Taxonomy and ecology of Botryosphaeriaceae associated with *Acacia karroo* in South Africa

by

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PHILOSOPHIAE DOCTOR

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Dedicated to my dearest parents, Marziye and Javeed,
and to my dear friend, Seonju

Declaration

I, the undersigned, hereby declare that the thesis submitted herewith for the degree *Philosophiae Doctor* to the University of Pretoria contains my own independent work. This work has hitherto not been submitted for any degree at any other University.

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Fahimeh Jami

January 2014

CONTENTS

"……My Parents gave me a wing……Mike gave me the other one……Marieka taught me how to fly……Bernard navigated me……my sisters, brothers and friends accompanied me in this journey……"

Fahimeh

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Fungi in the Botryosphaeriaceae (Botryosphaeriales), includes a large number of species that have been characterized as saprophytes, endophytes and plant pathogens. This family has a cosmopolitan distribution and occurs on a very wide range of hosts, including gymnosperms, monocotyledons and dicotyledons. The Botryosphaeriaceae infect mostly woody plants, many of which display high levels of endophytic colonization by these fungi. A number of studies have recently been conducted on these fungi infecting a variety of native woody plants across South Africa, showing that they occur widely in these hosts as endophytes. They have also been shown to be potential pathogens, and have often been recovered from die-back symptoms on these native tree species.

Die-back that is typical of Botryosphaeriaceae-associated diseases has been noticed on *Acacia karroo* (Sweet thorn, or Soetdoring) in various areas in southern Africa. The aims of the studies in this thesis were to characterise the Botryosphaeriales infecting this commonly occurring and important native tree across much of its natural range in South Africa. This tree species has one of the widest geographical ranges of any *Acacia* sp. in the region, spanning several different biomes in most geographical parts of South Africa. Infection by the Botryosphaeriaceae on *A. karroo* was investigated for both healthy plant material and die-back symptoms. The common occurrence of the Botryosphaeriaceae on this tree also provided the opportunity to study the geographical and temporal distribution of various species, as well as their potential tissue preference.

Initial surveys of Botryosphaeriaceae on native *A. karroo* trees were made in two previously unsampled regions of its distribution, namely Pretoria (Gauteng Province) and Bloemfontein (Free State Province). A number of novel taxa were discovered, and these are described in Chapter 1[1]. These species were defined based on the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS) region and large-subunit ribosomal RNA (LSU), translation elongation factor 1-α (TEF-1α) and β-tubulin loci, as well as supporting morphological characters. These tools and the emerging taxonomic framework provided the foundation for more comprehensive, systematic and targeted collections conducted to answer specific questions in subsequent chapters.

Common die-back symptoms have been observed on *A. karroo* trees in the Pretoria area (Gauteng province) for many years. These symptoms include rapid death of terminal branches giving rise to flagging symptoms, or individual branches with dead leaves. Botryosphaeriaceae species are known to be associated with die-back symptoms, and in Chapter 2[2], the presence and possible correlation of Botryosphaeriaceae with these symptoms was investigated. Due to the prominent endophytic nature of the Botryosphaeriaceae, healthy and diseased tissues on the same trees were compared to determine whether some species are exclusively associated with the diseased tissue or whether they also occur as asymptomatic endophytes. Isolations were made from both asymptomatic and die-back branches, and isolates identified based on ITS, TEF-1α and β-tubulin loci. The pathogenicity of all species was tested in inoculation trials on 1-year-old *A. karroo* trees in the greenhouse.

While some species of Botryosphaeriaceae appear to have wide host ranges, others are known only from single plant species. It is, however, not clear whether the apparently narrow host ranges reflect host specificity or if this is an artifact of incomplete sampling. In Chapter 3[3], host overlap by species of Botryosphaeriaceae was considered by isolating these fungi from native South African trees residing in four different families, namely *Acacia karroo* (Leguminosae), *Celtis africana* (Cannabaceae), *Searsia lancea* (Anacardiaceae) and *Gymnosporia buxifolia* (Celastraceae). This work was conducted at a single location and using one collection event. Isolates obtained, were characterized based on DNA sequence data and morphological comparisons.

In Chapter 4, the biogeographic structure of the Botryosphaeriaceae associated with *A. karroo* was considered across South Africa. This is the first time that such a large geographic survey across South Africa on a particular fungal group has been done. In addition, the temporal and spatial variation in Botryosphaeriaceae species, as well as occurrence across tissue types, were investigated by sampling *A. karroo* from a single location (the Pretoria area) over a three year period. Fungi were isolated as endophytes from healthy trees. Isolates were grouped based on morphology of cultures, and representatives were identified based on comparisons of sequence data for the ITS and TEF-1α loci.

Preface

Numerous studies have been done on the Botryosphaeriaceae in South Africa in recent years. This reflects their importance as pathogens and prominence in plant tissues. In fact, this family represents one of the best-studied fungal families in South Africa, with numerous other studies from other countries adding supporting data and knowledge. In Chapter 5 we review the recent literature, together with data from databases as well as our own data collected as part of this project, with regards to the host associations and geographic distribution of the Botryosphaeriaceae from South Africa. We also considered background information relating to the taxonomy, biology and pathology of these fungi, which is used throughout the thesis.

[1.] Jami F, Slippers B, Wingfield MJ, Gryzenhout M, 2012. Five new species of the Botryosphaeriaceae from *Acacia karroo* in South Africa. Cryptogamie Mycologie, 33, 245-266.

[2.] Jami F, Slippers B, Wingfield MJ, Gryzenhout M, 2013. Greater Botryosphaeriaceae diversity in healthy than associated diseased *Acacia karroo* tree tissues. Australasian Plant Pathology, 42, 421-430.

[3.] Jami F, Slippers B, Wingfield M, Gryzenhout M, 2013. The pattern of Botryosphaeriaceae on four unrelated native South African hosts. Fungal Biology, http://dx.doi.org/10.1016/j.funbio.2013.11.007.

Chapter 1

Five new species of the Botryosphaeriaceae from *Acacia karroo* in South Africa

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ABSTRACT

The Botryosphaeriaceae represents an important, cosmopolitan family of latent pathogens infecting woody plants. Recent studies on native trees in southern Africa have revealed an extensive diversity of species of Botryosphaeriaceae, about half of which have not been previously described. This study adds to this growing body of knowledge, by discovering five new species of the Botryosphaeriaceae on *Acacia karroo,* a commonly occurring native tree in southern Africa. These species were isolated from both healthy and diseased tissues, suggesting they could be latent pathogens. The isolates were characterized based on their morphology and compared to other species for which DNA sequence data are available, in phylogenetic analyses based on the ITS, TEF-1α, β-tubulin and LSU gene regions. The five new species were described as *Diplodia allocellula*, *Dothiorella dulcispinae*, *Do. brevicollis*, *Spencermartinsia pretoriensis* and *Tiarosporella urbis-rosarum*. Evidence emerging from this study suggests that many more species of the Botryosphaeriaceae remain to be discovered in the southern Africa.

INTRODUCTION

The Botryosphaeriaceae is a cosmopolitan family of fungi with a very wide host range of mostly woody plants. They can be primary or opportunistic pathogens, endophytes or saprobes, as reviewed by Slippers & Wingfield (2007). Many species have been reported from South Africa in recent years, isolated from trees in the Myrtaceae (*Eucalyptus* spp.), Proteaceae (*Protea* spp.), Fabaceae (*Acacia* spp.), Combretaceae (*Terminalia* spp.) and Pinaceae (*Pinus* spp.) (Smith et al. 1994; Denman et al. 2003; Smith et al. 2001; Slippers et al. 2004; Roux and Wingfield 1997; Van der Walt 2008; Pavlic et al. 2004; Begoude et al. 2010; Swart and Wingfield 1991). Most studies on members of the Botryosphaeriaceae in southern Africa have focused on their association with trees of agricultural or forestry importance.

