclerocarya birrea*, *Mangifera indica* and *Lannea schweinfurthii* in disturbed and undisturbed ecosystems in the Limpopo and Mpumalanga provinces was investigated. These tree species were selected because they are important agricultural commodities used as raw material in the cosmetic industry, as traditional medicine and as a source of extra income by surrounding rural communities.

4.2. Materials and Methods

4.2.1. Sample collection and isolation of fungi

Asymptomatic and symptomatic branches (20 – 30 cm long and 2 – 5 mm thick) were collected from *S. birrea, M. indica* and *L. schweinfurthii* trees in disturbed and undisturbed ecosystems of the Limpopo and Mpumalanga provinces in 2017 and 2018 (see Chapter 3 for a description of sites). Disturbed sites were found at Tshikundamalema and Tshipise in the Limpopo Province and undisturbed sites were found at Nwanedi and the Mapungubwe National Park in Limpopo and the Kruger National Park in Mpumalanga. A single branch showing symptoms of dieback and an asymptomatic branch were collected from each tree and transported to the laboratory for fungal isolations. A total of 404 *Anacardiaceae* trees were surveyed and sampled (see Chapter 3). Asymptomatic branches were collected from all trees and symptomatic branches were collected from 398 trees.

Fungal isolates from asymptomatic branch samples were obtained in Chapter 3 of this Thesis. Symptomatic branches were surface disinfected in 10% hydrogen peroxide, rinsed twice in sterile distilled water and de-barked to expose the interface between necrotic and healthy tissue. Small pieces (1 – 1.5 mm) were isolated from the border zone of necrotic and healthy wood and plated onto 2% MEA with 0.1 g Streptomycin. Primary isolates from both asymptomatic and symptomatic isolations were incubated at 25 °C and checked regularly for fungal growth. Cultures typical of the *Botryosphaeriaceae* (fast growth, grey-black aerial mycelium) were selected and purified by transferring single hyphal tips onto fresh 2% MEA.

Isolates from symptomatic tissue representing different sites and hosts were grouped based on culture morphology and colour using the colour chart of Rayner (1970). These grouped into seven morphogroups. Twelve isolates were selected as representatives and subjected to DNA extraction for preliminary identification. Isolates used in this study are lodged at the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

4.2.2. DNA extraction, amplification and sequencing reactions

Genomic DNA was extracted from the mycelium of 7-day-old cultures following the protocol published by Möller *et al.* (1992). DNA concentrations were quantified using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and adjusted to a working concentration of 50 ɳg $\mu L^{-}1$ using sterile SABAX water (SABAX; Adcock Ingram, Bryanston, S.A). DNA was stored at -20 °C until further use.

Regions from the ITS, *tef-1α*, *β-tub* and *rpb2* genes were amplified and sequenced for all the representative isolates. The ITS region, which includes the ITS-1 spacer, 5.8S and ITS-2 spacer, was sequenced using primer pairs ITS-1 and ITS-4 (White *et al.,* 1990), the *tef-1α* gene region was sequenced using primer pairs tef1-728F and tef1-986R (Carbone and Kohn, 1999), the *β-tub* gene region was sequenced with primers Bt2a and Bt2b (Glass and Donaldson, 1995) while the rpb2 gene was sequenced with primer pairs rpb2-LasF and rpb2-LasR (Cruywagen *et al.,* 2017) and rpb2bot6F and rpb2bot7R (Sakalidis *et al.,* 2011b).

Polymerase chain reactions (PCR) were conducted at a final volume of 25 µL, containing ~ 40 – 50 ɳg genomic DNA, 0.2 µM of each primer, 5 µL MyTaq PCR reaction buffer (10 mM Tris-HCL [pH 8.3], 3.0 mM $MgCl_2$, 50 mM KCl, Roche Diagnostics, Mannheim, Germany), 0.5 υ MyTaq™ DNA polymerase (Bioline, London, UK) and 16.5 µL sterile SABAX water (SABAX; Adcock Ingram, Bryanston, S.A). Amplification reactions were carried out in a thermal cycler (C1000, Bio-Rad, USA) using the following conditions; initial denaturation for 2 min at 94 °C, followed by 30 cycles for 30 sec at 94 °C, annealing for 30 sec at 54 °C (ITS and *rpb2*) and 56 °C (*tef-1α* and *β-tub*), extension for 1 min at 72 °C and final extension for 7 min at 72 °C. The resulting amplicons were separated by gel electrophoresis on 1% agarose gel stained with GelRed® and visualised under UV light. Exosap (Mixture of Exonuclease I and FastAP

Alkaline Phosphatase) (Thermo Fisher Scientific Inc. Waltham, MA, USA) was used to purify amplicons following manufacturer's specifications.

Sequencing reactions were conducted using the ABI Prism® Big Dye™ Sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing of the amplicons was conducted at the sequencing facility of the University of Pretoria. Sequence reads were obtained in both directions (forward and reverse) using the same primers that were amplified for the respective gene regions. Consensus sequences from the forward and reverse sequence reads were generated using CLC Main Workbench v.7.9 (QIAGEN, Aarhus, Denmark).

4.2.3. Phylogenetic analyses and identification of isolates

ITS, *tef-1α*, *β-tub* and *rpb2* sequences generated in this study were preliminarily identified by comparing them with sequences of previously published species on the GenBank database (http://www.ncbi.nlm.nih.gov/genbank) using BLASTn searches. Reference sequences showing similarity to query sequences on BLASTn searches were retrieved from GenBank.

Twenty isolates obtained from asymptomatic branches were selected based on their host differences and grouping in the phylogenetic trees presented in Chapter 3 (Table 4.1). Sequences from these isolates together with the selected isolates from symptomatic branches and reference sequences from GenBank were aligned using the online version of MAFFT v.7 (Katoh *et al.,* 2019). Manual adjustments of sequence alignment were carried out using BioEdit Sequence Alignment Editor v.7.2.5 (Hall, 1999). Sequence datasets for the four loci were analysed individually and in combination. The best nucleotide substitution model for the datasets was determined using jModelTest v.2.1.7 (Darriba *et al.,* 2012) under the Akaike Information Criterion (AIC) to select the model that optimally fits the data.

Phylogenetic analyses were conducted using Maximum likelihood and Bayesian methods. Maximum likelihood (ML) phylogenetic analyses were performed using raxmlGUI v.2.0 (Edler *et al.,* 2021), with the models HKY + I + G, TIM2 + I + G, TrN + I, TrN + G and TrN + I + G4 selected for the ITS, *tef-1α*, *β-tub, rpb2* and combined datasets, respectively. Bootstrap analyses were done using 1000 replicates to determine the robustness of the trees.

Bayesian inference (BI) of phylogenetic trees was performed using MrBayes v.3.2.6 (Ronquist and Huelsenbeck, 2003). Four simultaneous Markov chains were run for 3 x 10^6 generations and trees were sampled every 100th generation. The first 7500 trees representing the burn-in phase of the analyses were discarded and the remaining 22500 trees were used to calculate posterior probabilities (PP) based on a majority rule consensus tree. Effective sampling size (ESS) values were assessed using Tracer v.1.7.1 (http://tree.bio.ed.ac.uk/software/tracer/).

Trees were viewed in FigTree v.1.3.1 (Rambaut, 2009). The trees were rooted to sequences of *Melanops tulasnei* (*Botryosphaeriales*; *Melanopsaceae*) as the outgroup taxa.

All the isolates from asymptomatic and symptomatic tissue were assigned to a species based on their grouping within morphogroups and the identity of the representative isolates that were included in the phylogenetic analyses. Isolates that looked different from other isolates in the morphogroups were also sequenced to confirm their identity.

Table 4.1. Representative fungal isolates obtained from asymptomatic and symptomatic branches of *Anacardiaceae* included in the dataset for analyses. Isolates in **bold** were sequenced in this study. Isolates from asymptomatic tissue are those sequenced in Chapter 3.

Isolate No.	Identity	Host	Tissue type	Site	GenBank accession numbers			
					ITS	*tef-1α*	*β-tub*	*rpb2*
CMW54349	*Diplodia allocellula*	*L. schweinfurthii*	Asymptomatic branch	Nwanedi	OL441853	OL441909	OL441965	OM585579
CMW54351	*D. allocellula*	*S. birrea*	Asymptomatic branch	Kruger National Park	OL441855	OL441911	OL441967	OM585581
CMW54350	*D. allocellula*	*L. schweinfurthii*	Symptomatic branch	Nwanedi	OL441854	OL441910	OL441966	OM585580
CMW54352	*D. allocellula*	*S. birrea*	Symptomatic branch	Kruger National Park	OL441856	OL441912	OL441968	OM585582
CMW57630	*Dothiorella brevicollis*	*S. birrea*	Asymptomatic branch	Mapungubwe National Park	OL441859	OL441915	OL441971	-
CMW57631	*Do. brevicollis*	*L. schweinfurthii*	Symptomatic branch	Nwanedi	OL441860	OL441916	OL441972	-

Table 4.1. (Continued)

Isolate No.	Identity	Host	Tissue type	Site	GenBank accession numbers			
					ITS	*tef-1α*	*β-tub*	*rpb2*
CMW57462	*Do. dulcispinae*	*S. birrea*	Asymptomatic branch	Kruger National Park	OL441861	OL441917	OL441973	-
CMW57466	*Do. dulcispinae*	*S. birrea*	Asymptomatic branch	Mapungubwe National Park	OL441862	OL441918	OL441974	-
CMW57463	*Do. viticola*	*S. birrea*	Asymptomatic branch	Mapungubwe National Park	OL441863	OL441919	OL441975	-
CMW57569	*Do. viticola*	*S. birrea*	Symptomatic branch	Mapungubwe National Park	OL441865	OL441921	OL441977	-
CMW54318	*Lasiodiplodia crassispora*	*L. schweinfurthii*	Asymptomatic branch	Kruger National Park	OL441866	OL441922	OL441978	OL442026
CMW54319	*L. crassispora*	*S. birrea*	Asymptomatic branch	Tshikundamalema	OL441867	OL441923	OL441979	OL442022
CMW54322	*L. crassispora*	*S. birrea*	Symptomatic branch	Mapungubwe National Park	OL441871	OL441927	OL441983	OL442026

Table 4.1. (Continued)

Isolate No.	**Identity**	**Host**	**Tissue type**	**Site**	**GenBank accession numbers**			
					ITS	***tef-1α***	***β-tub***	***rpb2***
CMW54323	*L. crassispora*	*S. birrea*	Symptomatic branch	Mapungubwe National Park	OL441872	OL441928	OL441984	OL442027
CMW54314	*L. gonubiensis*	*S. birrea*	Asymptomatic branch	Kruger National Park	OL441873	OL441929	OL441985	OL442028
CMW54315	*L. gonubiensis*	*S. birrea*	Asymptomatic branch	Kruger National Park	OL441874	OL441930	OL441986	OL442029
CMW54312	*L. exigua*	*S. birrea*	Asymptomatic branch	Mapungubwe National Park	OL441875	OL441931	OL441987	OL442030
CMW54313	*L. exigua*	*S. birrea*	Asymptomatic branch	Mapungubwe National Park	OL441876	OL441932	OL441988	OL442031
CMW54329	*L. mahajangana*	*L. schweinfurthii*	Asymptomatic branch	Nwanedi	OL441878	OL441934	OL441990	OL442033
CMW54331	*L. mahajangana*	*S. birrea*	Asymptomatic branch	Mapungubwe National Park	OL441880	OL441936	OL441992	OL442035

Table 4.1. (Continued)

Isolate No.	Identity	Host	Tissue type	Site	GenBank accession numbers			
					ITS	***tef-1α***	***β-tub***	***rpb2***
CMW54330	*L. mahajangana*	*M. indica*	Symptomatic branch	Tshipise	OL441879	OL441935	OL441991	OL442034
CMW54335	*L. mahajangana*	*M. indica*	Symptomatic branch	Kruger National Park	OL441882	OL441938	OL441994	OL442037
CMW54337	*L. pseudotheobromae*	*S. birrea*	Symptomatic branch	Kruger National Park	OL441883	OL441939	OL441995	OL442038
CMW54339	*L. pseudotheobromae*	*L. schweinfurthii*	Symptomatic branch	Kruger National Park	OL441884	OL441940	OL441996	OL442039
CMW54357	*Neofusicoccum parvum*	*S. birrea*	Asymptomatic branch	Tshipise	OL441892	OL441948	OL442004	OL442047
CMW54360	*N. parvum*	*M. indica*	Asymptomatic branch	Tshikundamalema	OL441894	OL441950	OL442006	OL442049
CMW54361	*N. parvum*	*M. indica*	Symptomatic branch	Tshipise	OL441895	OL441951	OL442007	OL442050

Table 4.1. (Continued)

Isolate No.	**Identity**	**Host**	**Tissue type**	**Site**	**GenBank accession numbers**			
					ITS	***tef-1α***	***β-tub***	***rpb2***
CMW54362	*N. parvum*	*M. indica*	Symptomatic branch	Tshipise	OL441896	OL441952	OL442008	OL442051
CMW57467	*Oblongocollomyces ednahkunjekuae*	*S. birrea*	Asymptomatic branch	Mapungubwe National Park	OL441905	OL441961	OL442017	OL442060
CMW57572	*Oblongocollomyces ednahkunjekuae*	*S. birrea*	Asymptomatic branch	Mapungubwe National Park	OL441906	OL441962	OL442018	OL442061
CMW57465	*Oblongocollomyces* sp. 1	*S. birrea*	Asymptomatic branch	Mapungubwe National Park	OL441907	OL441963	OL442019	OL442062
CMW57573	*Oblongocollomyces* sp. 1	*S. birrea*	Asymptomatic branch	Mapungubwe National Park	OL441908	OL441964	OL442020	OL442063

4.3. Results

4.3.1. Sample collection and tree health status

Most trees with symptoms of dieback did not appear to be seriously affected, except *M. indica* trees at Tshikundamalema and Tshipise which showed severe symptoms of dieback (Figure 4.1). It is not clear whether the dieback and severe decline symptoms on *M. indica* trees are only due to infection by species of the *Botryosphaeriaceae* or other factors such as nutrient deficiency and unfavourable abiotic conditions.

Figure 4.1. *Mangifera indica* trees with severe dieback surrounded by healthy *M. indica* trees at Tshipise.

4.3.2. Fungal isolates

A total of 575 isolates with typical culture morphology and colour characteristics of the *Botryosphaeriaceae* were obtained from asymptomatic and symptomatic samples. Isolations from asymptomatic samples yielded 404 isolates (see Chapter 3), while isolations from symptomatic samples yielded 171 isolates. Most isolates from both asymptomatic and symptomatic samples were obtained from *S. birrea*, followed by *L. schweinfurthii* and then *M. indica* (Table 4.2).

Table 4.2. Number of *Botryosphaeriaceae* isolates obtained from asymptomatic and symptomatic branches of *Sclerocarya birrea*, *Lannea schweinfurthii* and *Mangifera*.

Tree species	**No. of isolates (asymptomatic branches)**	**No. of isolates (symptomatic branches)**
S. birrea	192	71
L. schweinfurthii	112	65
M. indica	100	35

4.3.3. DNA Extraction, amplification and sequencing

DNA amplification and sequencing were successful for the ITS, *tef-1α* and *β-tub* loci of all isolates. The *rpb*2 gene region could not be amplified for some isolates. Isolates were preliminarily identified through Blastn searches. BLASTn analyses of the sequences showed the highest similarity to species of *Botryosphaeriaceae.*

4.4. Phylogenetic analyses and identification of isolates

Alignment of sequences yielded 582 bp, 546 bp, 450 bp, 573 bp and 2152 bp for the ITS, *tef-1α*, *β-tub, rpb2* and combined dataset, respectively. The phylogenetic trees generated from ML and BI analyses of the ITS (Figure 4.2), *tef-1α* (Figure 4.3), *β-tub* (Figure 4.4), *rpb2* (Figure 4.5), as well as the combined (Figure 4.6) datasets, were similar in the separation of clades representing species identified. Isolates included in phylogenetic analyses were separated into five main clades corresponding to five genera of *Botryosphaeriaceae*; *Diplodia*, *Dothiorella, Lasiodiplodia*, *Neofusicoccum* and *Oblongocollomyces/Sphaeropsis*.

4.4.1. ITS phylogeny

The phylogeny emerging from analyses of the ITS sequence data separated the isolates into five clades. The five clades represented *Lasiodiplodia* (clade I)*,* *Diplodia* (clade II), *Oblongocollomyces/Sphaeropsis* (clade III), *Dothiorella* (clade IV) and *Neofusicoccum* (clade

V) (Figure 4.2).

Isolates in the *Lasiodiplodia* clade were further separated into five sub-clades. Isolates CMW54337 and CMW54339 formed a monophyletic clade with *L. pseudotheobromae* (BS = 78%; PP = 0.99). Isolates CMW54329, CMW54330, CMW54331 and CMW54335 together with *L. vaccinii* and *L. magnoliae* formed a polytomy within the genus based on the ITS sequence data. Isolates CMW54312 and CMW54313 grouped with *L. americana* and *L. exigua* (BS = 97%; PP = 1). Isolates CMW54314 and CMW54315 formed a highly supported monophyletic clade with *L. gonubiensis* (BS = 99%; PP = 1). Isolates CMW54318, CMW54319, CMW54322 and CMW54323 grouped with *L. crassispora* and *L. pyriformis* (BS = 100%; PP = 1), but with *L. pyriformis* forming a subclade within the bigger clade (BS = 97%).

The *Diplodia* clade included *D. allocellula*, *D. eriobotryicola* and *D. sapinea* sequences. Isolates CMW54349, CMW54350, CMW54351 and CMW54352 formed a polytomy with *D. allocellula* (BS = 97%), but without PP support. Isolates CMW57465, CMW57467, CMW57572 and CMW57573 in clade III did not group with any previously described species of *Botryosphaeriaceae.* These isolates formed two highly supported sister clades (CMW57465 and CMW57573) (BS = 96%; PP = 0.99) and (CMW57467 and CMW57572) (BS = 96%; PP = 0.96) indicating that they possibly represent two closely related but distinct species. The *Dothiorella* clade included three sub-clades, with isolates CMW57463 and CMW57569 grouping with *Do. plurivora* and *Do. viticola* (BS = 93%), but without PP support. Two isolates, CMW54630 and CMW57631 formed a polytomy within the genus based on the ITS sequence data. Isolates CMW57462 and CMW54466 grouped with *Do. dulcispinae* and *Do. oblonga* (BS = 96%; PP = 0.95). Clade V included *N. parvum, N. ribis* and *N. umdonicola* sequences. Isolates CMW54357, CMW54360, CMW54361 and CMW54362 formed a monophyletic clade with *N. parvum* (BS = 80%; PP = 0.99).

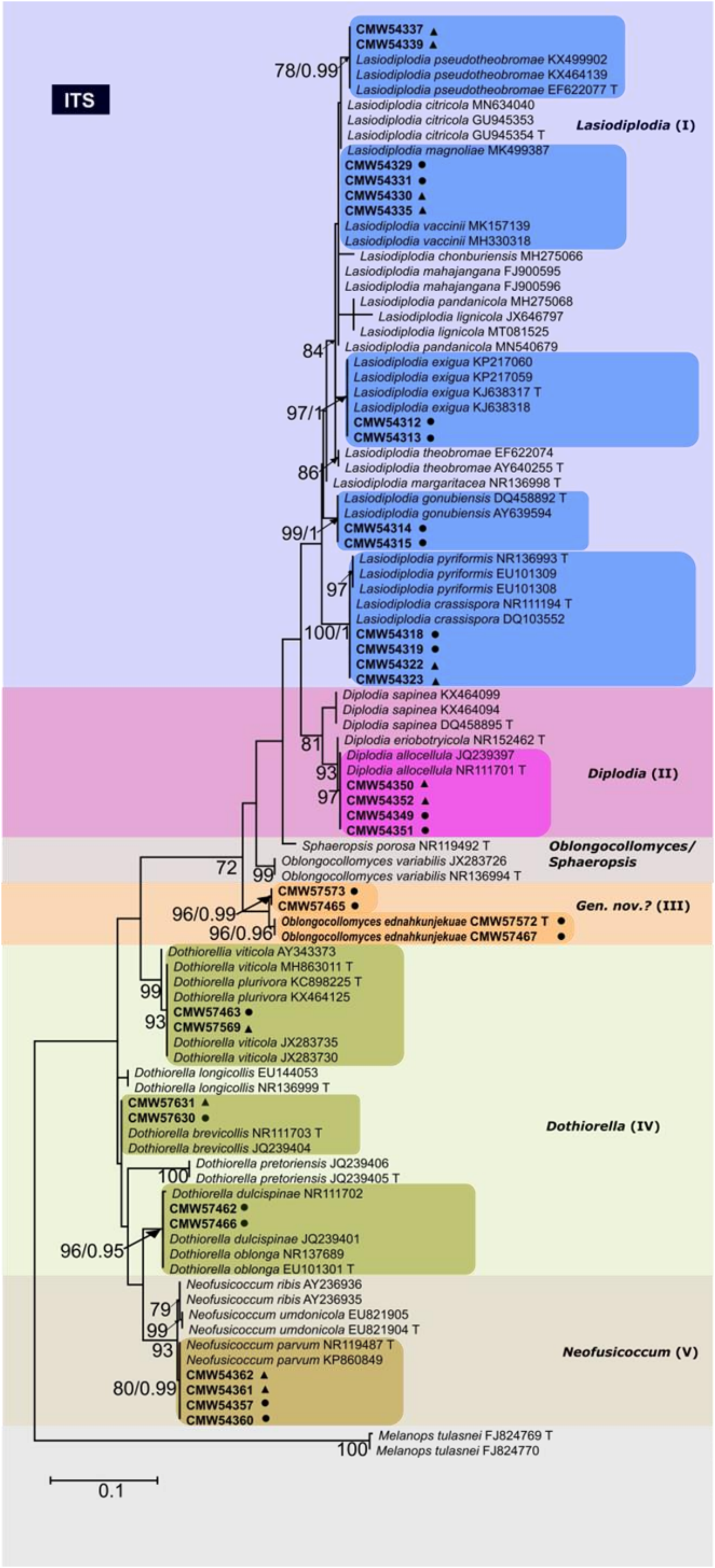

Figure 4.2. Phylogenetic tree generated from ML and BI analyses of the ITS dataset. Isolates from asymptomatic branches are indicated with (●) and those from symptomatic branches are indicated with (▲). Bootstrap support values above 70% and PP values above 0.95 are shown on the tree. *Melanops tulasnei* sequences are rooted to the tree as outgroup taxa.

4.4.2. *Tef-1α* phylogeny

Maximum likelihood and BI analyses of the *tef-1α* dataset separated isolates included in this study into five clades. The clades represented the genera *Lasiodiplodia* (clade I), *Diplodia* (clade II), *Oblongocollomyces/Sphaeropsis* (clade III), *Neofusicoccum* (clade IV) and *Dothiorella* (clade V) (Figure 4.3). Most clades did not receive BS and PP support.

The *Lasiodiplodia* clade included isolates corresponding to *L. crassispora*, *L. exigua*, *L. gonubiensis*, *L. mahajangana* and *L. pseudotheobromae*. Isolates CMW54329, CMW54330, CMW54331 and CMW54335 grouped with *L. mahajangana* (BS = 76%), but without PP support. Isolates CMW54312 and CMW54313 together with *L. exigua* formed a polytomy within the genus clade. Isolates CMW54337 and CMW54339 grouped with *L. pseudotheobromae* but without BS and PP support. Isolates CMW54318, CMW54319, CMW54322 and CMW54323 grouped with *L. crassispora,* separate from *L. pyriformis* but without BS and PP support. Two isolates, CMW54314 and CMW54315 grouped with *L. gonubiensis* (BS = 100%), but without PP support. Clade II included *D. allocellula, D. eriobotryicola* and *D. sapinea* sequences. Isolates CMW54349, CMW54350, CMW54351 and CMW54352 grouped with *D. allocellula* (BS = 96%; PP = 0.99), the group also included sequences of *D. eriobotryicola* as a subgroup.

Clade III included *O. variabilis* and *S. porosa* sequences and four isolates from the sampled trees clustered in this clade. The four isolates grouped into two sub-clades, with isolates CMW57465 and CMW57573 forming a sister clade with *O. variabilis* (BS = 100%), but without PP support and isolates CMW57467 and CMW57572 forming a sister clade with both *O. variabilis* and the sub-clade with isolates CMW57465 and CMW57573 (BS = 81%), but without PP support. The *Neofusicoccum* clade included *N. parvum, N. ribis* and *N. umdonicola* sequences. Isolates CMW54357, CMW54360, CMW54361 and CMW54362 grouped with *N. parvum* (BS = 90%), but without PP support. Clade V included *Dothiorella* sequences. Isolates CMW57463 and CMW57569 grouped with *Do. viticola* (BS = 98%), but without PP support. Two isolates, CMW57630 and CMW54631 grouped with *Do. brevicollis* (BS = 86%; PP = 1). Isolates CMW57462 and CMW57466 grouped with sequences of *Do. oblonga* (BS = 100%; PP = 1), and separate from *Do. dulcispinae*.

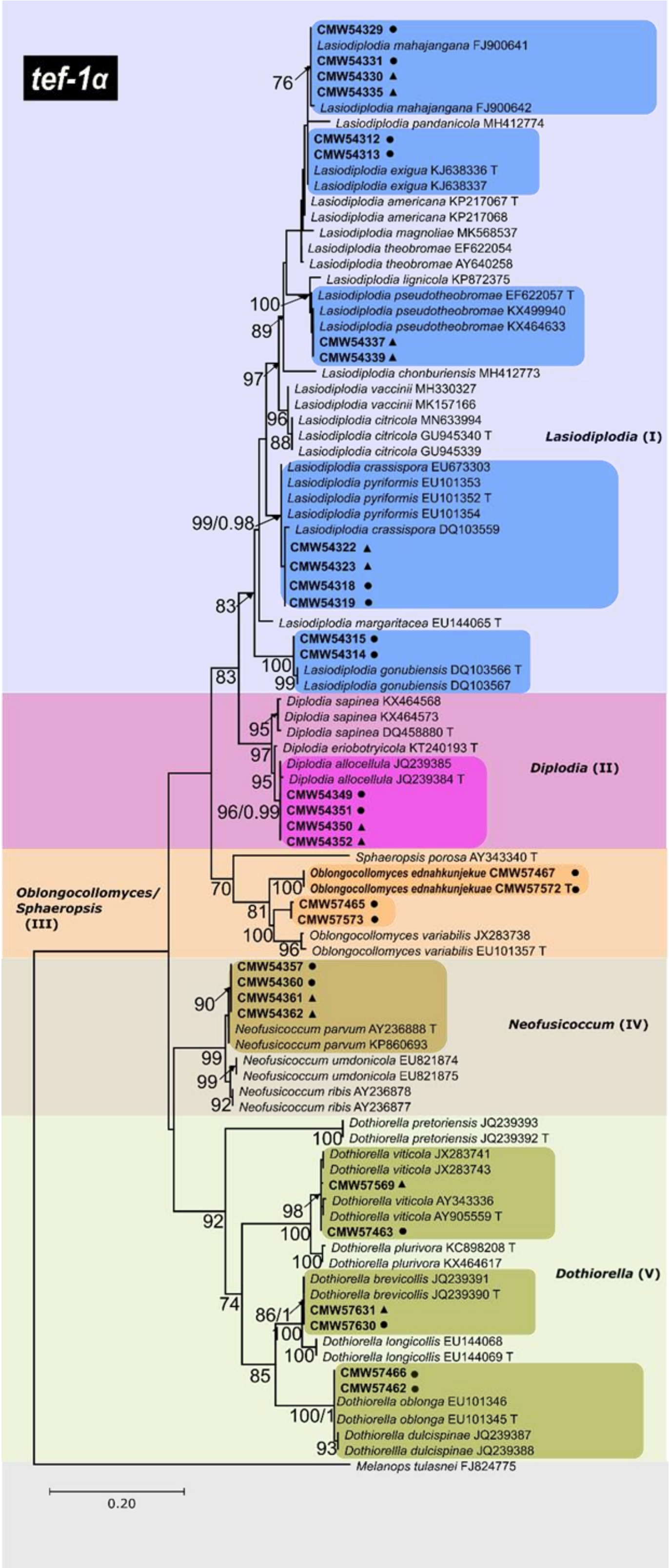

Figure 4.3. Phylogenetic tree based on analyses of the *tef-1α* dataset. Isolates from asymptomatic branches are indicated with (●) and those from symptomatic branches are indicated with (▲). Bootstrap support values (> 70%) and PP values (> 0.95) are shown on the tree. *Melanops tulasnei* sequences are rooted to the tree as outgroup taxa.

4.4.3. *β-tub* phylogeny

The *β-tub* phylogeny separated isolates from the sampled branches into five main clades corresponding to five genera of *Botryosphaeriaceae* (Figure 4.4). The clades represented *Dothiorella* (clade I), *Neofusicoccum* (clade II), *Oblongocollomyces/Sphaeropsis* (clade III), *Diplodia* (clade IV) and *Lasiodiplodia* (clade V).

The *Dothiorella* clade included three sub-clades, with isolates CMW57463 and CMW57569 grouping with *Do. plurivora* and *Do. viticola* (BS = 81%), but without PP support. Isolates CMW57630 and CMW57631 grouped with *Do. brevicollis* (BS = 98%; PP = 1). Isolates CMW57462 and CMW57466 grouped with *Do. dulcispinae* and *Do. oblonga* (BS = 100%; PP = 1). The *Neofusicoccum* clade included *N. parvum, N. ribis* and *N. umdonicola* sequences. Isolates CMW54357, CMW54360, CMW54361 and CMW54362 formed a monophyletic clade with *N. parvum* (BS = 80%), but without PP support. Clade III included four isolates that clustered with *Oblongocollomyces* and *Sphaeropsis* sequences. Two isolates, CMW57465 and CMW57573 formed a sister clade with *S. porosa,* while isolates CMW57467 and CMW57572 formed a sister clade with both *S. porosa* and isolates CMW57465 and CMW57573.

Clade IV included *D. allocellula*, *D. eriobotryicola* and *D. sapinea* sequences, with isolates CMW54349, CMW54350, CMW54351 and CMW54352 grouping with *D. allocellula* and *D. eriobotryicola* (BS = 100%; PP = 0.99). The *Lasiodiplodia* clade included isolates corresponding to *L. crassispora*, *L. gonubiensis*, *L. mahajangana* and *L. pseudotheobromae*. Isolates CMW54318, CMW54319, CMW54322 and CMW54323 grouped with *L. crassispora* and *L. pyriformis* (BS = 78%), but without PP support. Two isolates, CMW54337 and CMW54339 grouped with *L. pseudotheobromae* and *L. theobromae* but without BS and PP support. Isolates CMW54312 and CMW54313 together with *L. exigua* formed a polytomy within the genus based on the *β-tub* sequence data. Isolates CMW54329, CMW54330, CMW54331 and CMW54335 formed a monophyletic clade with *L. mahajangana* (BS = 88%; PP = 0.99). Isolates CMW54314 and CMW54315 formed a highly supported monophyletic clade with *L. gonubiensis* (BS = 99%; PP = 0.99).

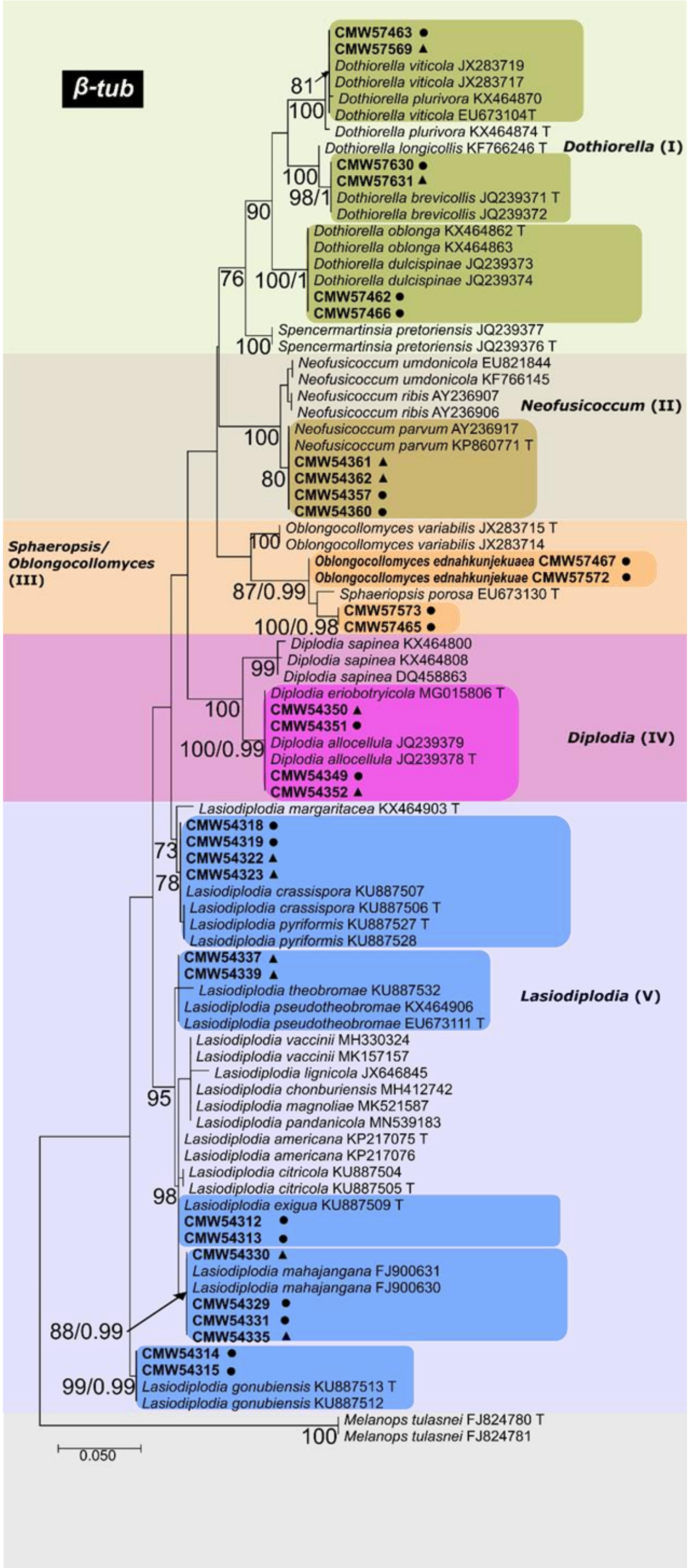

Figure 4.4. Phylogenetic tree generated from maximum likelihood and bayesian inference analyses of the *β-tub* dataset. Isolates from asymptomatic branches are indicated with (●) and those from symptomatic branches are indicated with (▲). Bootstrap support values (> 70%) and PP values (> 0.95) are shown on the tree. *Melanops tulasnei* sequences are rooted to the tree as outgroup taxa.