A few recent studies of Botryosphaeriaceae on native southern African trees, such as *Syzygium cordatum* (Myrtaceae), *Pterocarpus angolensis* (Leguminosae), *Terminalia catappa* (Combretaceae), *Acacia* spp. especially *A. melifera* (Fabaceae), and woody species of *Leucadendron*, *Leucospermum* and *Protea* (Proteaceae), have revealed large numbers of species in these native environments, many of which have represented new species and genera. In total, 32 species of Botryosphaeriaceae have been identified from native woody hosts in South Africa, 12 species of which have been from native African *Acacia* spp., eight species from *S. cordatum*, seven species from *Pt. angolensis* and five species from *T. catappa*. Among these 32 species, 15 were new taxa (Pavlic et al. 2007; Begoude et al. 2010; Van der Walt 2008; Mehl et al. 2011). The number and distribution of new species in these studies suggest that there probably are many more Botryosphaeriaceae species in South Africa on unsampled hosts and in unexplored regions.

The ecological relevance of this diversity of the Botryosphaeriaceae discovered recently in native environments of South Africa, is not well understood. Endophytism is common to most species of the family. For example *Diplodia pterocarpi* Cooke, *Lasiodiplodia crassispora* T. Burgess & Barber, *L. mahajangana* Begoude, Jol. Roux, Slippers, *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous, *Neofusicoccum*

kwambonambiense Pavlic, Slippers & M.J. Wingf., *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *N. batangarum* Begoude, Jol. Roux & Slippers, and *Pseudofusicoccum olivaceum* J.W.M. Mehl & B. Slippers have been isolated from asymptomatic tissues of *T. catappa* in South Africa (Begoude et al. 2010). Many of the Botryosphaeriaceae are, however, best described as latent pathogens that can cause significant damage to woody agricultural crops and forestry species typically when host plants are under stress (Slippers and Wingfield 2007). This is also evident from their association with die-back of native trees such as those from *S. cordatum* in South Africa (Pavlic et al. 2004), namely *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not., *L. gonubiensis* Pavlic, Slippers & M.J. Wingf., *L. theobromae* (Pat.) Griffon & Maubl., *N. australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, *N. luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *N. mangiferae* (Syd. & P. Syd.) Crous, Slippers & A.J.L. Phillips, *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips and *N. ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips. Pathogenicity studies with species of Botryosphaeriaceae have shown that they often differ in pathogenicity on a particular host, or between hosts. For example, *N. vitifusiforme* causes die-back on *Vitis vinifera* (Van Niekerk et al. 2004), but was isolated as an endophyte from healthy tissue of *Terminalia* spp. in southern Africa (Begoude et al. 2011). *Lasiodiplodia pseudotheobromae* was isolated from both healthy tissues and those with symptoms of die-back on *T. catappa* trees, but isolates showed varying levels of pathogenicity on that host (Begoude et al. 2010). These studies have shown that much work will be required to fully understand the ecology of the Botryosphaeriaceae.

Little is known regarding species of the Botryosphaeriaceae on *Acacia karroo* in South Africa. This tree species is the most widespread and ecologically important native *Acacia* in southern Africa (Timberlake et al. 1999; Barnes et al. 1996). Previous studies on Botryosphaeriaceae species of native *Acacia* have focused mostly on *A. mellifera* (black thorn) and were limited to the Prieska area (Northern Cape province) in South Africa and Namibia (Van der Walt 2008). Furthermore, surveys on non-native *A. mearnsii* or black wattle were conducted in Pietermaritzburg (KwaZulu/Natal Province), Piet Retief (Mpumalanga Province) and Alexandria (Eastern Cape Province) (Roux and Wingfield 1997). Samples of *A. karroo* were limited and yielded only three Botryosphaeriaceae

species, namely *B. dothidea*, and a new species in *Phaeobotryosphaeria* and *Spencermartinsia*, respectively (Van der Walt 2008). There thus appears to be substantial scope to more thoroughly sample this important and widespread host, especially in previously unsampled areas.

This study is part of a larger effort to document the diversity of the Botryosphaeriaceae associated with South African native trees in general, and on *A. karroo* in particular. Here we report on two previously unsampled regions of its distribution, namely Pretoria (Gauteng Province) and Bloemfontein (Free State Province) in South Africa. As in previous studies on native hosts, surveys revealed a high level of diversity of new Botryosphaeriaceae species. The isolated species were characterized here based on morphology and DNA sequences of the ITS, TEF1-α, β-tubulin and large subunit rDNA gene regions.

MATERIALS AND METHODS

Collection of samples

Sixty four asymptomatic branches and 40 branches showing die-back were collected from 48 trees at various sites around the city of Pretoria, Gauteng Province, and from the Glen area, Bloemfontein, Free State Province, South Africa, in June 2008 and February 2010 (Table 1). Plant tissues were surface sterilized in 10 % hydrogen peroxide for two minutes, rinsed three times in sterile water and placed on 2 % malt extract agar (Biolab, Midrand, South Africa). Cultures showing typical morphology of the Botryosphaeriaceae (fast growing, white to black cultures with aerial hyphae) were isolated after 4-5 days. Single hyphal-tip cultures of these isolates were made and these are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, with

duplicates of type cultures deposited in the Centraalbureau voor Schimmelcultures (CBS), The Netherlands.

DNA sequence analyses

Isolates obtained in this study were initially grouped based on culture morphology. DNA was extracted from the mycelium of 5-day-old single hyphal-tip cultures (Lee and Taylor 1990) of three representatives for each morphological group. Sequence data from the following genome regions were used in phylogenetic analyses, namely the internal transcribed spacer (Kirisits 2004) region of the ribosomal RNA (rRNA) operon using primers ITS-1 (Gardes & Bruns, 1993) and ITS-4 (White *et al*., 1990), the translation elongation factor 1-α (TEF-1α) gene using primers EF1-728F and EF1-986R (Carbon & Kohn, 1999), the β-tubulin gene using primers Bt2a and Bt2b (Glass & Donaldson, 1995), and the large subunit rDNA (LSU) gene region using primers LR0 and LR5 (Vilgalys & Hester, 1990).

The 25 µl PCR reaction mixtures contained 2.5 µl of 10 mM PCR buffer (PCR buffer with $MgCl_2$), 1 µl of 25 mM $MgCl_2$, 2.5 µl of 100 mM of each deoxynucleotide triphosphate, 1 µl of 10 nM of each primer, 2 ng DNA template and 1 U Taq polymerase (Biotech International, Needville, TX, USA). Non-template controls of sterile water were used with every PCR reaction. The amplification conditions were as follows: initial denaturation of 5 min at 95 °C, followed by 35 cycles of 30 seconds at 95 °C, 30 seconds at 56 °C, and 1 min at 72 °C, and a final extension of 5 min at 72 °C. Five µl of each PCR product were separated by electrophoresis in 1 % agarose gels in 5 % TAE buffer (40 mM Tris, 40 mM acetate, 2 mM EDTA, pH 8.0). The amplified PCR fragments were purified with Sephadex (Sigma, Steinheim, Germany) and sequenced with the BigDye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, California, USA) in both directions, with the same primers used for the PCR reactions. Products were separated with an ABI 3730 48 capillary sequencer (Perkin-Elmer Applied Biosystems).

Sequences of the isolates were edited using Vector NTI 11 (Lu and Moriyama 2004). DNA sequences for species previously published were retrieved from GenBank (http://www.ncbi.nlm.gov). The resulting data matrices for each gene were rooted with *Pseudofusicoccum stromaticum* (Mohali, Slippers & M.J. Wingf.) Mohali, Slippers & M.J. Wingf. following the example of Phillips *et al.* (2008). The data matrices were aligned online using MAFFT (http://align.bmr.kyushuu.ac.jp/mafft/online/server/) version 6 (Katoh et al. 2005) and checked manually for alignment errors.

Phylogenetic analyses of sequence data for Maximum Parsimony (MP) and Maximum Likelihood (ML) were made using PAUP* v.4.0b10 (Swofford 2001). Maximum parsimony genealogies for single genes were constructed with the heuristic search option (100 random taxa additions), tree bisection and reconstruction (TBR) in PAUP. The uninformative aligned regions within each dataset were removed from the analyses, gaps were treated as fifth character and all characters were unordered and of equal weight. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the tree(s) obtained was evaluated by 1000 bootstrap replications. Congruence between the different datasets was tested using the partition homogeneity test (PHT) in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Farris et al. 1995; Huelsenbeck et al. 1996), with the uninformative characters removed before analysis. Other measures such as tree length (TL), consistency index (CI), rescaled consistency index (RC), and the retention index (RI) (Hillis and Huelsenbeck 1992) were recorded.