4.4.4. *Rpb2* phylogeny

The phylogeny emerging from ML and BI analyses of the *rpb2* sequence data separated the isolates included in this study into four clades. The four clades represented *Lasiodiplodia* (clade I), *Diplodia* (clade II)*, Oblongocollomyces*/*Sphaeropsis* (clade III) and *Neofusicoccum* (clade IV) (Figure 4.5).

Isolates in the *Lasiodiplodia* clade were distinguished as *L. gonubiensis, L. mahajangana, L. crassispora* and *L. pseudotheobromae.* Isolates CMW54329, CMW54330, CMW54331 and CMW54335 grouped with *L. mahajangana* (BS = 77%), but without PP support. Isolates CMW54312 and CMW54313 formed a polytomy within the clade based on the *rpb2* sequence data. Isolates CMW54337 and CMW54339 formed a highly supported monophyletic clade with *L. pseudotheobromae* (BS = 98%; PP = 1). Isolates CMW54314 and CMW54315 corresponding to *L. gonubiensis* formed a highly supported monophyletic clade (BS = 100%; PP = 1). Four isolates, CMW54318, CMW54319, CMW54322 and CMW54323 formed a polytomy within a clade that included *L. crassispora* (BS = 100%; PP = 1), and separate from *L. pyriformis* that was also included in the group.

The *Diplodia* clade included *D. sapinea* sequences only because *rpb2* sequences for other *Diplodia* species are unavailable on public databases. Four isolates in the *Diplodia* clade (CMW54349, CMW54350, CMW54351 and CMW54352) formed a monophyletic group (BS = 98%). This group placed sister to the *D. sapinea* clade, indicating that they are members of the same genus (BS = 98%). These isolates were identified as *D. allocellula* based on analyses of the ITS, *tef-1α* and *β-tub* sequence data*.* The four isolates (CMW57465, CMW57467, CMW57572 and CMW57573) that could not be assigned to a species based on analyses of the ITS, *tef-1α* and *β-tub* sequence datasets could not be identified to species level based on analyses of the *rpb2* sequence data. Two of the isolates, CMW57465 and CMW57573 formed a sister clade with *O. variabilis* (BS = 100%; PP = 1), while isolates CMW57467 and CMW57572 formed a sister clade with both *O. variabilis* and isolates CMW57465 and CMW57573 (BS = 100%; PP = 1). The *Neofusicoccum* clade included *N. parvum, N. ribis* and *N. umdonicola* sequences, with isolates CMW54357, CMW54360, CMW54361 and CMW54362 grouping with *N. parvum* (BS = 88%; PP = 1).

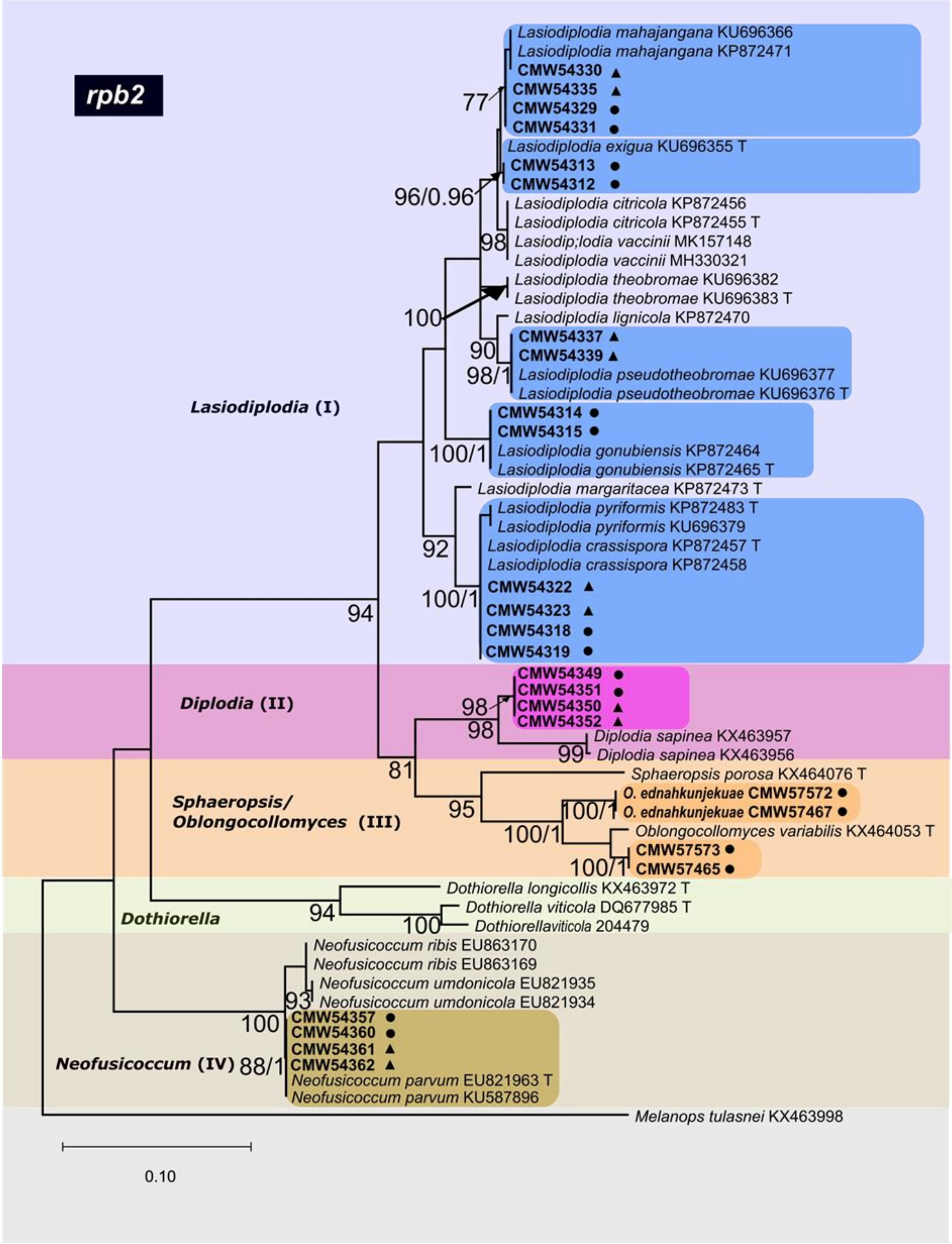

Figure 4.5. Phylogenetic tree based on ML and BI analyses of the *rpb*2 dataset. Isolates from asymptomatic branches are indicated with (●) and those from symptomatic branches are indicated with (▲). Bootstrap values above 70% and PP values above 0.95 are shown at the nodes. The tree was rooted to *Melanops tulasnei.*

4.4.5. Combined phylogeny

The phylogeny emerging from ML and BI analyses of the combined dataset separated isolates from the sampled trees into five clades. The clades represented the genera *Lasiodiplodia* (clade I), *Diplodia* (clade II), *Oblongocollomyces/Sphaeropsis* (clade III), *Neofusicoccum* (clade IV) and *Dothiorella* (clade V) (Figure 4.6).

The *Lasiodiplodia* clade included five sub-clades, with isolates CMW54329, CMW54330, CMW54331 and CMW54335 forming a polytomy with *L. mahajangana* (BS = 99%; PP = 0.95). Isolates CMW54312 and CMW54313 formed a monophyletic clade with *L. exigua* (BS = 77%), but without PP support. Two isolates, CMW54337 and CMW54339 formed a highly supported monophyletic clade with *L. pseudotheobromae* (BS = 100%; PP = 1). Isolates CMW54314 and CMW54315 formed a polytomy with *L. gonubiensis* (BS = 100%; PP = 1). Isolates CMW54318, CMW54319, CMW54322 and CMW54323 grouped with *L. crassispora,* separate from *L. pyriformis* but without BS and PP support. Clade II included *D. allocellula, D. eriobotryicola* and *D. sapinea* sequences. Isolates CMW54349, CMW54350, CMW54351 and CMW54352 grouped with *D. allocellula* (BS = 97%; PP = 0.95), the group also included sequences of *D. eriobotryicola* as a subgroup.

Clade III included *O. variabilis* and *S. porosa* sequences. Four isolates, CMW57572, CMW57573, CMW57465 and CMW57467 clustered in this clade. Isolates CMW57465 and CMW57573 formed a highly supported sister clade with *O. variabilis* (BS = 100%; PP = 1), while isolates CMW57467 and CMW57572 formed a sister clade with both *O. variabilis* and the sub-clade with isolates CMW57465 and CMW57573 (BS = 100%; PP = 0.99). Clade IV included *N. parvum, N. ribis* and *N. umdonicola* sequences, with isolates CMW54357, CMW54360, CMW54361 and CMW54362 forming a polytomy with *N. parvum* (BS = 98%; PP = 0.95), separate from *N. ribis* and *N. umdonicola.* The *Dothiorella* clade included three sub-clades, with isolates CMW57463 and CMW57569 grouping with *Do. plurivora* and *Do. viticola* (BS = 81%), but without PP support. Isolates CMW57462 and CMW57466 formed a polytomy with *Do. oblonga* (BS = 100%; PP = 1), separate from *Do. dulcispinae.* Isolates CMW57630 and CMW57631 formed a highly supported monophyletic clade with *Do. brevicollis* (BS = 100%; PP = 0.99).

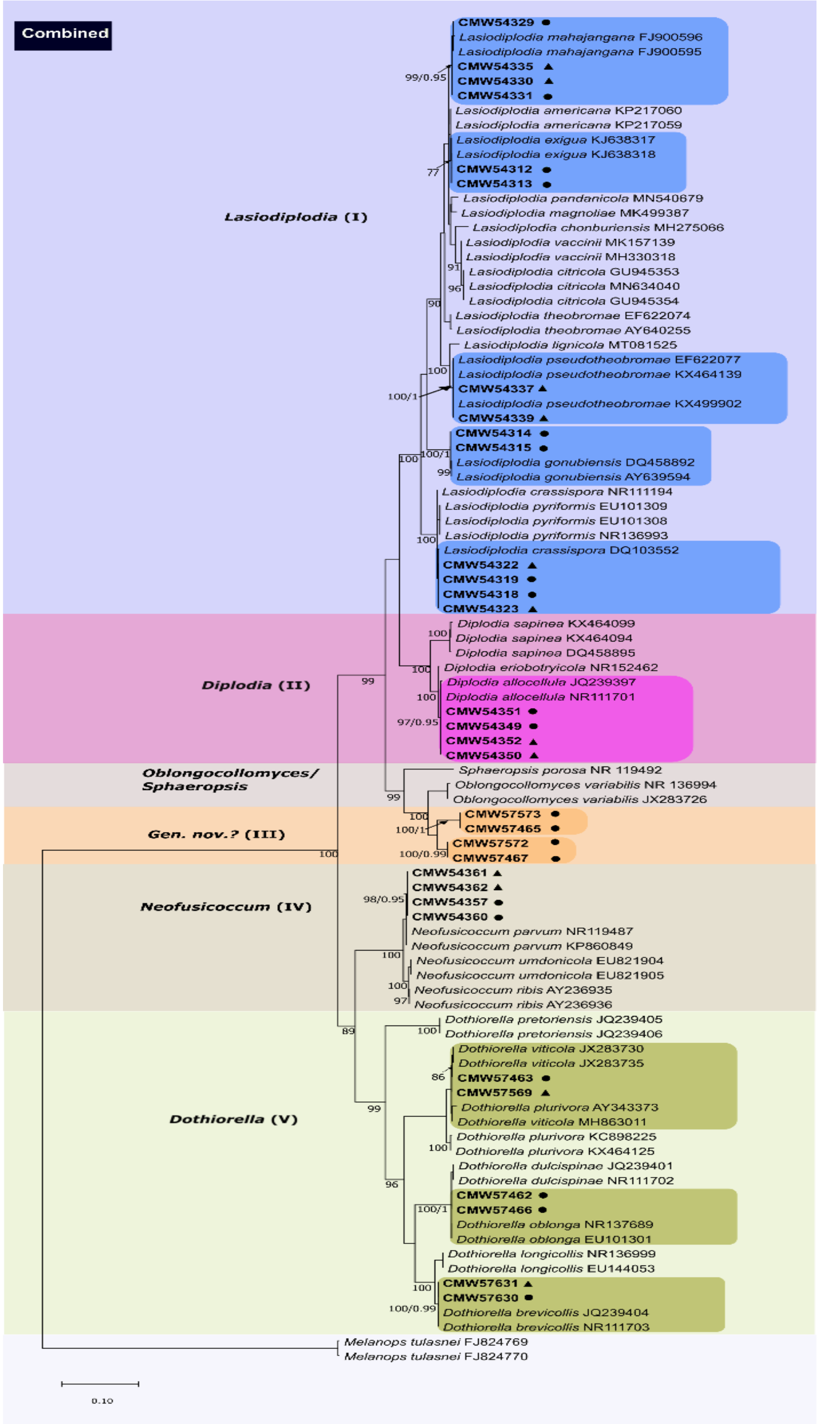

Figure 4.6. Phylogenetic tree based on ML and BI analyses of the combined dataset including the ITS, *β-tub*, *tef-1α* and *rpb2* gene regions. Isolates from asymptomatic branches are indicated with (●) and those from symptomatic branches are indicated with (▲). Bootstrap values above 70% and PP values above 0.95 are shown at the nodes. *Melanops tulasnei* sequences are rooted to the tree as outgroup taxa.

4.5. Concordance between phylogenies for species identification

The topologies of the phylogenetic trees from ML and BI analyses of the individual and combined datasets were similar in the separation of clades representing genera of *Botryosphaeriaceae* identified in this study. Evaluation of the four loci in resolving species of *Botryosphaeriaceae* revealed that the robustness of the locus is related to the genus in question. None of the loci provided 100% resolution on the separation of isolates. This was mainly due to some of the isolates not clustering into monophyletic clades with previously described species of *Botryosphaeriaceae*. Some phylogenies could not distinguish between *Do. plurivora* and *Do. viticola, Do. dulcispinae* and *Do. oblonga*, *L. magnoliae, L. mahajangana* and *L. vaccinii.* In addition, four isolates (CMW57465, CMW57467, CMW57572 and CMW57573) obtained from *S. birrea* at Mapungubwe could not be assigned to previously published sequences of a species. In phylogenetic analyses, these isolates grouped within a clade that included *Oblongocollomyces* and *Sphaeropsis* species but were phylogenetically close to *O. variabilis*. Two of the four isolates were described as *Oblongocollomyces ednahkunjekuae* based on morphological characteristics and the other two isolates remains undescribed and they are referred to as *Oblongocollomyces* sp. 1.

Based on phylogenetic concordance of the five datasets, twelve species, namely *D. allocellula*, *Do. brevicollis*, *Do. dulcispinae*, *Do. viticola*, *L. crassispora*, *L. exigua*, *L. gonubiensis*, *L. mahajangana*, *L. pseudotheobromae*, *N. parvum*, *O. ednahkunjekuae* and *Oblongocollomyces* sp. 1 were identified from asymptomatic and symptomatic samples of the three tree species sampled.

4.6. Species diversity and co-occurrence on healthy and infected tissue

Diplodia allocellula, *Do. brevicollis*, *Do. dulcispinae*, *Do. viticola*, *L. crassispora*, *L. exigua*, *L. gonubiensis*, *L. mahajangana*, *L. pseudotheobromae*, *N. parvum*, *Oblongocollomyces ednahkunjekuae* and *Oblongocollomyces* sp. 1 were identified as endophytes and as potential pathogens on the three tree species of *Anacardiaceae* in the Limpopo and Mpumalanga provinces.

Six species; *D. allocellula, Do. brevicollis, Do. viticola, L. crassispora, L. mahajangana* and *N. parvum* occurred in both asymptomatic and symptomatic branches of the three tree species. Five species, *Do. dulcispinae*, *L. exigua*, *L. gonubiensis*, *O. ednahkunjekuae* and *Oblongocollomyces* sp. 1 were exclusive to asymptomatic branches. It is noteworthy that *L. pseudotheobromae* was the only species unique to symptomatic branches (Figure 4.7).