For ML analyses, the best nucleotide substitution models for each dataset separately, were found with Modeltest 3.7 (Posada & Buckley, 2004). The models K81uf+I+G (G=0.528, I=0.476), TrNef+I+G (G=0.819, I=0.759), TrN+I+G (G=1.807, I=0.144), TrN+I+G (G= 1.476, I= 0.562) and GTR+I+G (G=0.2910, I=0.0) were chosen for the ITS, LSU, TEF-1α, β-tubulin and combined datasets, respectively. The analyses were also performed in PAUP 4.0b10.

Morphological characteristics

To induce sporulation, cultures were inoculated onto sterilized twigs of *A. karroo* placed on the surface of 2% MEA (Biolab), and these were incubated at 25 °C under near-UV light. Fifty released conidia, and 20 pycnidia and conidiogenous cells were measured for the isolates chosen to represent holotypes for each putative new species, and the ranges and averages were computed. These measurements were augmented with 20 measurements obtained from additional isolates. Measurements and digital images were made with an HRc Axiocam digital camera and accompanying Axiovision 3.1 software (Carl Zeiss Ltd., Munich, Germany). The specimens were deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

Colony morphology and colour were determined from cultures grown on MEA at 5–35 °C, at 5 °C intervals, in the dark. For these, 6 mm diam. mycelial plugs were taken from the edges of actively growing 5-day-old single conidial cultures, and transferred to the centers of 25 ml MEA in 90 mm diam. Petri dishes. Three replicate plates were used for each isolate per temperature. Two perpendicular measurements were taken of the colony diameter daily until the mycelium of the fastest growing isolates had covered the plates and averages were computed. Colony colours were assigned using the designations of Rayner (1970).

RESULTS

Collection of samples

Several known as well as previously uncharacterized species of Botryosphaeriaceae were recovered from both healthy and diseased *A. karroo* and the focus of this study will only be on those not previously described. Twenty three isolates from all those recovered were determined as belonging to one of several new species. Six isolates of undescribed

species were from samples collected in Bloemfontein and 17 were from the Pretoria area. These could be grouped into five morphotypes that were selected for sequencing.

DNA sequence analyses

The datasets for the ITS, TEF-1α, β-tubulin and LSU rDNA sequences were analyzed individually and in combination. The ITS sequence dataset contained 522 characters (4 parsimony-uninformative, 148 parsimony-informative, 370 constant characters) with CI = 0.602, RI = 0.902, RC = 0.543, HI = 0.398 and TL = 321. The TEF-1α dataset contained 362 characters (3 parsimony-uninformative, 224 parsimony-informative, 135 constant characters) with CI = 0.617, RI = 0.891, RC = 0.550, HI = 0.383 and TL = 523. The β-tubulin dataset contained 471 characters (0 parsimony-uninformative, 142 parsimony-informative, 329 constant characters) with CI = 0.698, RI = 0.901, RC = 0.629, HI = 0.302, and TL=304. The LSU dataset contained 848 characters (7 parsimony-uninformative, 74 parsimony-informative and 767 constant characters) with CI = 0.652, RI = 0.885, RC = 0.577, HI = 0.348 and TL = 128. The tree statistics for the combined dataset were CI = 0.487, RI = 0.854, RC = 0.416, HI = 0.513, TL = 2148 (TreeBase Accession No. S12358), and the partition homogeneity test (PHT) on the datasets produced a P-value of 0.01.

The topology of the trees emerging from the ML and MP analyses were similar for the individual gene regions as well as in the combined analysis with regards to the clades representing species isolated in this study. However, clades representing genera occasionally collapsed in individual analyses. Fixed alleles could be identified in the datasets for the clades of the species identified in this study (Tables 2-4). Seven clades were identified in the MP and ML analyses representing *Lasiodiplodia*, *Diplodia*, *Tiarosporella, Dothiorella*, *Spencermartinsia, Phaeobotryosphaeria* and *Botryosphaeria.*

Isolates resided in four genera (Figure 1). Isolates CMW36468, CMW36469 and CMW36470 (from healthy branches of *A. karroo* in Pretoria) formed a distinct clade together with other *Diplodia* species. Isolates CMW36477, CMW36478 and CMW36479

(from healthy *A. karroo* in Pretoria and Bloemfontein) formed a distinct clade with *Tiarosporella* species. Isolates from healthy branches of *A. karroo* in Pretoria (CMW36480 and CMW36481) grouped separately with *Spencermartinsia* species, while the clade containing species of *Dothiorella* included two sub-clades (isolates CMW36460, CMW36461 and CMW36462 from branches with die-back from Pretoria, and isolates CMW36463 and CMW36464 from healthy branches in Pretoria, respectively). There was considerable sequence variation in the four gene regions (ITS, TEF-1α, β-tubulin and LSU rDNA) among isolates representing the undescribed species and those of their sister species, which included an undescribed *Dothiorella* species (Van der Walt *et al*. 2008) from *Acacia mellifera* (Table 2), *Dothiorella longicollis* Pavlic, T.I. Burgess & M.J. Wingf. (Table 3), and an undescribed *Spencermartinsia* species (Van der Walt *et al*. 2008) from *Acacia erioloba* (Table 4).

Morphological characteristics

Isolates were divided into five groups based on culture morphology, conidial shape and colour. The isolates in the fifth group corresponded to *Tiarosporella* in the DNA sequence comparisons and had white, raised aerial mycelium at the centers of the cultures with grey edges and undersides of the plates, and produced large hyaline conidia different from those of the other taxa. Isolates in the other four groups had dark grey or olivaceous colonies with aerial hyphae and dark conidia. *Diplodia*, *Dothiorella* and *Spencermartinsia* isolates could be distinguished by differences in growth and colour of the isolates. The isolates residing in *Diplodia* had grey mycelium with dark, regular edges and mostly aseptate conidia while those in *Dothiorella* had dark grey mycelium with irregular edges. The *Spencermartinsia* isolates were similar to the *Dothiorella* isolates and had similar conidia, but the colonies had regular edges. The species distinguished by phylogenetic inference could also be distinguished from other species in the respective genera based on differences in pycnidium shape and conidial size.

The use of *A. karroo* twigs on MEA to induce fruiting structures resulted in only anamorph states that were similar in characteristics between genera. The substantial

overlap in these characters allowed only limited comparisons with herbarium specimens and previous descriptions. Differences in ascospore septation distinguish *Spencermartinsia* (2-celled ascospores with an apiculus at either end of the ascospore) and *Dothiorella* (1-septate ascospores and apiculi absent), but ascospores were not observed in this study.

TAXONOMY

The following are descriptions of new species based on morphology:

Tiarosporella urbis-rosarum Jami, Gryzenh., Slippers & M.J. Wingf. sp. nov. MB564139 (Figure 2)

Etymology: The name refers to the city Bloemfontein, popularly called 'The city of roses', where this species was first isolated.

No teleomorph produced.

Pycnidia produced on *Acacia karroo* twigs on MEA in 6-10 weeks, solitary, globose, dark black (29`````m), up to 200 µm wide, immersed, unilocular, with long necks (4-9 mm); wall 5-7 cell layers thick, outer layers composed of dark-brown *textura angularis*, becoming thin-walled and hyaline towards the inner region. *Conidiogenous cells* holoblastic, hyaline, cylindrical, (5-) 5.5-9.5 (-11) × (3-) 3.2-4 (-5) µm. *Conidia* ovoid, smooth with fine granular content, thin-walled, hyaline, aseptate, apices rounded, (21-) 23.5-29.5 (-34) × (8-) 9-10 (-11) µm.

Colonies on MEA with appressed mycelial mats, pycnidia emerging after 2 months under near-ultraviolet light on *A. karroo* twigs. Mycelium grey, white at the centers, becoming dark grey at the regular edges, reverse dark grey to black. Growth at 5-35 °C. Growth rate 14.4 mm per day at an optimal temperature of 25 °C; covering the agar surface in a 90 mm diam. Petri dish after 6 days in the dark.