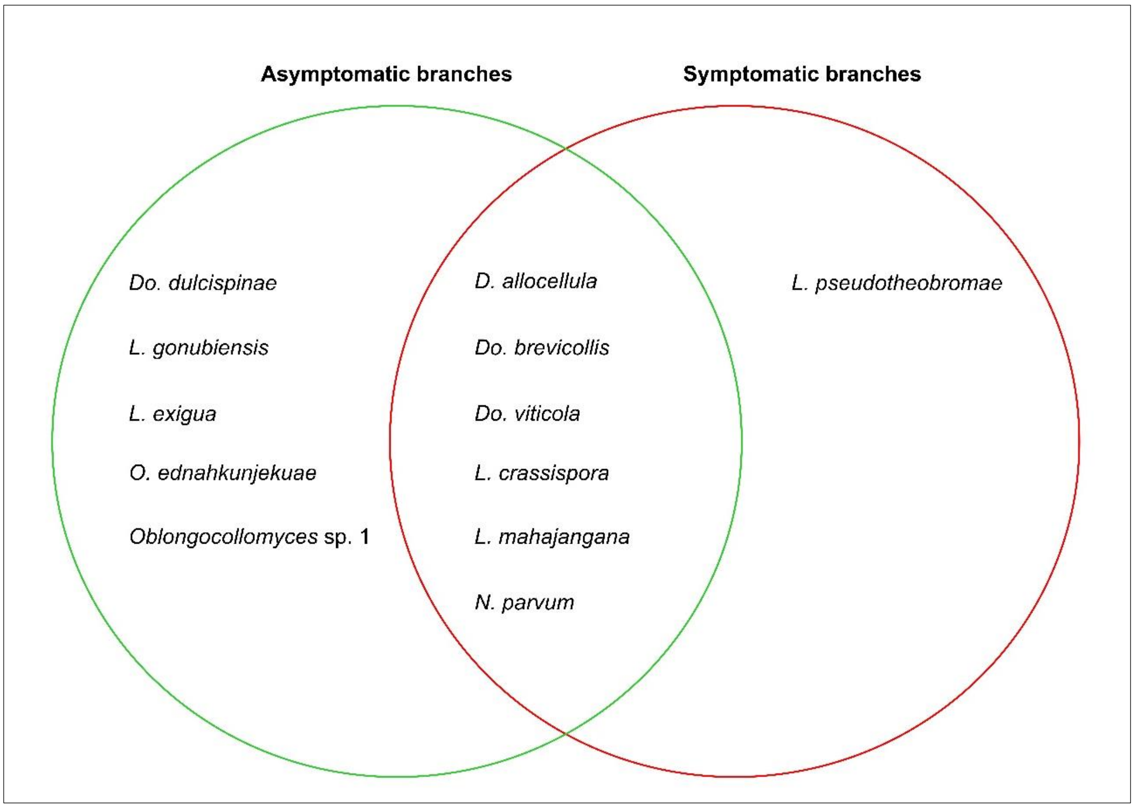

Figure 4.7. Venn diagram showing species of *Botryosphaeriaceae* occurring in both asymptomatic and symptomatic tree species of *Anacardiaceae.*

There was variation in the number of species identified as endophytes and as pathogens on the three tree species sampled (Figure 4.8). Twelve species were identified on *S. birrea* trees, but they differed in the tissue type from which they were isolated. *Dothiorella dulcispinae, L. exigua, L. gonubiensis, Oblongocollomyces ednahkunjekuae* and *Oblongocollomyces* sp. 1 occurred in asymptomatic branches only, *D. allocellula*, *Do. brevicollis, Do. viticola, L. crassispora, L. mahajangana* and *N. parvum* occurred in both asymptomatic and symptomatic branches, and *L. pseudotheobromae* occurred in symptomatic branches only. Five species, *D. allocellula, Do. brevicollis, L. crassispora, L mahajangana* and *L. pseudotheobromae* were isolated from *L. schweinfurthii. Diplodia allocellula, Do. brevicollis* and *L. crassispora* occurred in both asymptomatic and symptomatic branches, *L. mahajangana* occurred in asymptomatic branches only and *L. pseudotheobromae* occurred in symptomatic branches. *Lasiodiplodia crassispora, L. mahajangana* and *N. parvum* occurred in both asymptomatic and symptomatic branches of *M. indica*.

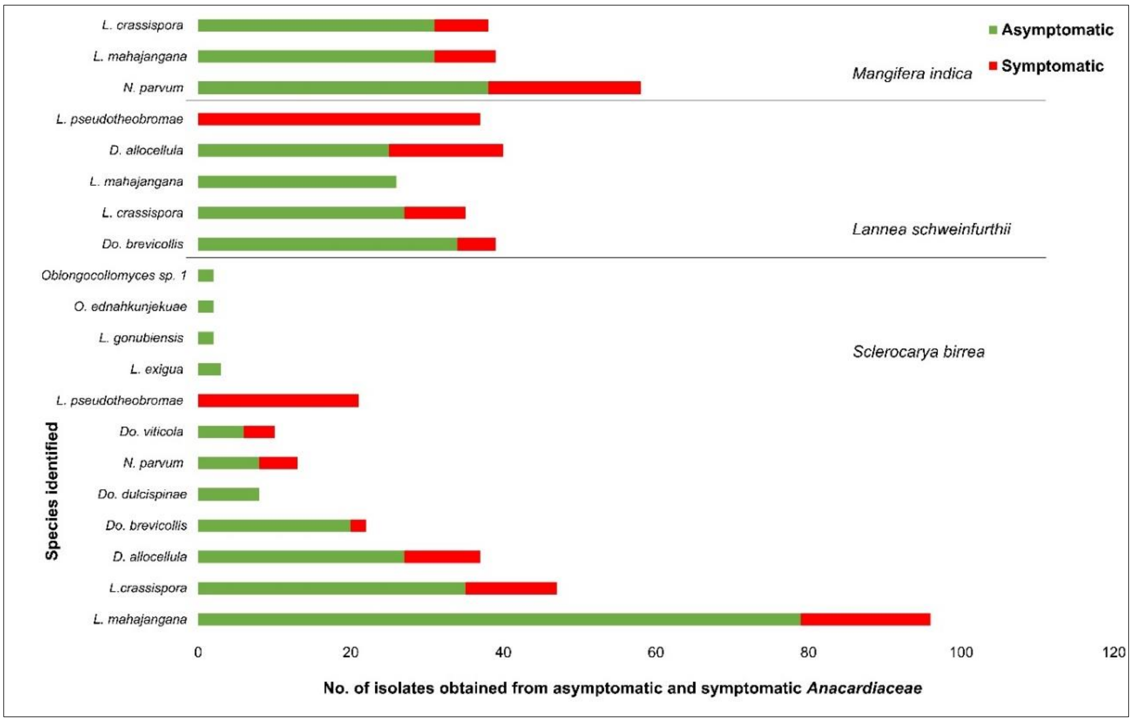

Figure 4.8. *Botryosphaeriaceae* species occurring in asymptomatic and symptomatic branches of *Sclerocarya birrea*, *Lannea schweinfurthii* and *Mangifera indica.* Isolates that were not sequenced were assigned to a specific species based on their grouping within the different morphogroups from which representative isolates were sequenced.

Species diversity and dominance of the *Botryosphaeriaceae* occurring in both asymptomatic and symptomatic branches of the same tree species varied across the different sampling sites in disturbed and undisturbed ecosystems (Figure 4.9). On trees in disturbed ecosystems, *L. mahajangana* and *N. parvum* occurred in both asymptomatic and symptomatic branches of *S. birrea* at Tshikundamalema, *L. crassispora* was only isolated from asymptomatic branches. *Lasiodiplodia crassispora* and *L. mahajangana* occurred in both asymptomatic and symptomatic branches of the same host at Tshipise, but *N. parvum* was not obtained from *S. birrea* trees at Tshipise. Similar variations occurred on hosts in undisturbed ecosystems. Four species, *Do. brevicollis*, *Do. viticola*, *L. crassispora* and *L. mahajangana* occurred in both asymptomatic and symptomatic branches of *S. birrea* at Mapungubwe National Park, while *L. crassispora* and *L. mahajangana* occurred in asymptomatic and symptomatic branches of the same host at Kruger National Park, *Do. brevicollis* and *Do. viticola* were therefore not associated with dieback of *S. birrea* at Kruger National Park. It is notable that *N. parvum* only occurred in disturbed sites, where it was fairly commonly isolated.

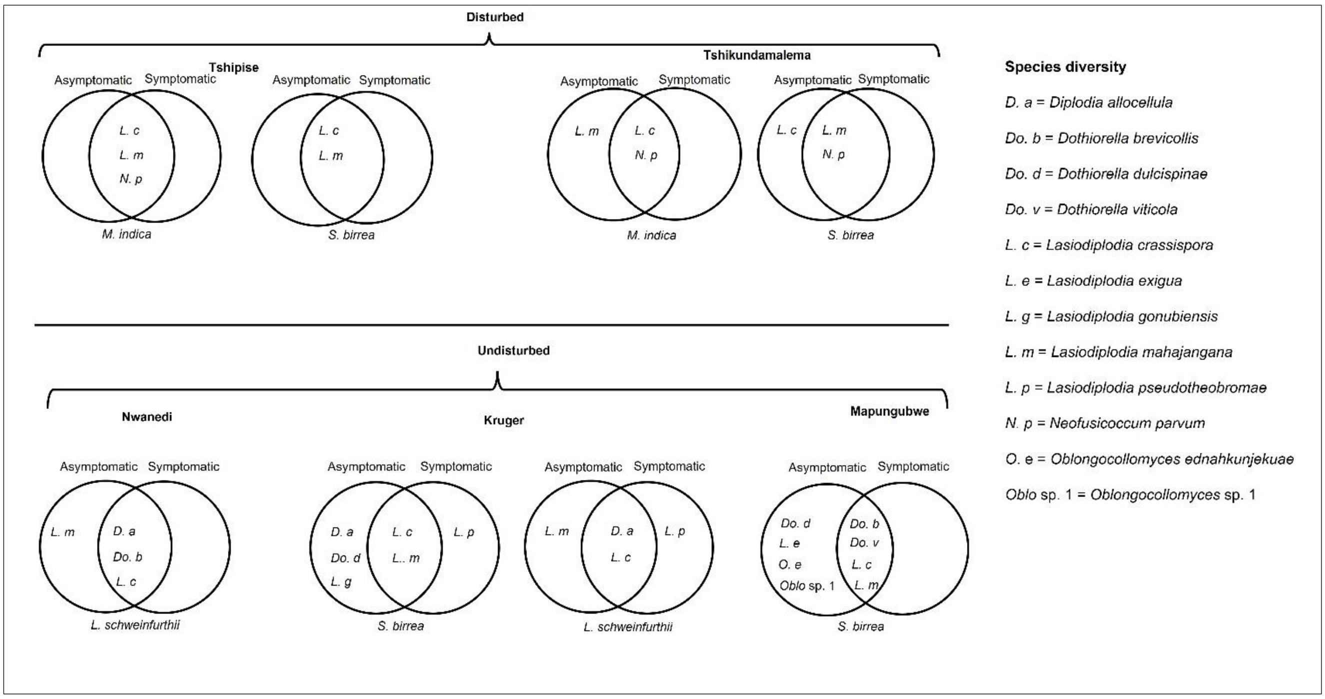

Figure 4.9. Species diversity and overlap of the *Botryosphaeriaceae* on healthy and diseased *Anacardiaceae* in disturbed and undisturbed ecosystems.

4.7. Discussion

Twelve species of *Botryosphaeriaceae* were identified as endophytes and as possible pathogens on *S. birrea*, *M. indica* and *L. schweinfurthii* trees in the Limpopo and Mpumalanga provinces. Ten species identified in this study, *D. allocellula, Do. brevicollis, Do. dulcispinae, Do. viticola, L. crassispora, L. gonubiensis, L. exigua, L. mahajangana, L. pseudotheobromae* and *N. parvum* represent known taxa of *Botryosphaeriaceae.* A previously unknown species was described in this study as *Oblongocollomyces ednahkunjekuae* sp. nov and another species could not be induced to sporulate and it is referred to as *Oblongocollomyces* sp. 1. Six species, *D. allocellula*, *Do. brevicollis*, *Do. viticola*, *L. crassispora*, *L. mahajangana* and *N. parvum,* occurred in both asymptomatic and symptomatic branches. *Dothiorella dulcispinae, L. exigua, L. gonubiensis, O. ednahkunjekuae* and *Oblongocollomyces* sp. 1 were only isolated from asymptomatic branches, while *L. pseudotheobromae* was the only species unique to symptomatic branches of *Anacardiaceae*. There were also clear differences between species diversity in disturbed and undisturbed sites, with undisturbed sites having a higher number of species, while *N. parvum* only occurred in disturbed sites.

Most species identified in this study were isolated from asymptomatic samples, demonstrating how common species of *Botryosphaeriaceae* are as endophytes in healthy plant material (Slippers and Wingfield, 2007; Jami *et al.,* 2013). The discrepancy in the number of isolates and species obtained between asymptomatic and symptomatic branches might be because other fungi (including saprophytes) outcompete the *Botryosphaeriaceae* once the dieback symptoms progress, while these fungi are amongst the more common endophytic ones (Slippers and Wingfield, 2007; Begoude *et al.,* 2010; Heath *et al.,* 2011; Jami *et al.,* 2013; Marques *et al.,* 2013). The ecological role of the *Botryosphaeriaceae* as endophytes of woody trees remains unclear. Studies suggest that *Botryosphaeriaceae* as a prominent group of endophytes are more likely to cause disease outbreaks on stressed hosts if the agent of stress is prolonged and widespread (Desprez-Loustau *et al.,* 2006, 2007).

The six species, *D. allocellula*, *Do. brevicollis*, *Do. viticola*, *L. crassispora*, *L. mahajangana* and *N. parvum*, occurring in both asymptomatic and symptomatic samples indicate their ability to occupy healthy tissue as latent opportunistic pathogens. Such pathogens are predicted to become important in the era of climate change (Desprez-Loustau *et al.,* 2007; Piskur *et al.,* 2011). The pathogenicity of some species identified, including *Do. viticola*, *L. crassispora*, *L. mahajangana* and *N. parvum,* has been well established (van der Linde *et al.,* 2011; Mayorquin *et al.,* 2012; Correia *et al.,* 2016). *Lasiodiplodia crassispora* was first described as a canker pathogen on *Santalum album* in Australia (Burgess *et al.,* 2006). The fungus has also been isolated from infected *Eucalyptus urophylla,* grapevine and mango in Uruguay, California and Brazil (Pérez *et al*., 2010; Úrbez-Torres *et al*., 2011; Marques *et al.,* 2013; Correia *et al.,* 2016). In South Africa, the fungus is known to cause dieback on *Pterocarpus angolensis* and *S. birrea (*Mehl *et al.,* 2011, 2017). In the present study*, L*. *crassispora* was isolated from asymptomatic and symptomatic branches of *S. birrea*, *L. schweinfurthii* and *M. indica*.

Dothiorella viticola occurred in both asymptomatic and symptomatic *S. birrea* trees at Mapungubwe National Park but was not present at other sites or in other hosts. *Dothiorella viticola* was first described as a saprophyte from declining *V. vinifera* in Spain (Luque *et al.,* 2005). Since its description, the fungus has been isolated from healthy and infected tissues causing dieback, canker and gummosis on various hosts including citrus in California and Tunisia, *Populus cathayana* in China, and *V. vinifera* in Chile, Australia and USA (Úrbez-Torres *et al.,* 2007; Zhang *et al.,* 2009; Qiu *et al.,* 2011; Adesemoye *et al.,* 2011; Díaz *et al.,* 2013; Valencia *et al.,* 2015; Hamrouni *et al.,* 2018). In South Africa, *Do. viticola* is known to occur on healthy and infected *Celtis africana*, *Gymnosporia buxifolia*, *Prunus persica, Podocarpus henkelii, Senegalia mellifera, V. karroo* and *V. vinifera* (Jami *et al.,* 2017).

In the current study, *L. mahajangana* was the dominant endophyte occurring on tree species of *Anacardiaceae*, which is consistent with the study of Cruywagen *et al.* (2017) who reported *L. mahajangana* as the dominant species isolated from baobabs in Africa (Benin, Namibia, Senegal, South Africa and Zimbabwe). *Lasiodiplodia mahajangana* was first described in Madagascar on *Terminalia catappa* (Begoude *et al.,* 2010). The second report of the fungus was in South Africa on *Euphorbia ingens* trees with blue stain symptoms (van der Linde *et al.,* 2011). The fungus appears to be widely distributed in Africa, with a diverse host range. Other hosts known for this fungus in South Africa include *S. birrea* and *M. indica* (Mehl *et al.,* 2017).

Neofusicoccum parvum was the most abundant species isolated from *M. indica* branches. The fungus is a common endophyte and a well-known pathogen associated with dieback, canker, fruit rot and many other diseases of woody tree species in plantations, orchards and natural ecosystems (Iturritxa *et al.,* 2011; Pillay *et al.,* 2013; Sakalidis *et al.,* 2013). *Neofusicoccum parvum* has a global or near-global distribution (Slippers *et al.,* 2017; Burgess *et al.,* 2019; Batista *et al.,* 2021). The global distribution of this fungus suggests that it is invasive and has expanded its distribution, and potentially will have undesirable consequences in terms of plant health of both native and non-native plant communities in natural and managed ecosystems. This fungus has been recorded as the most widespread species, with the ability to infect a broad range of native and non-native hosts in South Africa (Jami *et al.,* 2017). *Neofusicoccum parvum* has previously been reported to cause diseases on mangoes globally (de Oliveira Costa *et al.,* 2010; Trakunyingcharoen *et al.,* 2014; Li *et al.,* 2021).

Results from the current study indicate that not all species of *Botryosphaeriaceae* are associated with dieback of the three tree species of *Anacardiaceae* sampled. *Dothiorella dulcispinae*, *L. exigua, L. gonubiensis, O. ednahkunjekuae* and *Oblongocollomyces* sp. 1 were not associated with disease symptoms on any tree species at the time when samples were collected. This indicates that the capacity to colonize living wood does not translate to causing disease under all circumstances. Furthermore, some species identified were only associated with disease symptoms on specific hosts and not on others. For example, *L. mahajangana* was associated with dieback of *S. birrea* and *M. indica*, but not on *L. schweinfurthii* trees. The reason for this phenomenon has not yet been investigated, but the pattern of infection observed in this study could be due to factors associated with host specialization and defense mechanisms. These results provide useful baseline data for further studies seeking to understand host-pathogen interactions between species of *Botryosphaeriaceae* and their hosts.

Lasiodiplodia pseudotheobromae was the only species unique to symptomatic branches of the three tree species of *Anacardiaceae*. The fungus was the most common species on symptomatic *S. birrea* and *L. schweinfurthii* trees at Kruger National Park. *Lasiodiplodia pseudotheobromae* was thought to have a limited distribution and a narrow host range, but recent studies have reported a broad host range and occurrence in tropical environments, like most other species in the genus (Mehl *et al.,* 2011; Begoude *et al.,* 2012; Machado *et al.,* 2014; Netto *et al.,* 2014). *Lasiodiplodia pseudotheobromae* has been reported as a pathogen on hosts in Australia, Brazil, Cameroon, China, Egypt and Mexico (Begoude *et al.,* 2010; Mehl *et al.,* 2011; Ismail *et al.,* 2012; Machado *et al.,* 2014; Netto *et al.,* 2014; Countinho *et al.,* 2016; Pipattanapuckdee *et al.,* 2019; Chen *et al.,* 2021). The fungus is also common in South Africa where it has been reported to cause dieback and canker on *Adansonia digitata, P. angolensis*, *S. birrea, Syzygium cordatum*, *Senegalia mellifera, Terminalia sericea, T. catappa* and *V. karroo* (Jami *et al.,* 2017).