Specimens examined: South Africa, Free State Province, Bloemfontein, June 2008, M. Gryzenhout, from healthy wood section of *Acacia karroo*, holotype PREM 60698 resulting from inoculations of living isolate to *A. karroo* twigs, living cultures CMW 36477 = CBS 130405.

Additional specimens: South Africa, Free State Province, Bloemfontein, June 2008, M. Gryzenhout, from healthy wood section of *Acacia karroo.* Paratype PREM 60699 (living cultures CMW 36478 = CBS 130406). South Africa, Gauteng Province, Pretoria, June 2008, M. Gryzenhout & F. Jami, from healthy wood section of *Acacia karroo*. Paratype PREM 606700 (living cultures CMW 36479 = CBS 130407).

Notes: Eight species of this genus have been described (Karadzic 2003). DNA sequences of only *Tiarosporella madreeya*, *T. tritici* and *T. graminis* var. *karroo* are, however, available (Crous et al. 2006) and the DNA sequences of *T. urbis-rosarum* differ from these species. The sizes of conidia of *T. urbis-rosarum* are very similar to those of *T. tritici* (Crous et al. 2006), but conidia of *T. urbis-rosarum* are slightly smaller (23.5-29.5 × 9-10 µm) than those of *T. tritici* (29-38 × 12-17 µm).

Diplodia allocellula Jami, Gryzenh., Slippers & M.J. Wingf. sp. nov. MB 564140 (Figure3)

Etymology: The name refers to the variability in length of the conidiogenous cells in this fungus.

No teleomorph produced.

Pycnidia immersed on MEA in 6-10 weeks, solitary, globose, chestnut, covered by hyphal hairs, up to 100 µm diam, without necks. *Conidiogenous cells* holoblastic, smooth with fine granular contents, unicellular, cylindrical to sub-cylindrical, hyaline and truncate

at base, (4-) 4.2-5 (-5.5) × (10.3-) 13.4-23.6 (-27.6) µm. *Conidia* ovoid to ellipsoid, smooth with fine granular content, apices rounded and truncated at the base, thick-walled, aseptate, initially hyaline, becoming dark brown or sepia (13``k), aseptate, (9-) 10-12.5 (-14.5) × (20-) 21.5-25 (-30) µm.

Colonies on MEA initially white turning grey from the middle of colonies within 5-7 d, aerial mycelium slightly fluffy, becoming dense, cottony with age, turning smoke grey to dark grey (23````d) toward the edges after 7-9 days, reverse olivaceous-black (33```` m) and with regular edges. Growth in culture from 5-35 °C. Growth rate of 18.2 mm per day optimal at 25 °C and covering 90 mm diam. Petri dishes after 5 days in the dark.

Specimens examined: South Africa, Gauteng Province, Pretoria (George Storrar avenue, Fountain circle), Nov. 2009, M. Gryzenhout & F. Jami, from branch of *Acacia karroo* showing die-back, holotype PREM 60701 resulting from inoculations of living isolates onto *A. karroo* twigs, living cultures CMW 36468 = CBS 130408.

Additional specimens: South Africa, Gauteng Province, Pretoria (George Storrar avenue, Fountain circle), Nov. 2009, M. Gryzenhout & F. Jami, from branch of *Acacia karroo* showing die-back. Paratype PREM 60702 (living cultures CMW 36469 = CBS 130409) and PREM 60703 (living cultures CMW 36470 = CBS 130410).

Notes*: Diplodia allocellula* grouped with *D. mutila* (Fr.) Mont., *D. rosulata* Gure, Slippers & Stenlid and *D. africana* Damm & Crous as sister species in the phylogenetic analyses of other *Diplodia* species. *Diplodia allocellula* differ phylogenically from its closest *Diplodia* relatives and morphologically in its smaller conidia (10-12.5 × 21.5-25 µm), compared to those of *D. mutila* (13.2-13.5 × 25.1-25.7 µm), *D. rosulata* (11-17.5 × 25-32 µm) and *D. africana* (10-15 × 25.5-33 µm). *Diplodia rosulata* has been found from seeds of *Prunus africana* in Ethiopia (Gure et al. 2005), *D. mutila* is known from diseased *Vitis vinifera*, *Pyrus communis*, *Quercus suber* and *Malus pumila* in Spain, Portugal and USA (Alves et al. 2004), while *D. africana* has been described from wounds on *Prunus persica* in South Africa (Western Cape Province) (Damm et al. 2007). In contrast, *Diplodia*

allocellula was obtained from healthy *A. karroo* tissue in this study in Pretoria, South Africa.

Dothiorella dulcispinae Jami, Gryzenh., Slippers & M.J. Wingf. sp. nov. MB 564141 (Figure 4)

Etymology: The name refers to the host, *Acacia karroo* (Sweet Thorn), dulcis=sweet and spina= a thorn.

No teleomorph produced.

Pycnidia produced on *Acacia karroo* twigs on MEA after 2-4 weeks, solitary, globose, dark brown, up to 200 µm wide, semi-immersed, unilocular, with a short necks (100-300 µm); wall 6-8 cell layers thick, outer layers composed of dark-brown *textura angularis*, becoming thin-walled and hyaline toward the inner region. *Conidiogenous cells* 1-2 celled, holoblastic, hyaline, cylindrical, proliferating percurrently near the apex. *Conidia* ovoid, smooth with fine granular content, rounded apices, thick-walled, initially hyaline and aseptate, becoming dark brown or sepia (13``k) and 1-septate, with 2 cells of unequal length, apices rounded, (6-) 7-10 (-11) × (14-) 16-22 (-24) µm.

Colonies on MEA developing dense aerial mycelium with age, olivaceous-grey (23````l), surface pale olivaceous-grey (21````i) to olivaceous black (27````m), reverse olivaceous-black, umbonate with irregular zonation and lobate edges. Growth at 5-35 °C, 17.9 mm per day and optimal at 25 °C, covering the surface of 90 mm diam. Petri dishes after 5 days in the dark.

Specimens examined: South Africa, Gauteng Province, Pretoria, Nov. 2009, F. Jami, from die-back wood section of *Acacia karroo*, holotype PREM 60706 resulting from inoculations of living isolates onto *A. karroo* twigs (living cultures CMW 36460= CBS

130413).

Additional specimens: South Africa, Gauteng Province, Pretoria, Nov. 2009, F. Jami, from die-back wood section of *Acacia karroo*, paratype PREM 60707 (living cultures CMW 36461= CBS 130414) and PREM 60708 (living cultures CMW 36462= CBS 130415).

Note*:* Based on phylogenetic analyses, isolates of *Do. dulcispinae* are most closely related to those of a undescribed species of *Dothiorella*, which is known as an endophyte of *A. mellifera* in South Africa (Van der Walt 2008). It differs from the latter species in the morphology of pycnidia, conidiogenous cells and conidia which are all smaller than those of the undescribed *Dothiorella* species.

Dothiorella brevicollis Jami, Gryzenh., Slippers & M.J. Wingf. sp. nov. MB 564142 (Figure 5)

Etymology: The name refers to the fact that the pycnidia have short necks.

No teleomorph produced.

Pycnidia, produced on *Acacia karroo* twigs on MEA after 2-4 weeks, brown (7``m), up to 200 µm wide, semi-immersed, unilocular, globose with a short neck; wall 5-7 cell layers thick, outer layers composed of dark-brown *textura angularis*, becoming thin-walled and hyaline toward the inner region. *Conidiogenous cells* holoblastic, hyaline, cylindrical, (3-) 3.2-3.7 (-4) × (3-) 3.2-7.5 (-9) µm. *Conidia* ovoid, smooth with fine granular content, rounded apices, thick-walled, initially hyaline and aseptate, becoming dark brown and 1-septate, with 2 cells of equal length, apices rounded, (8-) 9-12 (-13) × (20-) 21.5-26 (-27) µm.

Colonies on MEA appressed, pycnidia emerging after 9–10 d under near-ultraviolet light. Mycelium olivaceous-grey (21````b), surface pale olivaceous-grey to dark olivaceous-grey (23````l), reverse olivaceous-black (29````m) to iron grey (29`````k), irregular edges. Growth observed from 5-35 °C, reaching 17.6 mm per day and optimal at 25 °C, covering the surface of a 90 mm diam. Petri dish after 6 days in the dark.

Specimens examined: South Africa, Gauteng Province, Pretoria, Nov. 2009, F. Jami, from healthy wood section *Acacia karroo,* holotype PREM 60704 resulting from inoculations of living isolates onto *A. karroo* twigs (living cultures CMW 36463 = CBS 130411).

Additional specimens: South Africa, Gauteng Province, Pretoria, Nov. 2009, F. Jami, from healthy wood section *Acacia karroo,* paratype PREM 60705 (living cultures CMW 36464 = CBS 130412).

Notes*: Do. brevicollis* has shorter pycnidial necks than those of *Do. longicollis*. It can also be distinguished from *Do. dulcispinae* described in this study based on phylogenetic analyses and by its larger conidia.

Spencermartinsia pretoriensis Jami, Gryzenh., Slippers & M.J. Wingf. sp. nov. MB 564143 (Figure 6)

Etymology: The name refers to Pretoria, where samples yielding the species were collected.

No teleomorph produced.

Pycnidia produced on *Acacia karroo* twigs on MEA after 2-4 weeks, brown, up to 200 µm wide, semi-immersed, unilocular, with short necks; wall 5-7 cell layers thick, outer

layers composed of dark-brown *textura angularis*, becoming thin-walled and hyaline towards the inner regions. *Conidiophores* absent and reduced to *conidiogenous cells*, 1-2 celled, hyaline, holoblastic, cylindrical, proliferating percurrently near the apex, (3-) 3.2-7.5 (-9) × (3-) 3.2-3.7 (-4) µm. *Conidia* ovoid, smooth with a fine granular content, rounded apices, thick-walled, initially hyaline and aseptate, becoming dark brown (7``m) and 1-septate, with 2 cells of equal length, apices rounded, (18-) 20-28 (-33) × (6.5-) 7-14 (-11) µm.

Colonies on MEA appressed, and pycnidia emerging after 8-10 days. Mycelium pale olivaceous, surface pale olivaceous to dark greenish olivaceous, reverse olivaceous-black (29````m), regular zonation and lobate edges. Growth from 5-35 °C; up to 22.5 mm per day and optimal at 25 °C, covering the surface of 90 mm diam. Petri dish after 4 days in the dark.

Specimens examined: South Africa, Gauteng Province, Nov. 2009, F. Jami, from die-back wood section *Acacia karroo,* holotype PREM 60709 resulting from inoculations of living isolates onto *A. karroo* twigs (living cultures CMW 36480 = CBS 130404)

Additional specimens: South Africa, Gauteng Province, Nov. 2009, F. Jami, from die-back wood section *Acacia karroo,* paratype PREM 60710 (living cultures CMW 36481 = CBS 130405).

Note*: Spencermartinsia pretoriensis* is not distinguishable from an undesrcibed *Spencermartinsia* species from *Acacia erioloba* based on morphology and could be differentiated from this species only with DNA sequence comparisons (Table 4).

DISCUSSION

Extensive sampling for the Botryosphaeriaceae on native trees has been undertaken in South Africa in recent years. Despite this fact, this study yielded a large number of new taxa, which suggests that many undescribed species remain to be isolated and identified. Here we considered only tree species (*A. karroo*), in two areas where were not previously sampled. In the process we discovered five new species in four genera of the Botryosphaeriaceae. Identifications of the species collected were primarily based on DNA sequence comparisons of four loci. It was not possible to compare the species with the plethora of those previously described in these genera and for which no sequence data are available. However, morphological data were useful to distinguish these five species from each other and from other species described from the region.

Three of the five newly discovered species are relatively closely related and reside in the *Spencermartinsia* and *Dothiorella* clades. These two genera have only recently been split and were previously treated in the single genus *Dothiorella*. When *Do. viticola* was described as a new species, it could not be distinguished from *Do. iberica* and *Do. sarmentorum* based on anamorphic features, thus differences in teleomorph characters were used to differentiate the three species (Luque et al. 2005) where *Do. viticola* has 2-celled ascospores with apiculi at either end. Later studies introduced *Do. viticola* as the new genus *Spencermartinsia* (Phillips et al. 2008). The teleomorph for other members of *Spencermartinsia* spp. is still unknown, and their placement in the genus rests only on gene sequence similarities. In this study *S. pretoriensis* was placed in the genus because of its grouping with other *Spencermartinsia* spp. based on DNA sequences alone, and separate from the clade containing *Dothiorella* spp. These analyses, however, also show distinct clades within both the *Dothiorella* and *Spencermartinsia* clades, which might later be interpreted as representing distinct genera. As in the case of *Dothiorella* and *Spencermartinsia*, discovery of a teleomorph may also lead to changes in the taxonomy of this group.

Spencermartinsia species appear to be diverse on *Acacia* in southern Africa and the

discovery of *S. pretoriensis* on *A. karroo* in this study adds to this view. All four *Spencermartinsia* spp. that are currently known were recently isolated from native *Acacia* spp. in southern Africa, such as *A. erioloba, A. mellifera* and *A. tortillis* (Van der Walt 2008). *Spencermartinsia viticola,* the type species of the genus, was described from grape vines in Spain (Luque et al. 2005) and from citrus in California (Adesemoye and Eskalen 2011). It was also found on *A. melifera* in Pretoria, South Africa (Van der Walt 2008). Interestingly it is the only species thus far known from hosts other than *Acacia* and from the countries other than South Africa. Further studies of the Botryosphaeriaceae on native *Acacia* will test the emerging hypothesis that this group evolved on this host and in Africa.

Tiarosporella spp. have a distinct culture and conidial morphology compared to other genera of the Botryosphaeriaceae. Species have a woolly mycelium that is white at the center and grey at the colony edges. Pycnidia have long necks and produce ovoid hyaline conidia. Three species, *T. tritici* B. Sutton & Marasas from *Triticum aestiuum* (Poaceae), *T. graminis* var. *karroo* B. Sutton & Marasas, and *T. graminis* var. *gamines* (Piroz. & Shoemaker) Nag Raj from *Tribulus terrestris* (Zygophyllaceae), *Eriocephalus* sp. (Asteraceae) and *Nestlera* sp. (Poaceae) have been described from Heilbron (Free State province) and Middelburg (Eastern Cape province) in South Africa (Sutton and Marasas 1976). Crous et al. (2006) found that *Tiarosporella* grouped in an unresolved clade with *Lasiodiplodia*/*Diplodia.* Subsequent studies using greater numbers of samples showed that *Diplodia* and *Lasiodiplodia* group closely together (Phillips et al. 2008), but the placement of *Tiarosporella* was not addressed. Results of this study support the distinct grouping of *Tiarosporella* from species of *Lasiodiplodia* and *Diplodia*.

Species of the Botryosphaeriaceae are well-known as latent pathogens that can cause disease under conditions of stress (Slippers and Wingfield 2007). Two of the five species described in this study (*Do. dulcispinae* and *S. pretoriensis*) were isolated from diseased tissue, while three (*Do. brevicollis, T. urbis-rosarum* and *D. allocellula*) were associated only with healthy *A. karroo* tissue. These patterns of isolation do not allow for deductions to be made regarding the ability of the fungi to cause disease on *A. karroo,* and pathogenicity trials will be needed to better understand the role of these fungi as possible disease agents.