The abundance and isolation frequency of *L. pseudotheobromae* on infected native *Anacardiaceae* indicates that it could play a role in the dieback disease. *Lasiodiplodia pseudotheobromae* is an important pathogen in agriculture and forestry (Ismail *et al.,* 2012; Marques *et al.,* 2013; Pipattanapuckdee *et al.,* 2019; Chen *et al.,* 2021). *Lasiodiplodia pseudotheobromae* has been reported to cause stem-end rot and dieback on mango (Kwon *et al.,* 2017), papaya stem-end rot (Netto *et al.,* 2014), English walnut stem canker (Li *et al.,* 2016), blueberry dieback (Wang *et al.,* 2016), pedicel and peduncle discoloration of grapes (Dissanayake *et al.,* 2015) and cankers on *Anacardium occidentale, Eucalyptus, Citrus*, *Coffea* and *Gmelina* species (Phillips *et al.,* 2008; Pérez *et al.,* 2010; Chen *et al.,* 2011; Slippers *et al.,* 2014; Trakunyingcharoen *et al.,* 2015; Countinho *et al.,* 2016; Cruz *et al.,* 2019).

The declining symptoms of *M. indica* trees at Tshipise might reflect a combination of biotic and abiotic factors. Mango decline is a serious problem in mango-growing countries around the world (Sandhu and Gill, 2013; Kumar and Kumar, 2016). The complexity of events leading to mango decline makes it difficult to ascertain the causative agent as a pathogen, pest or abiotic condition. Other than severe dieback from which we isolated species of *Botryosphaeriaceae*, other symptoms, including stunted growth and leaf necrosis were observed on *M. indica* trees. These symptoms are often associated with nutrient deficiency and other abiotic stressors (Sandhu and Gill, 2013; Kumar and Kumar, 2016). *Mangifera indica* is a non-native tree species and is not always grown in areas with optimal growing conditions. Mango trees thrive well in areas characterised by clay-loamy soil with good water and nutrient-holding capacity (Normand *et al.,* 2015). The sites sampled in this study are characterised by sandy soil with limited nutrient and water-holding capacity and we assume that the non-optimal growing conditions might have contributed to the decline of *M. indica* trees.

4.8. Conclusions

This study demonstrates the common occurrence of the *Botryosphaeriaceae* as potential latent opportunistic pathogens on native and non-native tree species of *Anacardiaceae*. The presence and dominance of known pathogens in the *Botryosphaeriaceae* such as *L. mahajangana, L. pseudotheobromae* and *N. parvum* on symptomatic samples support suggestions by Slippers and Wingfield (2007) that the most damaging species in the family are widely distributed with a broad host range. These results raise concerns about the future health status of these trees and other woody tree species in the context of climate change. To understand the impact of climate change on disease expression by species of *Botryosphaeriaceae*, it is important to implement long-term monitoring of these trees.

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CHAPTER 5

Genetic diversity of a species in the *Botryosphaeriaceae* occurring on the *Anacardiaceae*

Abstract

Species of *Botryosphaeriaceae* are increasingly moved around the world. Populations of these species are often genetically diverse and appear to have originated from different sources and have been introduced multiple times. One suspected source of such spread is infected fruit, as *Botryosphaeriaceae* can infect fruits asymptomatically. In the present study, we investigated the species diversity of *Botryosphaeriaceae* occurring on asymptomatic *Mangifera indica* and *Sclerocarya birrea* fruits. We then determined genetic diversity of the most dominant species isolated from the fruits. Unripe mature fruits were collected from *M. indica* and *S. birrea* trees at Tshikundamalema and Tshipise in 2017 and 2018. Isolates obtained from endophytic fruit isolations were identified through DNA sequencing of the RNA polymerase II subunit (*rpb2*), the internal transcribed spacer (ITS), translation elongation factor (*tef-1α*) and β-tubulin (*β-tub*). Five species, namely *Neofusicoccum kwambonambiense, Neofusicoccum mediterranuem, Neofusicoccum parvum, Neofusicoccum umdonicola* and *Neofusicoccum ursorum* were identified from *M. indica* fruits, while no *Botryosphaeriaceae* was identified from *S. birrea* fruits. *Neofusicoccum parvum* was the dominant species occurring on *M. indica* fruits. Previously designed microsatellite markers were used to assess the genetic diversity and population structure of *N. parvum* isolates. *Neofusicoccum parvum* populations were characterized by high levels of genetic diversity. Bayesian clustering analyses in STRUCTURE revealed the presence of three distinct genetic clusters amongst the *N. parvum* samples, but the three clusters were not correlated with the host or geography. The study highlights the potential for fruit to serve as a source of international spread of *Botryosphaeriaceae*, especially for *N. parvum*.

Keywords: *Botryosphaeriaceae*, *Anacardiaceae*, genetic diversity, endophytic fungi, tree pathogens

5.1. Introduction

Pathogen invasion and emergence are important research questions in plant pathology (Sakai *et al.,* 2001; Gladieux *et al.,* 2015). Therefore, identifying the source and pathways of biological invasion is important, but complicated for many plant pathogens, especially when they occur very widely and on indigenous and introduced hosts (Robert *et al.,* 2012; Sakalidis *et al.,* 2013). Increased international trade and travel are considered the main drivers of invasive alien fungi (Ghelardini *et al.,* 2017; Wingfield *et al.,* 2017; Paap *et al.,* 2022). *Botryosphaeriaceae* are known to have a near-global distribution, occupying various ecological niches and occurring on numerous plants (Slipper *et al.,* 2017; Batista *et al.,* 2021). Some globally distributed *Botryosphaeriaceae* appear to have genetically diverse introduced populations and complex population structures, presumably due to multiple introductions of unique genotypes from diverse sources (Bihon *et al.,* 2012; Pavlic *et al.,* 2015; Salahlou *et al.,* 2016; Marsberg *et al.,* 2017; Mehl *et al.,* 2017a).

Multiple introductions of invasive fungi often result in increased genetic diversity and population substructures across the invasive range (Burgess *et al.,* 2004; Bihon *et al.,* 2012; Sakalidis *et al.,* 2013). Populations resulting from multiple introductions often show high genetic diversity, consisting of alleles from both endemic and introduced populations (Facon *et al.,* 2008). Fungal populations are also expected to exhibit higher levels of genetic diversity in their native range than in introduced environments due to the accumulation of mutations over time and recombinations (Burgess *et al.,* 2004; Bihon *et al.,* 2011). Bihon *et al.* (2011), for example, reported high levels of genetic diversity in the introduced *Diplodia sapinea* population in South Africa most likely linked to the trade of living plants or plant products. In contrast, populations resulting from a single introduction are often genetically homogeneous, exhibiting genetic variation similar to a subset of the source population (Facon *et al.,* 2008; Burgess *et al.,* 2004; Bihon *et al.,* 2011).

Members of *Botryosphaeriaceae* produce sexual and asexual structures under field conditions but are most commonly encountered in their asexual spore stage (Phillips *et al.,* 2013; Pavlic *et al.,* 2015; Slippers *et al.,* 2017). Certain species of *Botryosphaeriaceae*, with an unknown sexual state, might still reproduce sexually in a cryptic state. For example, Bihon *et al.* (2014) observed evidence of sexual reproduction in *D. sapinea* populations, for which no sexual state had ever been previously observed. A follow-up mating-type study showed that *D. sapinea* populations contained two mating-type idiomorphs indicating the presence of a heterothallic sexual cycle (Bihon *et al.,* 2014). These mixed modes of reproduction influence the population genetic diversity and distribution of *Botryosphaeriaceae* species (Bihon *et al.,* 2011, 2012, 2014; Nagel *et al.,* 2018; Zlatkovic *et al.,* 2019).

Botryosphaeriaceae are known to infect fruits of various commercially important species, including mango, apple, kiwi and others (Slippers *et al.,* 2005, 2007; McDonald and Eskalen, 2011; Jurick *et al.,* 2013; Lopes *et al.,* 2014) causing fruit rots, which lead to spoilage and economic loss (Trakunyingcharoen *et al.,* 2014; Jurick *et al.,* 2013; Lopes *et al.,* 2014). Moreover, these infections can be latent and can thus be transported between countries around the world in healthy fruit. Once sold, rotten fruit is likely to end up in waste dumps, next to the road or discarded elsewhere; from where these fungi can then spread to surrounding vegetation because of their wide host ranges (Marsberg *et al.,* 2017). Therefore, knowledge of the diversity of fungi infecting fruits is important to understand the potential spread of these fungi around the world.

In the current study, (i) the diversity of *Botryosphaeriaceae* infecting healthy-looking fruit of two *Anacardiaceae* species in South Africa, namely the non-native, commercially grown *Mangifera indica* (mango) and the highly prized native, *Sclerocarya birrea* (marula), was investigated; and subsequently (ii) the population genetic diversity of the most commonly isolated species from mango fruits, namely *Neofusicoccum parvum*, was explored using previously designed microsatellite markers.

5.2. Materials and Methods

5.2.1. Sample collection and fungal isolations

Unripe asymptomatic fruits were collected from 25 *M. indica* and 25 *S. birrea* trees at Tshikundamalema and another 25 of each tree species at Tshipise resulting in a total of 100 trees sampled. Mango fruits were collected from three mango cultivars, including Tommy Atkins, Kensington and Valencia Pride which were randomly spread at the two sites. Two fruits were collected from each tree and a total of 200 fruit samples for the two tree species were transported to the laboratory for fungal isolations.

The fruits were surface-sterilised in 70% ethanol, rinsed twice in sterile distilled water and air-dried under a laminar flow for 10 to 15 min. Isolations were made by cutting 0.5 - 1 mm of internal fruit tissue below the peduncle and plating unto Petri dishes containing 2% MEA with 0.1 g streptomycin. Primary isolates were incubated at 25 °C and monitored regularly for fungal growth. Isolates resembling species of *Botryosphaeriaceae* (fast growth, grey-black aerial mycelium) were selected and purified by transferring single hyphal tips onto fresh 2% MEA. Isolates were grouped based on colony morphology and colour characteristics using the colour chart of Rayner (1970). Isolates were grouped into five morphogroups, from which 1 to 5 isolates were selected from each group as representatives and their DNA was extracted for preliminary identification. Isolates used in the current study are lodged at the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

5.2.2. DNA extractions, PCR amplification and sequencing

Genomic DNA was extracted from the mycelium of 7-day-old cultures made from single hyphal tips following the protocol published by Möller *et al.* (1992). The extracted DNA pellets were re-suspended in 50 µL^{-}1 using sterile SABAX water (SABAX; Adcock Ingram, Bryanston, S.A). DNA concentrations were determined using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and adjusted to a working concentration of 50 ŋg µL^{-}1 using sterile water. Purified DNA was stored at -20 °C until further use.

The internal transcribed spacer, including the 5.8S RNA gene (ITS), and regions from the translation elongation factor (*tef-1α*), β-tubulin (*β-tub*) and RNA polymerase II subunit (*rpb2*) genes were amplified using polymerase chain reactions (PCR). The ITS gene was amplified using primer pairs ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.,* 1990). Part of the *β-tub* gene was amplified using primers Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2b (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') (Glass and Donaldson, 1995). The *tef-1α* loci was amplified with primers tef1-728F (5'-CATCGAGAAGTTCGAGAAGG-3') and tef1-986R (5'-TACTTGAAGGAACCCTTACC-3') (Carbone and Kohn, 1999) and part of the RNA polymerase II subunit (*rpb2*) using primer pairs rpb2bot6F (5'-GGTAGCGACGTCACTCCC-3') and rpb2bot7R (5'-GGATGGATCTCGCAATGCG-3') (Sakalidis *et al.,* 2011). DNA amplifications were conducted in a thermal cycler using the following cycling conditions; initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 sec, annealing at 54 °C (ITS and *rpb*2) and 56 °C (*tef-1α* and *β-tub*) for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 7 min. The resulting amplicons were separated on a 1% agarose gel stained with GelRed® (Biotium, Hayward, US) and visualised under ultraviolet light (UV) (Bio-Rad, Germany).

Amplicons were purified with Exosap (Thermo Fisher Scientific Inc., USA) before sequencing. DNA fragments were sequenced in both directions using the same primer pairs used for amplification reactions. Sequencing was carried out using an ABI Prism® Big Dye™ Sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing was done at the sequencing facility of the University of Pretoria, South Africa. Forward and reverse sequences were assembled in one sequence using CLC Main Workbench v.7.9 (QIAGEN, Aarhus, Denmark).

5.2.3. Phylogenetic analyses and species identification

Nucleotide sequences generated in this study were identified using BLASTn searches by comparing them with sequences maintained on the GenBank database (http://www.ncbi.nlm.nih.gov/genbank). Reference sequences retrieved from GenBank were combined with sequences generated in this study and aligned using MAFFFT online v.7 (Katoh *et al.,* 2019). Manual adjustments of sequences were made where necessary using BioEdit Sequence Alignment Editor v.7.2.5 (Hall, 1999). The best nucleotide substitution model was determined using jModelTest v.0.1.1 (Darriba *et al.,* 2012) with the corrected Akaike Information Criterion (AIC) to select the model that optimally fits the data.

Phylogenetic analyses were computed using Maximum likelihood and Bayesian methods. Maximum likelihood (ML) analyses were conducted using raxmlGUI v.2.0 (Edler *et al.,* 2021). The models TIM2 + G, HKY, TrN + G and TIM3 + I were selected for the respective ITS, *tef-1*α, *β-tub* and *rpb2* datasets. Branch support for ML analyses was determined using 1000 bootstrap (BS) replicates.

Bayesian inference (BI) of phylogenetic trees was conducted using MrBayes v.3.2.6 (Ronquist and Huelsenbeck, 2003). Four simultaneous Markov chains were run for 3 million generations, with trees sampled every 100th generation. The first 7500 trees representing the burn-in phase of the analyses were discarded and the remaining 22500 trees were used to obtain posterior probabilities (PP) following the majority rule consensus tree. Effective sampling size (ESS) values were assessed using Tracer v.1.7.1 (http://tree.bio.ed.ac.uk/software/tracer/). Trees were viewed using FigTree v.1.3.1 (Rambaut, 2009). The trees were rooted to sequences of *Melanops tulasnei* (CBS 116805 and CBS 116806) as the outgroup taxa.

5.3. *Neofusicoccum parvum* isolates used in the population study

Neofusicoccum parvum isolates used in the population study were obtained from asymptomatic *M. indica* fruits. *Neofusicoccum parvum* isolates obtained from asymptomatic and symptomatic branches in Chapters 3 and 4 of this Thesis were included in the population study to assess the influence of tissue type (fruit and branch samples) and occurrence in healthy and infected tissues on the genetic diversity of the fungus. Confirmation of species identity was based on analyses of four loci datasets. A total of 52 *N. parvum* isolates were assessed, of these, 33 isolates were obtained as endophytes on *M. indica* fruit samples in this chapter. The remaining 19 isolates were obtained from asymptomatic and symptomatic *M. indica* and *S. birrea* branch samples in previous chapters (Chapters 3 and 4, this Thesis).

5.4. Microsatellite PCR amplification

Eleven previously designed microsatellite markers were used to assess the genetic diversity of the 52 *N. parvum* isolates obtained from *M. indica* and *S. birrea* (Slippers *et al.,* 2004). PCR reaction mixtures consisted of ~ 50 ŋg DNA, 0.4 μM of each primer, 2.5 mM of dNTP, 1 x KAPA Taq Buffer (KAPA Biosystems, Cape Town, South Africa), 1 U KAPA Taq Polymerase KAPA Biosystems, Cape Town, South Africa) and 16.3 μL sterile SABAX water (SABAX; Adcock Ingram, Bryanston, S.A). The PCR conditions were as follows; initial denaturation for 1 min at 95 °C, followed by 35 cycles for 15 sec at 95 °C, annealing for 15 sec at 60 °C, extension at 72 °C for 1 min and final extension at 72 °C for 7 min. Amplified microsatellite products were separated and visualised under ultraviolet light against a 100 bp molecular weight marker.

The SSR amplicons were diluted (1:150) in sterile SABAX water. Each sample mixture contained 1 μL DNA, 0.14 μL formamide and 1 μL Genescan™ 500-LIZ® (Applied Biosystems, Warrington, UK) size standard. Fluorescent labelled-SSR PCR products were separated on an ABI Prism® 3500xl Genetic Analyser (Applied Biosystems, Warrington, UK). Allele sizes were scored by comparing the mobility of the SSR products with those of the internal size standards (LIZ-500) using GeneMapper v.5.0 (Applied Biosystems, Warrington, UK).

5.5. Genetic diversity analyses

To determine genetic diversity of the *N. parvum* population, the isolates were arranged according to host and geographic location. The inclusion of clonal genotypes is known to distort the estimates of population statistics parameters (Frantz *et al.,* 2006). Therefore, the data were clone-corrected by identifying identical genotypes using the Assign Clones option in GenoDive. v.1.0 (Meirmans and Van Tienderen, 2004). Clone correction reduced the sample size from 52 isolates to 25 isolates. The program GenAlEx v.6.5 (Peakall and Smouse, 2012) was used to calculate allele frequencies of the clone-corrected dataset at each microsatellite loci. The observed number of alleles (n_a) and the number of private alleles were also calculated using GenAlEx v.6.5 (Peakall and Mouse, 2012). Each allele was assigned a different letter at the loci (e.g. ABCDE), thus providing each isolate with a multilocus haplotype. Genetic diversity (*H*) between the Tshipise and Tshikundamalema populations was calculated using GenAlEx v.6.5 (Peakall and Mouse, 2012).