The host ranges of the new species described in this study are unknown. Very few Botryosphaeriaceae appear to be host specific when they are sampled widely. For example, *N. eucalyptorum* (Crous, H. Smith & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips and *N. eucalypticola* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips were thought to be specific to *Eucalyptus* spp. (Slippers et al. 2004), but *N. eucalyptorum* was subsequently found on other Myrtaceae (Pérez et al. 2009). A number of apparently host specific species are found among *Dothiorella* species, such as *Do. moneti* K. Taylor, Barber & T.I. Burgess from *Acacia rostellifera*, *Do. santali* K. Taylor, Barber & T.I. Burgess from *Santalum acuminatum* in Western Australia (Taylor et al. 2009), an undescribed species of *Dothiorella* from *A. melifera* in South Africa (Van der Walt 2008), and *Do. casuarini* J. de Wet, Slippers & M.J. Wingf. from a *Casuarina* sp. in Australia (De Wet et al. 2009). Other species in *Dothiorella* have been reported from different hosts such as *Do. longicollis* Pavlic, Burgess & M.J. Wingf. described from both *Lysiphyllum cunninghamii* and a *Terminalia* sp. in Western Australia (Pavlic et al. 2008), *Do. sarmentorum* from *Malus, Ulmus, Pyrus, Prunus, Menispermum* and almond, and *Do. iberica* from *Quercus* and *Malus* in Europe and USA (Phillips et al. 2005; Inderbitzin et al. 2010). A number of species in the Botryosphaeriaceae such as *L. theobromae, B. dothidea*, and *N. parvum* are also known to have wide host ranges (Slippers and Wingfield 2007). Similarly on *Acacia*, of the 12 Botryosphaeriaceae species isolated by Van der Walt et al. (2008) from *Acacia*, three species (*B. dothidea* and undescribed species in *Spencermartinsia* and *Pheaobotryosphaeria*) were found on *A. karroo* and other *Acacia* species. It is thus expected that the species described here may also be found on other members of *Acacia* and perhaps even other tree genera and their complete host ranges of the new species described in this study are still unknown.

The two different areas sampled in this study revealed different Botryosphaeriaceae species. Only one of five species was found in Bloemfontein, while all five occurred in Pretoria. Van der Walt et al. (2008) found that geography influenced the structure of Botryosphaeriaceae diversity on *Acacia*. In that study, which included samples from diverse areas of southern Africa, only three of 12 species overlapped among the regions sampled, while the others were found at only one or two sites. Other studies on the Botryosphaeriaceae have shown similar patterns. For example, seven species of the

Botryosphaeriaceae from *Adansonia gibbosa* sampled at five different sites in Western Australia (Pavlic et al. 2008), and six Botryosphaeriaceae species from *Prunus dulcis* from seven different sites in California, USA (Inderbitzin et al. 2010) revealed largely different species at the different sites. Thorough and repeated sampling targeting the various Botryosphaeriaceae species on *A. karroo* will, however, be needed to delimit geographic boundaries for the various species on this host.

Recent studies on the Botryosphaeriaceae from diverse native trees in South Africa (Pavlic et al. 2007; Begoude et al. 2010; Van der Walt 2008; Mehl et al. 2011) have resulted in the discovery of a large number of Botryosphaeriaceae species in this region. We, therefore, expected that the rate of new species descriptions in the family would decrease with additional sampling. The discovery of five new species in this study from a previously sampled host, and from only a few samples and sites, indicates that this expectation is unfounded and may not be realistic until a greater diversity of hosts have been sampled in many more locations across the region. *Acacia karroo* might be an ideal host, given its wide incidence across the region, to target for sampling alongside other hosts that are more geographically isolated. Such future work would provide a baseline of data on the geographical and host influence on structuring diversity in this important family of latent tree pathogens.

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Table 1. Representative isolates from *Acacia karroo* used in the phylogenetic analyses.

Isolate No.	Identity	Location	Collector	GenBank			
				ITS	EF1-α	LSU	β-tubulin
CMW36477 CBS 130405	*Tiarosporella urbis-rosarum*	Bloemfontein, SA	M. Gryzenhout	JQ239407	JQ239394	JQ239420	JQ239381
CMW36478 CBS 130406	*T. urbis-rosarum*	Bloemfontein, SA	M. Gryzenhout	JQ239408	JQ239395	JQ239421	JQ239382
CMW36479 CBS 130407	*T. urbis-rosarum*	Pretoria, SA	F. Jami & M. Gryzenhout	JQ239409	JQ239396	JQ239422	JQ239383
CMW36468 CBS 130408	*Diplodia allocellula*	Pretoria, SA	"	JQ239397	JQ239384	JQ239410	JQ239378
CMW36469 CBS 130409	*D. allocellula*	Pretoria, SA	"	JQ239398	JQ239385	JQ239411	JQ239379
CMW36470 CBS 130410	*D. allocellula*	Pretoria, SA	"	JQ239399	JQ239386	JQ239412	JQ239380
CMW36460 CBS 130413	*Dothiorella dulcispinae*	Pretoria, SA	"	JQ239400	JQ239387	JQ239413	JQ239373
CMW36461 CBS 130414	*Do. dulcispinae*	Pretoria, SA	"	JQ239401	JQ239388	JQ239414	JQ239374
CMW36462 CBS 130415	*Do. dulcispinae*	Pretoria, SA	"	JQ239402	JQ239389	JQ239415	JQ239375
CMW36463 CBS 130411	*Do. brevicollis*	Pretoria, SA	"	JQ239403	JQ239390	JQ239416	JQ239371
CMW36464 CBS 130412	*Do. brevicollis*	Pretoria, SA	"	JQ239404	JQ239391	JQ239417	JQ239372
CMW36480 CBS 130404	*Spencermartinsia pretoriensis*	Pretoria, SA	"	JQ239405	JQ239392	JQ239418	JQ239376
CMW36481 CBS 130405	*S. pretoriensis*	Pretoria, SA	"	JQ239406	JQ239393	JQ239419	JQ239377

Table 2. Polymorphic nucleotides fixed between an undescribed *Dothiorella* species from *Acacia mellifera* and *Dothiorella dulcispinae* based on sequence data of the ITS, TEF-1α, LSU and β-tubulin. Polymorphisms unique to *Dothiorella dulcispinae* are highlighted.

Identity	Isolate No.	ITS	TEF-1α				LSU		
		11	9	16	43	342	89	342	356
Undescribed *Dothiorella* species from *Acacia mellifera*	CBS121764	-	-	T	C	-	-	-	-
Undescribed *Dothiorella* species from *A. mellifera*	CBS121765	-	-	T	C	-	-	-	-
Dothiorella dulcispinae	CMW36460	T	A	-	-	T	G	T	G
Do. dulcispinae	CMW36461	T	A	-	-	T	G	T	G
Do. dulcispinae	CMW36462	-	A	-	-	T	G	T	G

Identity	Isolate No.	β-tubulin													
		43	49	56	61	139	157	166	171	180	267	333	345	348	410
Undescribed *Dothiorella* species from *Acacia mellifera*	CBS121764	T	A	A	-	G	C	-	C	C	A	G	T	T	-
Undescribed *Dothiorella* species from *A. mellifera*	CBS121765	T	A	A	-	G	C	-	C	C	A	G	T	T	-
Dothiorella dulcispinae	CMW36460	-	-	-	T	-	-	C	G	T	G	-	-	-	C
Do. dulcispinae	CMW36461	-	-	-	T	-	-	C	G	T	G	-	-	-	C
Do. dulcispinae	CMW36462	-	-	-	T	-	-	C	G	T	G	-	-	-	C

Table 3. Polymorphic nucleotides from sequence data of the ITS, TEF-1α, LSU and β-tubulin to show the relationships between *Dothiorella longicollis* and *Dothiorella brevicollis*. Polymorphisms unique to *Dothiorella brevicollis* are highlighted. (There were no differences for LSU sequence data.)

Identity	Isolate no.	ITS	TEF-1α					β-tubulin				
		72	9	31	39	88	90	244	247	350	56	450
Dothiorella longicollis	CBS122066	**T**	A	**A**	**T**	**T**	**-**	**-**	**A**	**C**	**-**	**T**
Do. longicollis	CMW26164	**T**	A	**A**	**T**	**T**	**-**	**-**	**A**	**C**	**-**	**T**
Dothiorella brevicollis	CMW36463	**-**	G	**-**	**-**	**-**	**G**	**A**	**-**	**-**	**A**	**-**
Do. brevicollis	CMW36464	**-**	**G**	**-**	**-**	**-**	**G**	**A**	**-**	**-**	**A**	**-**

Table 4. Polymorphic nucleotides from sequence data of the ITS, TEF-1α, LSU and β-tubulin showing the relationships between an undescribed *Spencermartinsia* species from *Acacia erioloba* and *Spencermartinsia pretoriensis*. Polymorphisms unique to *Spencermartinsia pretoriensis* are highlighted.