5.6. Bayesian clustering analyses

The presence and number of populations (K) in the *N. parvum* sample were determined using the Bayesian clustering algorithm implemented in STRUCTURE v.2.3.4 (Pritchard *et al.,* 2000). STRUCTURE implements a clustering algorithm informed by the Bayesian MCMC (Markov Chain Monte Carlo) approach to assign individuals to distinct populations (K). Individuals in the same sample are probabilistically distributed to K clusters, or jointly to two or more clusters if their genotypes indicate that they are admixed, regardless of their region or host origin. To determine the most probable number of genetically distinct clusters (K) in the *N. parvum* sample, 20 independent runs for each K = 1 to K = 10 were carried out at 900 000 Markov Chain Monte Carlo (MCMC) generations and a burn-in period of 300 000 iterations. Allele frequencies were set as correlated and lambda was set at one. The possible number of clusters in the *N. parvum* population was obtained using Evanno's approach (Evanno *et al.*, 2005) based on ΔK implemented online in STRUCTURE HARVESTER (Earl and vonHoldt, 2012).

The spatial genetic structure of the *N. parvum* population was further analysed by distance-based methods using Principal Coordinates Analysis (PCoA) in GenAlEx v.6.5 (Peakall and Smouse, 2012). The multivariate analysis (PCoA) is based on distances between multilocus genotypes of individuals and does not make any assumption about the underlying population genetic model. Analysis of molecular variance (AMOVA) was carried out to differentiate percentage variations among and within populations in GenAlEx v.6.5 (Peakall and Smouse, 2012).

5.7. Results

5.7.1. Fungal isolates

Forty-three *Botryosphaeriaceae* isolates that actively grew on MEA were obtained as endophytes on 63 mango fruit samples. No isolates resembling species of *Botryosphaeriaceae* were obtained from marula fruits. The 16 representative isolates (Table 5.1) were identified by partial sequencing of the ITS, *tef-1α*, *β-tub* and *rpb2* gene regions.

Table 5.1. Isolates used for phylogenetic analyses, isolates in **bold** were obtained from asymptomatic *Mangifera indica* and sequences in *italics* were obtained from GenBank.

Species	**Culture No**	**Host**	**Location**	**GenBank accession numbers**			
				ITS	***tef- 1α***	***β-tub***	***rpb2***
Neofusicoccum australe	CBS 115502	Unknown	Portugal	*MT587457*	*MT592167*	*MT592659*	*MT592356*
N. australe	CBS 130996	*Mangifera indica*	Italy	*MT587458*	*MT592171*	*MT592663*	*MT592360*
N. cordaticola	CMW 14151	*Syzygium cordatum*	South Africa	*EU821922*	*EU821892*	*EU821862*	*EU821952*
N. cordaticola	CMW 14124	*S. cordatum*	South Africa	*EU821925*	*EU821895*	*EU821865*	*EU821955*
N. eucalyptorum	CBS 145975	*Eucalyptus* sp.	New Zealand	*MT587477*	*MT592190*	*MT592682*	*MT592381*
N. eucalyptorum	CMW 40038	Unknown	Greece	*KF923241*	*KF923274*	*KF923262*	None
N. kwambonambiense	CBS 102.17	*Carya illinoensis*	Florida, USA	*KX464169*	*KX464686*	*KX464964*	*KU587930*
N. kwambonambiense	CBS 123639	*S. cordatum*	South Africa	*NR137014*	*KX464687*	*KX464965*	*MT592383*
N. kwambonambiense	**CMW54304**	*M. indica*	Tshikundamalema	OL441885	OL441941	OL441997	OL442040
N. kwambonambiense	**CMW54305**	*M. indica*	Tshikundamalema	OL441886	OL441942	OL441998	OL442041
N. kwambonambiense	**CMW54306**	*M. indica*	Tshipise	OL441888	OL441944	OL442000	OL442043
N. kwambonambiense	**CMW54307**	*M. indica*	Tshipise	OL441887	OL441943	OL441999	OL442042

Table 5.1. (Continued)

Species	Culture No	Host	Location	GenBank accession numbers			
				ITS	***tef- 1α***	***β-tub***	***rpb2***
N. mediterraneum	CBS 121718	Unknown	Greece	*NR160225*	*GU799462*	*GU799461*	*KY855815*
N. mediterraneum	PD312	*Eucalyptus* sp.	Greece	*GU251176*	*GU251308*	None	None
N. mediterraneum	**CMW54308**	*M. indica*	Tshikundamalema	OL441891	OL441947	OL442003	OL442046
N. mediterraneum	**CMW54309**	*M. indica*	Tshikundamalema	OL441890	OL441946	OL442002	OL442045
N. mediterraneum	**CMW54310**	*M. indica*	Tshipise	OL441889	OL441945	OL442001	OL442044
N. parvum	CMW 41213	*Barringtonia racemosa*	South Africa	*KP860849*	*KP860693*	*KP860771*	*KU587896*
N. parvum	CBS 138823	*Populus nigra*	New Zealand	*NR119487*	*AY236888*	*AY236917*	*EU821963*
N. parvum	**CMW57574**	*M. indica*	Tshipise	OL441899	OL441955	OL442011	OL442054
N. parvum	**CMW57575**	*M. indica*	Tshipise	OL441900	OL441956	OL442012	OL442055
N. parvum	**CMW57576**	*M. indica*	Tshipise	OL441898	OL441954	OL442010	OL442053
N. parvum	**CMW57577**	*M. indica*	Tshikundamalema	OL441901	OL441957	OL442013	OL442056
N. parvum	**CMW57578**	*M. indica*	Tshikundamalema	OL441897	OL441953	OL442009	OL442052
N. ribis	CMW 7772	*Ribes* sp.	USA, New York	*AY236935*	*AY236877*	*AY236906*	*EU863170*

Table 5.1. (Continued)

Species	**Culture No**	**Host**	**Location**	**GenBank accession numbers**			
				ITS	***tef- 1α***	***β-tub***	***rpb2***
N. ribis	CMW 7773	*Ribes* sp.	USA, New York	*AY236936*	*AY236878*	*AY236907*	*EU863169*
N. umdonicola	CMW 14058	*S. cordatum*	South Africa	*EU821904*	*EU821874*	*EU821844*	*EU821934*
N. umdonicola	CBS 123646	*S. cordatum*	South Africa	*EU821905*	*EU821875*	*KF766145*	*EU821935*
N. umdonicola	**CMW54300**	*M. indica*	Tshikundamalema	OL441902	OL441958	OL442014	OL442057
N. umdonicola	**CMW54300**	*M. indica*	Tshikundamalema	OL441902	OL441958	OL442014	OL442057
N. umdonicola	**CMW54301**	*M. indica*	Tshipise	OL441903	OL441959	OL442015	OL442058
N. ursorum	CBS 131680	*Podocarpus henkelii*	South Africa	*MT587527*	*MT592253*	*MT592745*	*MT592443*
N. ursorum	CBS 131679	*Afrocarpus falcatus*	South Africa	*MT587526*	*MT592252*	*MT592744*	*MT592442*
N. ursorum	**CMW57571**	*M. indica*	Tshipise	OL441904	OL441960	OL442016	OL442059
N. vitifusiforme	CBS 130993	*M. indica*	Italy	*MT587530*	*MT592258*	*MT592750*	*MT592448*
N. vitifusiforme	CBS 130998	*M. indica*	Italy	*MT587531*	*MT592259*	*MT592751*	*MT592449*
Melanops tulasnei	CBS 116805	*Quercus robur*	Germany	*FJ824769*	None	*FJ824780*	None
M. tulasnei	CBS 116806	*Q. robur*	Germany	*FJ824770*	*FJ824775*	*FJ824781*	*KX463998*

5.7.2. DNA sequence and phylogenetic analyses

GenBank BLASTn searches of sequences generated in this study showed the highest similarity to *Neofusicoccum* species. Phylogenetic analyses of the four loci datasets placed the isolates into five highly supported monophyletic clades corresponding to five *Neofusicoccum* species, including *N. parvum*, *N. kwambonambiense*, *N. umdonicola*, *N. mediterraneum* and *N. ursorum*. The most variable loci were *β-tub* and *rpb2* which grouped all the isolates into monophyletic clades with *Neofusicoccum* species.

5.7.2.1. ITS phylogeny

Phylogenetic analyses of the ITS dataset separated the isolates into five clades corresponding to *N. umdonicola* (clade I), *N. kwambonambiense* (clade II), *N. parvum* (clade III), *N. ursorum* (clade IV) and *N. mediterraneum* (clade V) (Figure 5.1). Isolates CMW54300 and CMW54301 formed a monophyletic clade with *N. umdonicola* (BS = 72%; PP = 0.95). Four isolates, CMW54304, CMW54305, CMW54306 and CMW54307 formed a monophyletic clade with *N. kwambonambiense* (BS = 86%; PP = 0.99). Isolates CMW57574, CMW57575, CMW57576, CMW57577 and CMW57578 corresponding to *N. parvum* formed a highly supported monophyletic clade (BS = 99%; PP = 0.95). Isolate CMW57571 grouped with *N. ursorum* (BS = 98%; PP = 0.97). Three isolates, CMW54308, CMW54309 and CMW54310 formed a polytomy with *N. mediterraneum* but without BS and PP support.

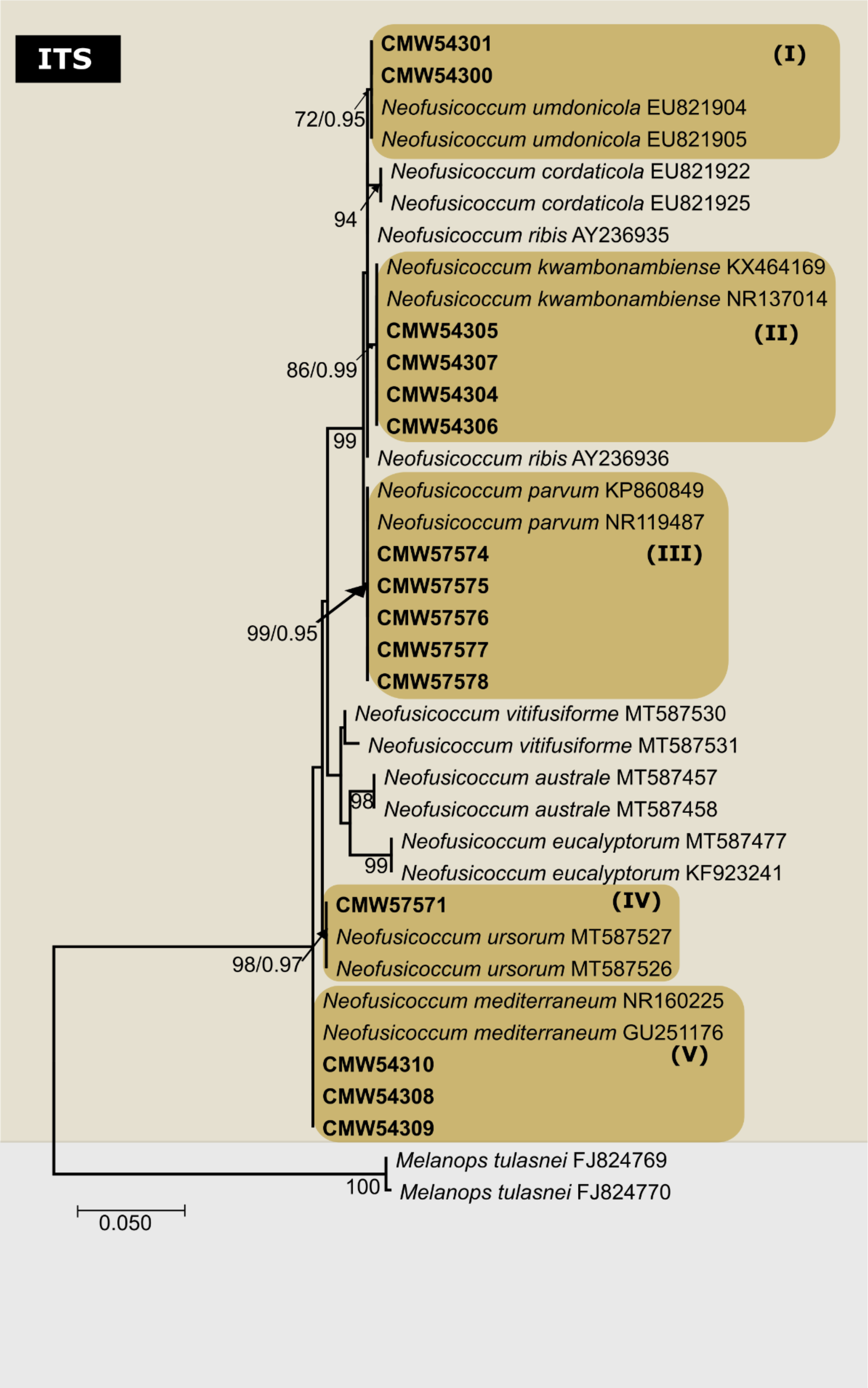

Figure 5.1. Phylogenetic tree based on analyses of the ITS dataset. Bootstrap values (> 70%) and PP values (> 0.95) appear at the nodes. Sequences generated in the current study are in **bold**. The tree was rooted to isolates of *Melanops tulasnei.*

5.7.2.2. *Tef-1α* phylogeny

The phylogeny emerging from ML and BI analyses of the *tef-1α* dataset separated isolates obtained in this study into five clades. The five clades accommodated isolates corresponding to *N. kwambonambiense* (clade I), *N. umdonicola* (clade II), *N. parvum* (clade III), *N. ursorum* (clade IV) and *N. mediterraneum* (clade V) (Figure 5.2). Isolates CMW54304, CMW54305, CMW54306 and CMW54307 together with *N. kwambonambiense* formed a polytomy within the genus without BS and PP support. Isolates CMW54300 and CMW54301 formed a polytomy with *N. umdonicola* based on the *tef-1α* sequence data. Isolates CMW57574, CMW57575, CMW57576, CMW57577 and CMW57578 grouped with *N. parvum* (BS = 97%; PP = 0.96). Isolate CMW57571 grouped with *N. ursorum* (BS = 95%) but without PP support. Isolates CMW54308, CMW54309 and CMW54310 grouped with *N. mediterraneum* (BS = 94%; PP = 0.99).

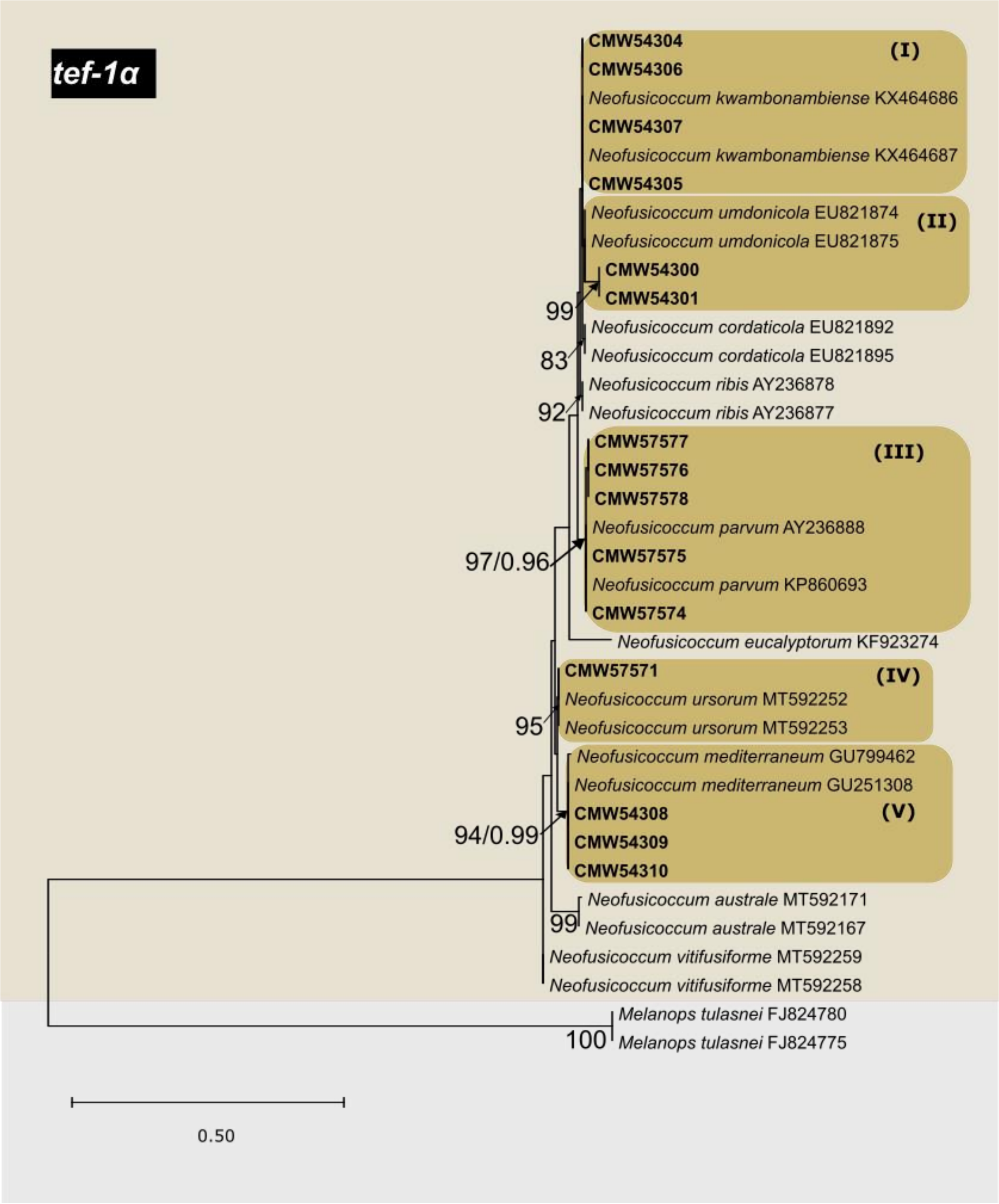

Figure 5.2. Phylogenetic tree based on analyses of the *tef-1α* sequence data. Bootstrap values above 70% and PP values above 0.95 are shown at the nodes. Isolates in **bold** were generated in this study. The tree was rooted to *Melanops tulasnei.*

5.7.2.3. *β-tub* phylogeny

Maximum likelihood and BI analyses of the *β-tub* dataset separated the isolates into five clades corresponding to *N. parvum* (clade I), *N. umdonicola* (clade II), *N. kwambonambiense* (clade III), *N. ursorum* (clade IV) and *N. mediterraneum* (clade V) (Figure 5.3). Isolates CMW57574, CMW57575, CMW57576, CMW57577 and CMW57578 corresponding to *N. parvum* did not receive BS and PP support. Isolates CMW54300 and CMW54301 formed a monophyletic clade with *N. umdonicola* (BS = 74%; PP = 0.96). Four isolates, CMW54304, CMW54305, CMW54306 and CMW54307 formed a monophyletic clade with *N. kwambonambiense* (BS = 93%; PP = 0.96). Isolate CMW57571 grouped with *N. ursorum* but without BS and PP support. Isolates CMW54308, CMW54309 and CMW54310 formed a highly supported monophyletic clade with *N. mediterraneum* (BS = 99%; PP = 0.99).