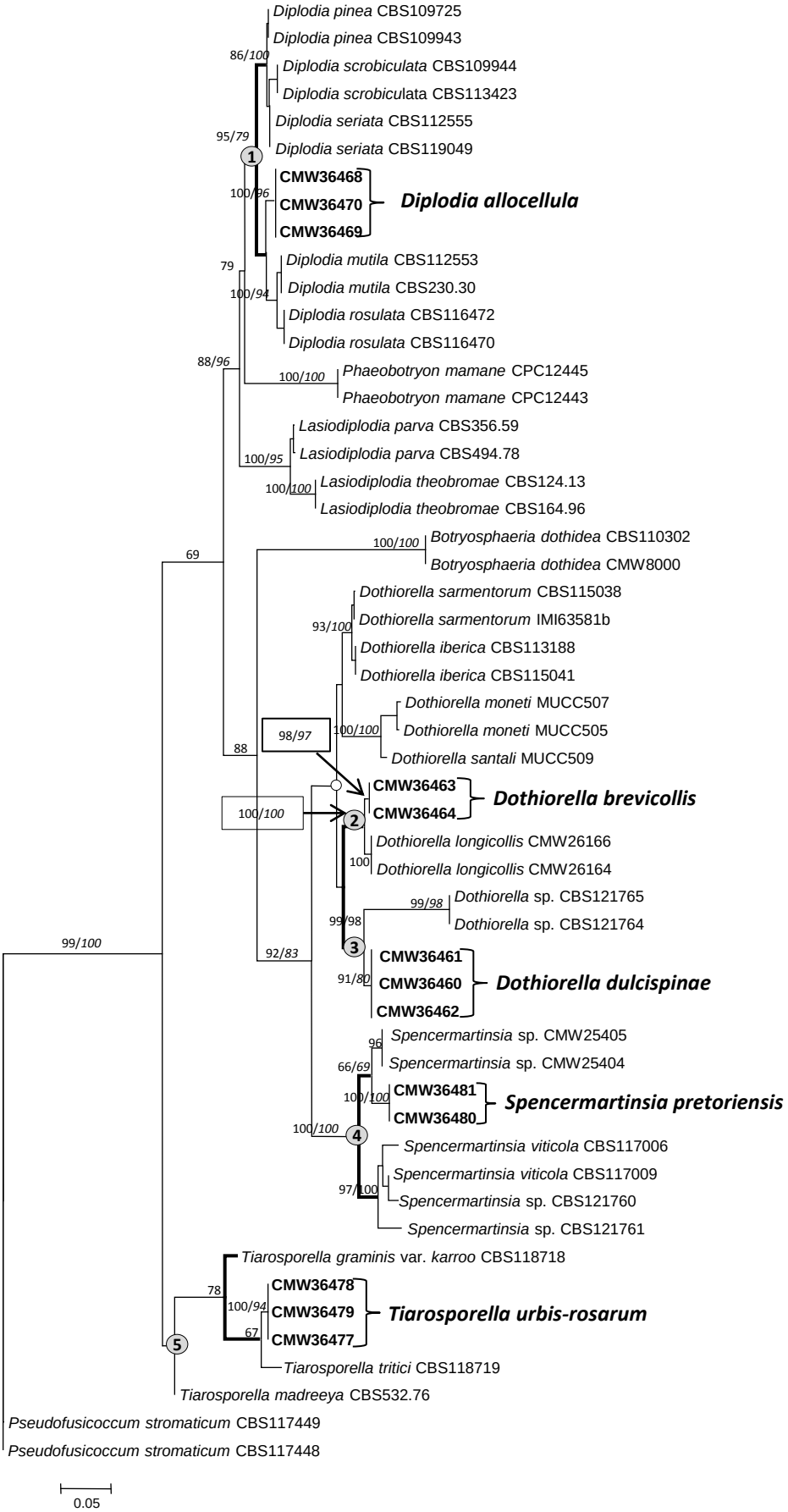
Identify	Isolate no.	ITS																				
		7	18	26	77	78	84	85	87	92	98	104	105	107-109	145	147	169-170	395	406	487	488
Undescribed *Spencermartinsia* species from *Acacia erioloba*	CMW25404	**A**	**T**	**-**	**C**	**-**	**T**	**C**	**C**	**-**	**-**	**-**	**-**	**- - -**	**A**	**-**	**A -**	**T**	**A**	**A**	**-**
Undescribed *Spencermartinsia* species from *A. erioloba*	CMW25405	**A**	**T**	**-**	**C**	**-**	**T**	**C**	**C**	**-**	**-**	**-**	**-**	**- - -**	**A**	**-**	**A -**	**T**	**A**	**A**	**-**
Spencermartinsia pretoriensis	CMW36480	**-**	**-**	**T**	**-**	**T**		**-**	**-**	**A**	**G**	**C**	**C**	**C C A**	**-**	**G**	**- C**	**-**	**-**	**-**	**C**
S. pretoriensis	CMW36481	**-**	**-**	**T**	**-**	**T**		**-**	**-**	**A**	**G**	**C**	**C**	**C C A**	**-**	**G**	**- C**	**-**	**-**	**-**	**C**

Table 4. Continued

Identify	Isolate no.	TEF-1α																							
		8	20	21	34	36	37	40	46	47	48	59	128	155	160	161	163	164	166	180	193	216	220	244	247
Undescribed *Spencermartinsia* species from *Acacia erioloba*	CMW25404	T	G	C	T	T	C	T	T	C	A	T	-	A	C	A	C	C	C	-	A	C	A	A	A
Undescribed *Spencermartinsia* species from *A. erioloba*	CMW25405	T	G	C	T	T	C	T		C	A	T	-	A	C	A	C	C	C	-	A	C	A	A	A
Spencermartinsia pretoriensis	CMW36480	C	C	G	-	C	-	C	C	G	T	-	C	G	G	G	T	-	T	A	T	G	C	G	C
S. pretoriensis	CMW36481	C	C	G	-	C	-	C	C	G	T	-	C	G	G	G	T	-	T	A	T	G	C	G	C

Identity	Isolate no.	β-tubulin		LSU									
		261	299	16	41	43	117	146	436	470	475	485	
Undescribed *Spencermartinsia* species from *Acacia erioloba*	CMW25404	-	A	T	-	-	-	T	A	T	-	A	
Undescribed *Spencermartinsia* species from *A. erioloba*	CMW25405	-	A	T	-	-	-	T	A	T	-	A	
Spencermartinsia pretoriensis	CMW36480	C	-	-	C	C	C	-	-	-	C	-	
S. pretoriensis	CMW36481	C	-	-	C	C	C	-	-	-	C	-	

Figure 1. ML tree of the combined data set of ITS ribosomal DNA, TEF-1α, β-tubulin and LSU loci. Bootstrap values for ML (Piano et al. 2005) and MP (italic) above 60% are given at the nodes. The numbers are shown in bold for isolates newly sequenced in this study. The tree was rooted to *Pseudofusicoccum stromaticum* (CBS117448 and CBS117449).


Diplodia pinea CBS109725
Diplodia pinea CBS109943
86/100
Diplodia scrobiculata CBS109944
Diplodia scrobiculata CBS113423
Diplodia seriata CBS112555
95/79
Diplodia seriata CBS119049
1
CMW36468
100/96
CMW36470
CMW36469
Diplodia allocellula
79
Diplodia mutila CBS112553
100/94
Diplodia mutila CBS230.30
Diplodia rosulata CBS116472
Diplodia rosulata CBS116470
88/96
100/100
Phaeobotryon mamane CPC12445
Phaeobotryon mamane CPC12443
Lasiodiplodia parva CBS356.59
100/95
Lasiodiplodia parva CBS494.78
100/100
Lasiodiplodia theobromae CBS124.13
Lasiodiplodia theobromae CBS164.96
100/100
Botryosphaeria dothidea CBS110302
69
Botryosphaeria dothidea CMW8000
Dothiorella sarmentorum CBS115038
93/100
Dothiorella sarmentorum IMI63581b
Dothiorella iberica CBS113188
Dothiorella iberica CBS115041
Dothiorella moneti MUCC507
98/97
100/100
Dothiorella moneti MUCC505
88
Dothiorella santali MUCC509
CMW36463
100/100
CMW36464
Dothiorella brevicollis
2
Dothiorella longicollis CMW26166
100
Dothiorella longicollis CMW26164
99/98
Dothiorella sp. CBS121765
99/98
Dothiorella sp. CBS121764
99/100
3
92/83
CMW36461
91/80
CMW36460
CMW36462
Dothiorella dulcispinae
Spencermartinsia sp. CMW25405
96
66/69
Spencermartinsia sp. CMW25404
CMW36481
100/100
CMW36480
Spencermartinsia pretoriensis
100/100
4
Spencermartinsia viticola CBS117006
Spencermartinsia viticola CBS117009
97/100
Spencermartinsia sp. CBS121760
Spencermartinsia sp. CBS121761
Tiarosporella graminis var. karroo CBS118718
78
CMW36478
100/94
CMW36479
Tiarosporella urbis-rosarum
67
CMW36477
5
Tiarosporella tritici CBS118719
Tiarosporella madreeya CBS532.76
Pseudofusicoccum stromaticum CBS117449
Pseudofusicoccum stromaticum CBS117448
0.05