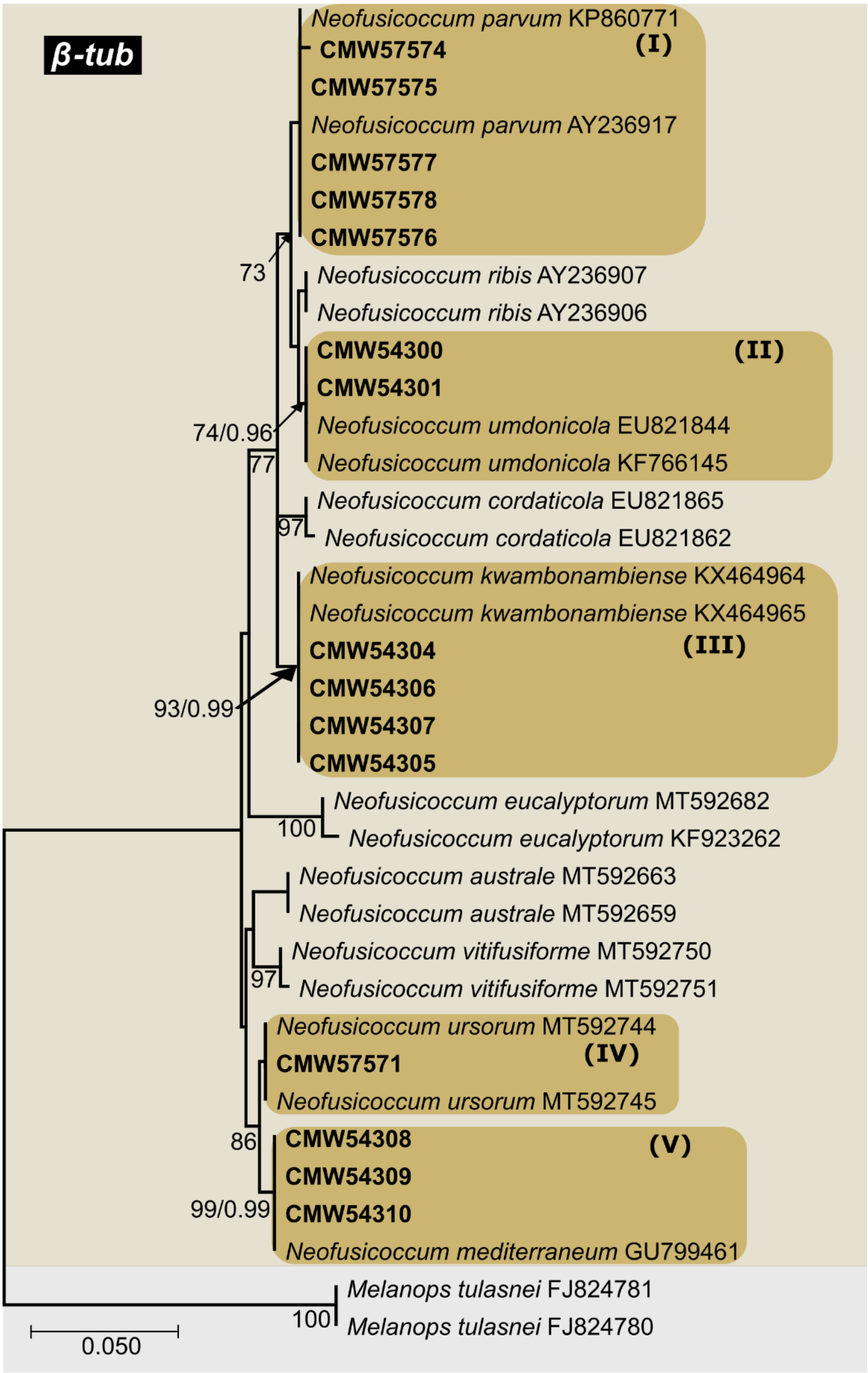

Figure 5.3. Phylogenetic tree based on ML and BI analyses of the *β-tub* sequence data. Bootstrap values above 70% and PP values above 0.95 are shown at the nodes. Isolates in **bold** were obtained in this study. The tree was rooted to *Melanops tulasnei.*

5.7.2.4. *Rpb2* phylogeny

The *rpb2* phylogeny separated isolates obtained in this study into five clades. The five clades represented *N. kwambonambiense* (clade I), *N. umdonicola* (clade II), *N. parvum* (clade III), *N. ursorum* (clade IV) and *N. mediterraneum* (clade V) (Figure 5.4). Isolates corresponding to *N. kwambonambiense* (CMW54304, CMW54305, CMW54306 and CMW54307) formed a highly supported monophyletic clade (BS = 100%; PP = 1). Clade II included isolates CMW54300 and CMW54301 that grouped with *N. umdonicola* (BS = 90%; PP = 0.99). Isolates in clade III formed a highly supported monophyletic clade with *N. parvum* (BS = 99%; PP = 0.99). Isolate CMW57571 formed a highly supported monophyletic clade with *N. ursorum* (BS = 100%; PP = 0.99). Isolates corresponding to *N. mediterraneum* (CMW54308, CMW54309 and CMW54310) formed a highly supported monophyletic clade (BS = 99%; PP = 0.99).

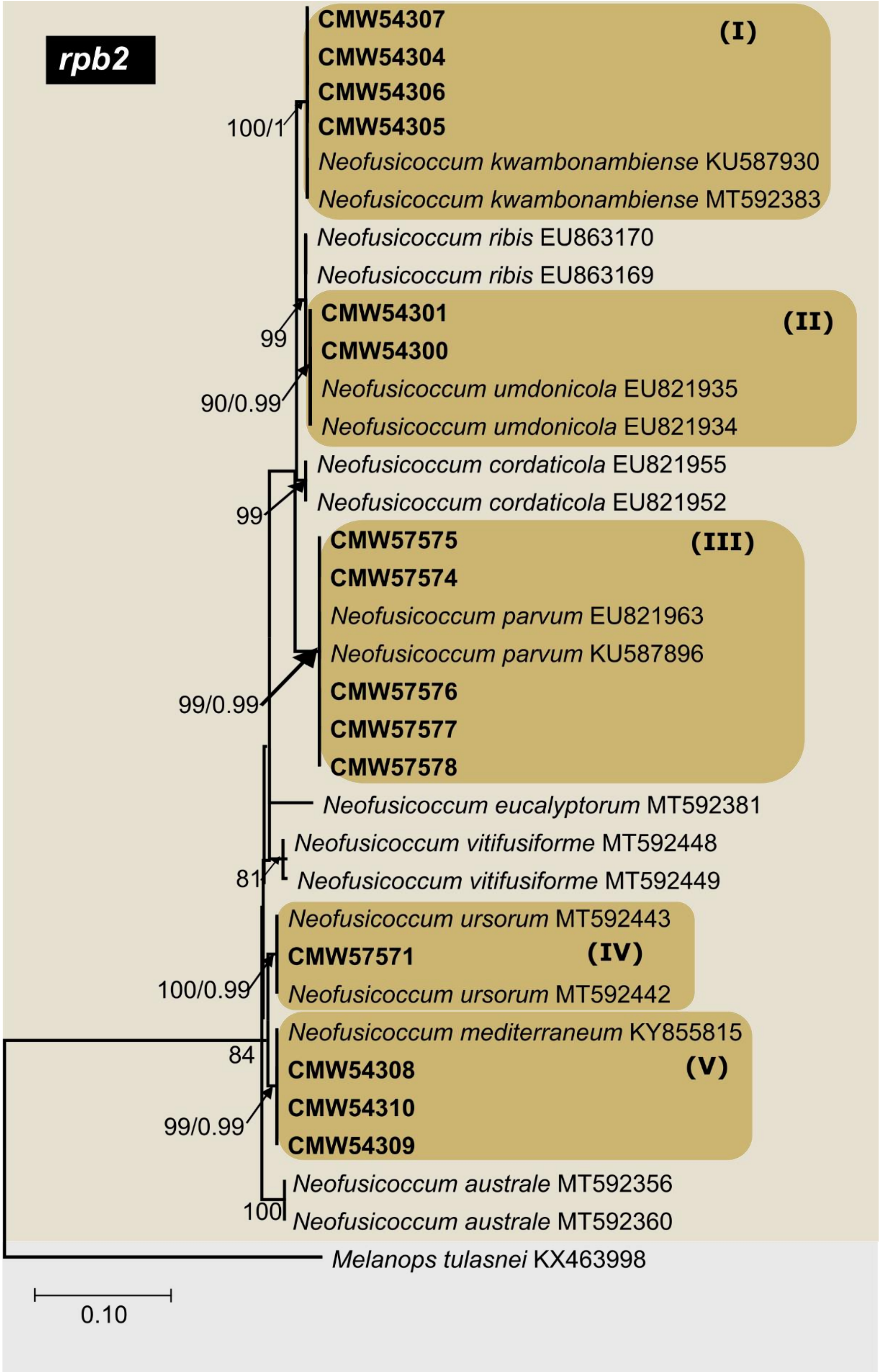

Figure 5.4. Phylogenetic tree based on maximum likelihood and Bayesian inference analyses of the *rpb2* sequence data. Bootstrap values (> 70%) and PP values (> 0.95) appear at the nodes. Isolates in **bold** were obtained in this study. The tree was rooted to *Melanops tulasnei.*

5.7.3. Species diversity

Five *Neofusicoccum* species including *N. umdonicola*, *N. parvum*, *N. kwambonambiense*, *N. ursorum* and *N. mediterraneum* were identified as endophytes on asymptomatic mango fruits. *Neofusicoccum parvum* was the dominant species (n = 33/43), followed by *N. kwambonambiense* (n = 4/43), *N. mediterraneum* (n = 3/43), *N. umdonicola* (n = 2/43) and *N. ursorum* (n = 1/43) (Figure 5.5).

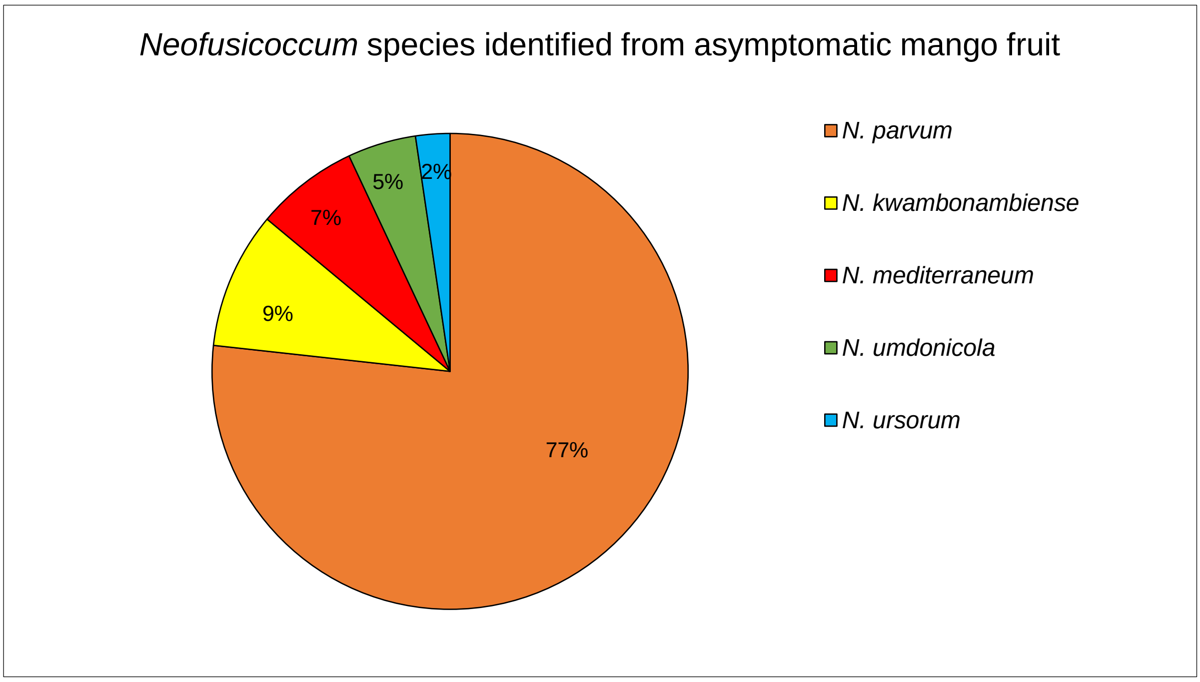

Figure 5.5. *Neofusicoccum* species identified as endophytes on asymptomatic *M. indica* fruit.

There was no variation in species diversity of the *Botryosphaeriaceae* occurring on the three mango cultivars and sites sampled in this study. Twenty-six *N. parvum* isolates were obtained from mango trees at Tshipise, while 7 isolates were obtained from trees at Tshikundamalema. Two *N. kwambonambiense* isolates were obtained from each site. Two *N. mediterraneum* isolates were obtained from trees at Tshikundamalema, while one isolate was obtained at Tshipise. A single *N. umdonicola* isolate was obtained from each site and one *N. ursorum* isolate was obtained from *M. indica* trees at Tshipise.

5.7.4. Microsatellite and genetic diversity

Nine of the eleven microsatellite markers successfully amplified microsatellite regions of the *N. parvum* isolates (Table 5.2). All nine markers were polymorphic among the 52 *N. parvum* isolates with a minimum number of two allele variants detected in each locus. Fragment allele sizes fell within the expected range. A total of 34 alleles were detected across the nine loci examined. Six alleles were detected in one locus (Neo08), five in one locus (Neo21), four in three loci (Neo07, Neo16 and Neo18), three in three loci (Neo05, Neo15 and Neo19) and two in one locus (Neo17).

Table 5.2. Allele sizes and frequencies at nine microsatellite loci for *Neofusicoccum parvum* isolates obtained from *Mangifera indica* and *Sclerocarya birrea* at Tshikundamalema and Tshipise. Alleles in **bold** indicate unique alleles.

Locus	Allele/n	Allele notation	Tshikundamalema	Tshipise
Neo05	405	A	0.167	0.154
	411	B	0.500	0.231
	414	C	0.333	0.615
Neo07	323	A	0.833	0.692
	327	B	_	**0.077**
	331	C	0.083	0.154
	335	D	0.083	0.077
Neo8	262	A	0.167	0.077
	268	B	_	**0.077**
	274	C	0.417	0.462
	280	D	0.083	0.154
	286	E	0.167	0.154
	292	F	0.167	0.077
Neo15	305	A	0.167	0.385
	311	B	0.083	0.154
	317	C	0.750	0.462
Neo16	364	A	0.583	0.231
	372	B	0.333	0.538
	376	C	_	**0.154**
	380	D	0.083	0.077
Neo17	301	A	0.500	0.538
	307	B	0.500	0.462
Neo18	179	A	0.500	0.231
	182	B	0.333	0.538
	191	C	0.083	0.154
	197	D	0.083	0.077
Neo19	306	A	0.750	0.692
	309	B	0.167	0.308
	312	C	**0.083**	_
Neo21	426	A	0.167	0.154
	429	B	0.167	0.385
	432	C	0.500	0.231
	435	D	0.167	0.154
	438	E	_	**0.077**

Of the 34 alleles detected in the entire *N. parvum* dataset, 29 alleles were shared between the Tshipise and Tshikundamalema populations (Table 5.3). Four alleles were unique to isolates obtained from Tshipise, while one allele was unique to isolates from Tshikundamalema. The overall genetic diversity (*H*) for the *N. parvum* sample was 0.576. Genetic diversity among the defined populations was slightly higher for the Tshipise population (*H* = 0.591) than the Tshikundamalema population (*H* = 0.560).

Table 5.3. Number of private alleles and genetic diversity detected from *Neofusicoccum parvum* isolates obtained from *Mangifera indica* and *Sclerocarya birrea* at Tshikundamalema and Tshipise.

Population site	**No. of isolates**	**No. of alleles**	**No. of private alleles**	**Genetic diversity (*H*)**
Tshikundamalema	12	30	1	0.560
Tshipise	13	33	4	0.591
Total	25	34	5	0.576

5.7.5. Bayesian clustering analyses

The results obtained from Bayesian clustering analyses in STRUCTURE and STRUCTURE HARVESTER revealed the presence of genetically structured groups in the *N. parvum* dataset (Figure 5.6). According to Evanno's statistics (Evanno *et al.*, 2005), the most likely number of populations (K) in the *N. parvum* sample was K = 3 (Figure 5.6a). Each population represented a mixture of isolates from *M. indica* and *S. birrea* from the two sites considered in this study (Figure 5.6b). Principal Coordinate Analyses (PCoA) also separated the data into three clusters as identified in STRUCTURE and STRUCTURE HARVESTER (Figure 5.7).

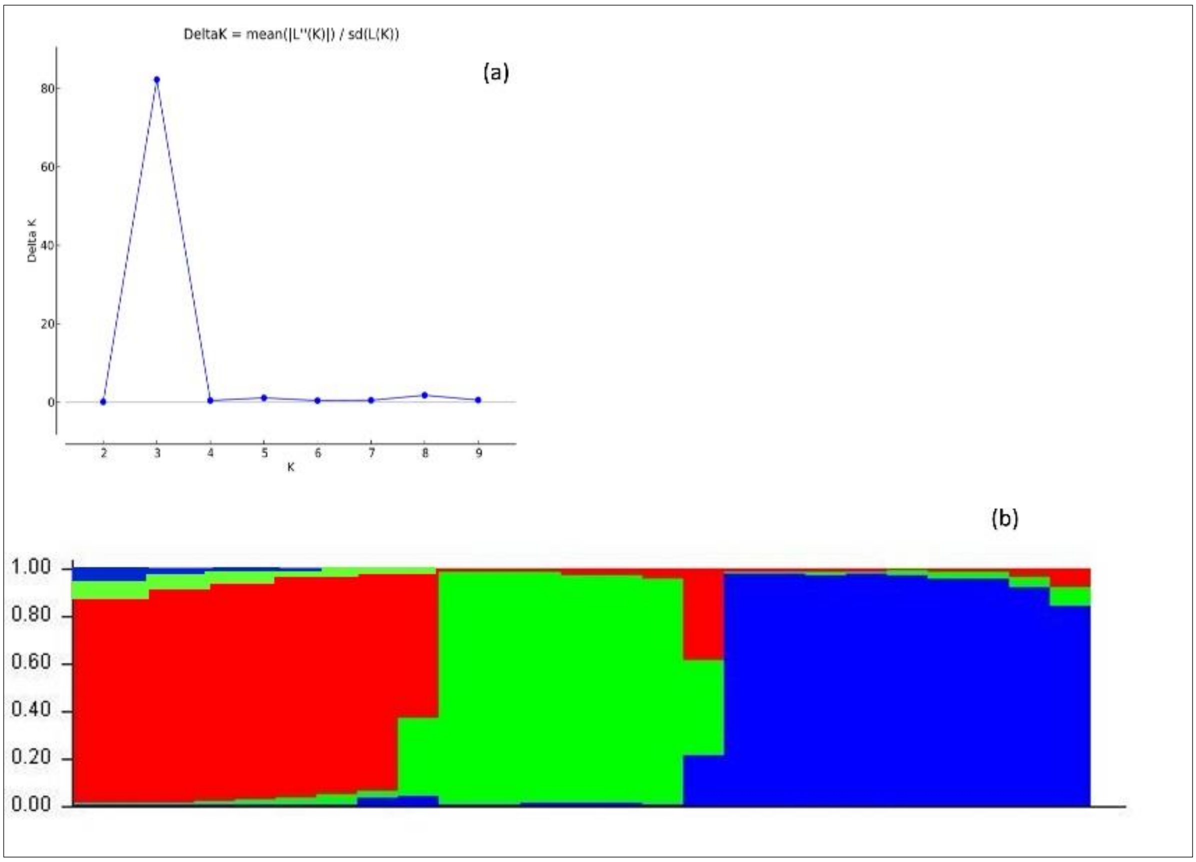

Figure 5.6. Bayesian clustering outcome from STRUCTURE and STRUCTURE HARVESTER for *Neofusicoccum parvum* isolates. (a) Schematic representation indicates the optimal number of clusters signaled by the highest ΔK (K = 3). (b) The number of clusters represented by a bar, with colours red, green and blue assigned to the populations.

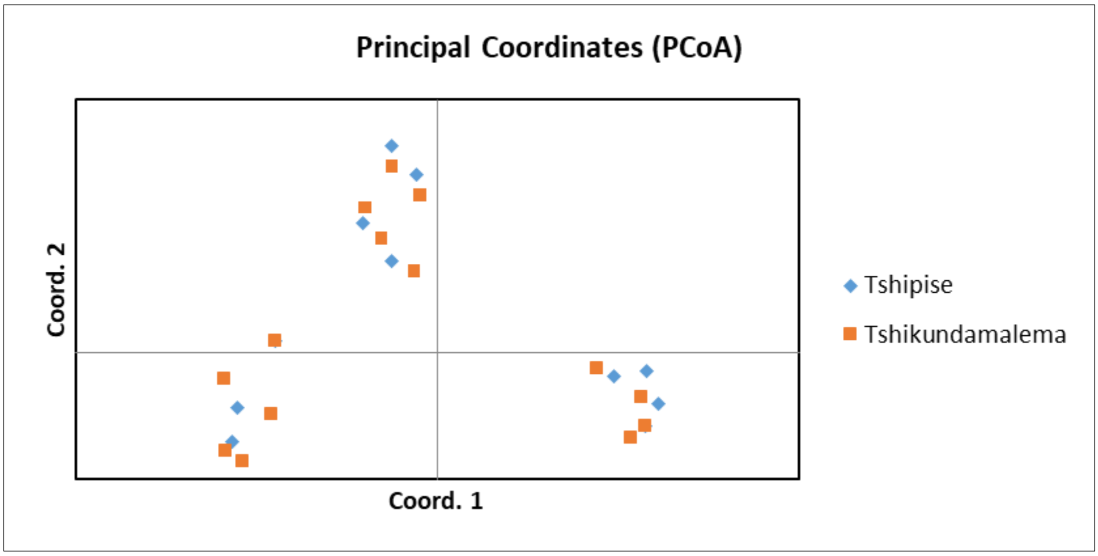

Figure 5.7. PCoA of *Neofusicoccum parvum* isolates from Tshipise and Tshikundamalema indicates the three clusters identified based on Nei's genetic distance.

Analyses of molecular variation (AMOVA) showed that all of the genetic variations were within populations and no genetic variation between host and geographic populations (Table 5.4). The lack of genetic variation between populations was confirmed by a negative value for Nei's unbiased genetic distance (-0.051).

Table 5.4. Analyses of molecular variance (AMOVA) for *N. parvum* populations obtained from Tshikundamalema and Tshipise.

Source of variation	***Df***	**Variance components**	**Estimated variation**	**Percentage variation %**
Among Populations	1	1.754	0.000	0%
Within Populations	23	64.686	2.812	100%
Total	24	66.440	2.812	100%

5.8. Discussion

Five species of *Botryosphaeriaceae* were identified as endophytes on mango fruits, while no *Botryosphaeriaceae* were isolated from marula fruit samples. *Neofusicoccum* was the only *Botryosphaeriaceae* genus amongst these endophytes occurring on mango fruit, despite previous studies showing the presence of other genera on other tissues of these trees (Mehl *et al.,* 2017b). The species identified included *N. parvum* (the dominant species), *N. kwambonambiense*, *N. mediterraneum*, *N. umdonicola* and *N. ursorum*. An assessment of genetic diversity and population structure of *N. parvum* isolates revealed high levels of genetic diversity that are reflective of diversity seen over broader areas and host samples in previous studies in the region (Pavlic *et al.,* 2015; Mehl *et al.,* 2017a)

The dominance of *Neofusicoccum* species on mango fruit suggests the presence of tissue specificity for certain species of *Botryosphaeriaceae*, as has been reported for other endophytes (Yuan *et al.,* 2009; Mishra *et al.,* 2012). In support of this, species from *Lasiodiplodia* were shown to occur on branches of these trees (Chapters 3 and 4). Of the five species identified as endophytes of mango fruit in the current study, only *N. parvum* was identified from branch samples of the *Anacardiaceae* in the broader study (Chapters 3 and 4). These results indicate that species diversity of *Botryosphaeriaceae* occurring on different parts of the same host differs, emphasizing the importance of examining different tissue types to understand the true diversity of *Botryosphaeriaceae* on a particular host. However, only two sites were sampled once. Therefore, more sampling should be done to support this observation.

Although a single isolate representing *N. ursorum* was obtained in the current study. This study represents the first report of *N. ursorum* on *M. indica* in South Africa. This species was similarly rare in previous studies where it has been identified. Maleme *et al.* (2009) first described the species from only two isolates from a *Eucalyptus* spp. In South Africa, *Neofusicoccum ursorum* has also been reported on *Podocarpus henkelii* (Ndove *et al.,* 2015). This species is not known in other countries suggesting that it might be native to South Africa.

The four other species identified on *M. indica* in this study, namely *N. kwambonambiense*, *N. mediterraneum, N. parvum* and *N. umdonicola* are known to occur on the same host from previous studies (Trakunyingcharoen *et al.,* 2014; Mehl *et al.,* 2017b). *Neofusicoccum kwambonambiense* and *N. umdonicola* were originally described as cryptic species in the *N. parvum* / *N. ribis* species complex on *S. cordatum* (Pavlic *et al.,* 2009). *Neofusicoccum kwambonambiense* is a known pathogen on tree species of *Anacardiaceae,* including *M. indica* and *S. birrea* (Mehl *et al.,* 2017b). It appears that *N. kwambonambiense* has a broad host range and a cosmopolitan distribution than other cryptic species in the *N. parvum* / *N. ribis* complex (i.e. *N. batangarum* and *N. cordaticola*). Other hosts for this fungus in South Africa include *Afrocarpus falcatus, Bruguiera gymnorrhiza, Celtis africana, E. grandis, Rhizophora mucronata, S. cordatum* and *Vachellia karroo* (Jami *et al.,* 2017). The other cryptic species identified in this study, *N. umdonicola*, appears to have a limited distribution when compared to *N. kwambonambiense*. The fungus has been reported on *S. birrea* and *M. indica* in Mpumalanga (Mehl *et al.,* 2017b), *Avicennia marina*, *B. gymnorrhiza* and *Lumnitzera racemosa* in Eastern Cape and KwaZulu-Natal (Osorio *et al.,* 2017) and *Schizolobium parahyba* in Ecuador (Mehl *et al.,* 2014).

Neofusicoccum mediterraneum is commonly isolated from dieback symptoms of various fruit and nut trees globally (Lazzizera *et al.*, 2008; Inderbitzin *et al.,* 2010; Moral *et al.,* 2010). It was first described as a dieback pathogen on a *Eucalyptus* spp. in Greece (Crous *et al.,* 2007). Other hosts for this fungus include olives, grapevines, almonds, pistachio and citrus (Lazzizera *et al.*, 2008; Inderbitzin *et al.,* 2010; Moral *et al.,* 2010). This species was first reported in South Africa on *M. indica* and *S. birrea* in Mpumalanga (Mehl *et al.,* 2017b). The presence of the fungus in the areas sampled indicates that the species is more widespread than previously understood. Other hosts across this distribution might thus be at risk, given the broad host range and known pathogenicity of this species.

Neofusicoccum parvum was the dominant endophyte on *M. indica* fruit samples in this study. The abundance of the fungus was not unexpected as it has previously been reported on *M. indica* in South Africa (Trakunyingcharoen *et al.,* 2014: Mehl *et al.,* 2017b). *Neofusicoccum parvum* is also known as a pathogen of *M. indica* in Australia (Slippers *et al.,* 2005), Brazil (de

Oliveira Costa *et al.,* 2010; Marques *et al.,* 2013) and Taiwan (Ni *et al.,* 2012). The distribution of *N. parvum* on fruit trees is not limited to *M. indica*, the fungus has been reported to cause canker, dieback, gummosis, fruit and stem-end rot on apples and kiwifruit (Pennycook and Samuels 1985; Mang *et al.,* 2022), pome and stone fruit trees (Slippers *et al.,* 2007), grapevines (Pitt *et al.,* 2013; Yan *et al.,* 2013), blueberry (Xu *et al.,* 2015; Guarnaccia *et al.,* 2020), citrus (Adesemoye and Eskalen, 2011; Bezerra *et al.,* 2021), strawberry (Lopes *et al.,* 2014), peach (Thomidis *et al.,* 2011) and avocado (McDonald and Eskalen, 2011), amongst others. *Neofusicoccum parvum* is one of the most widespread species of *Botryosphaeriaceae*. The common occurrence of *N. parvum* on healthy-looking fruits might have been a pathway for worldwide distribution during international trade.

High levels of genetic diversity were observed in the *N. parvum* populations considered in this study. Population genetics studies on this fungus often reflect a combination of sexual and asexual reproduction (Pavlic *et al.,* 2015; Mehl *et al.,* 2017b; Nagel *et al.,* 2018). Mixed reproduction allows for both increased genetic diversity through sexual reproduction and rapid dissemination of successful genotypes through asexual reproduction (Zhan *et al.,* 2000; Bihon *et al.,* 2012). Further studies are required to support evidence of recombination and the presence of sexual structures under field conditions. There was also no subdivision of diversity between the two sites, or amongst the hosts, although they are 90 km apart.

STRUCTURE and PCoA analyses for the samples identified three genetic clusters. This finding is consistent with those reported previously by Pavlic *et al.* (2015) and Mehl *et al.* (2017b) where three subpopulations were identified in the *N. parvum* populations in South Africa. These populations do not correlate with the host or geographic occurrence. A possible explanation for these separate populations, consistently identified across studies, is the existence of further cryptic species. These species are not clear in phylogenetic studies and will likely require a population genomics approach to test.

It is not clear from this and previous studies where *N. parvum* is native. The widespread occurrence, wide host range and high genetic diversity on both native and introduced hosts (Pérez *et al.,* 2010; Pavlic *et al.,* 2015; Jami *et al.,* 2017; Mehl *et al.,* 2017b) suggest that this species might be native to South Africa. It is also possible that it has been introduced multiple times through the international trade of plants and plant materials (such as fruit). The high levels of genetic diversity observed in the *N. parvum* population in this and other studies suggest the extent to which human-facilitated movement of the *Botryosphaeriaceae* has occurred around the world.

5.9. Conclusions

Only *Neofusicoccum* species were isolated from *M. indica* fruits, despite the fact that other genera of *Botryosphaeriaceae* have been isolated from the same trees. Amongst these, *N. parvum* was by far the most common species identified. This raises questions about tissue specificity of *Botryosphaeriaceae* and the potential role of fruit in the global distribution of this species. Moreover, no *Botryosphaeriaceae* was isolated from *S. birrea* fruit, highlighting the differences between host species. Apart from these ecological and phytopathological aspects, the study also points to issues of genetic diversity within *N. parvum* that ask for further study.

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GENERAL DISCUSSION

Studies presented in this Thesis investigated species diversity and distribution of the *Botryosphaeriaceae* on three tree species of *Anacardiaceae* in disturbed and undisturbed ecosystems. Focus was also placed on species variation and occurrence on asymptomatic versus symptomatic branch tissues. Finally, the species occurring on fruits of *Mangifera indica* (mango) and *Sclerocarya birrea* (marula) were investigated, and the genetic diversity and population structure of *N. parvum*, which was the most dominant species on mango fruits were determined. Overall results from these studies revealed that ecosystem practices that vary significantly between disturbed and undisturbed ecosystems influence species diversity. It was also found that species of *Botryosphaeriaceae* are more diverse on trees in undisturbed ecosystems. Some of the species identified in these studies are new to science, while others are new records from South Africa.

Knowledge of endophytes, potential pathogens and pathogenic species of *Botryosphaeriaceae* is essential to studying emerging and re-emerging fungal pathogens. Research focussing on the species overlap between asymptomatic and symptomatic tissue showed that not all species of *Botryosphaeriaceae* co-occur on the two tissue types. Six *Botryosphaeriaceae* species were found on both tissue types, while five species were identified from only healthy plant tissues. It is suggested that the latter species are latent pathogens on these trees since they are known to cause disease on other tree species. The latent opportunistic behaviour of *Botryosphaeriaceae* species explains why these fungi are overlooked in plants and woody plants showing decline symptoms.

This study highlights the importance of examining different host tissues to obtain a broader understanding of species diversity of the *Botryosphaeriaceae* on these hosts*.* Research on the fruits of *Mangifera indica* and *Sclerocarya birrea*, identified a single genus from the *Botryosphaeriaceae*, namely *Neofusicoccum,* on *M. indica* fruits. No species from this family was identified from the fruit of *S. birrea.* Five *Neofusicoccumm* species were identified, of which *N. parvum* was the dominant species. Assessment of the genetic diversity and population structure of *N. parvum* isolates revealed the presence of three populations. High levels of genetic diversity were observed suggesting multiple introductions of the fungus with plant materials. The widespread occurrence and high levels of genetic diversity of *N. parvum* and other cosmopolitan species of *Botryosphaeriaceae* as shown in earlier studies indicate the extent to which human-mediated movement of these fungi has occurred and the inability of quarantine techniques to detect these fungi as latent pathogens during their endophytic phase.