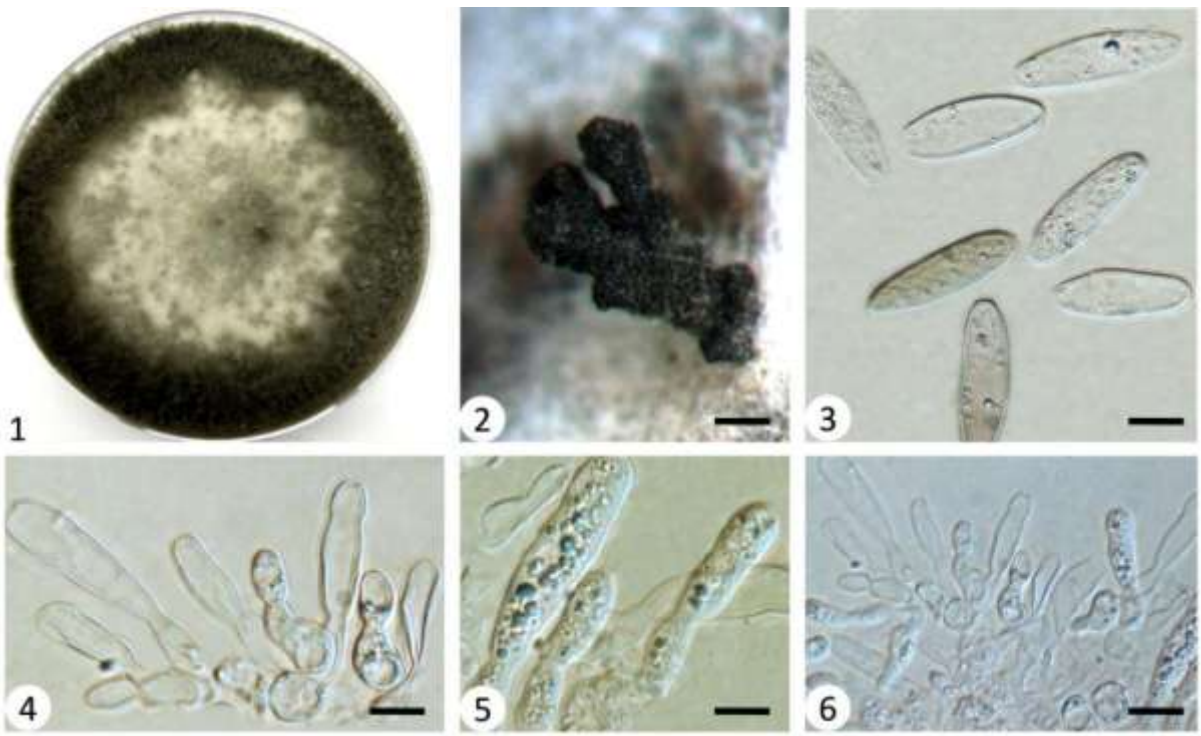


Figure 2. Micrographs of *Tiarosporella urbis-rosarum*. **1.** Culture morphology on MEA (scale bar = 100 μm); **2.** Pycnidium; **C.** Conidia (scale bar = 10 μm); **4-6.** Conidiogenous cells (scale bar 4,5 = 10 μm & scale bar 6 = 5 μm).

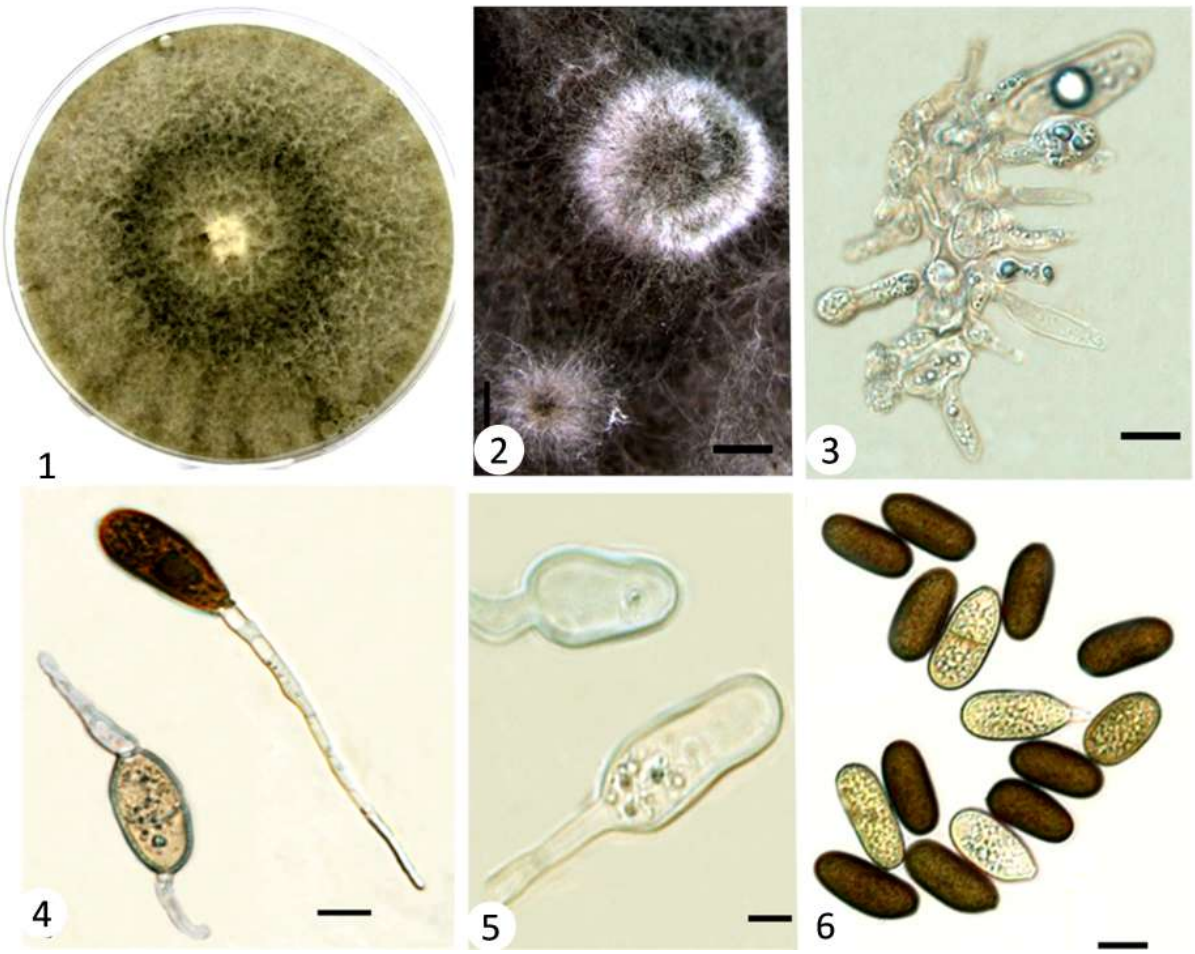


Figure 3. Micrographs of *Diplodia allocellula* **1.** Culture morphology on MEA; **2.** Pycnidium (scale bar = 100 μm); **3.** Conidiogenous cells (scale bar = 10 μm); **4.** Germinating conidia (scale bar = 10 μm); **5.** Young conidia with attached conidiogenous cells (scale bar = 5 μm; **6.** Maturing conidia at various stages (scale bar = 10 μm).

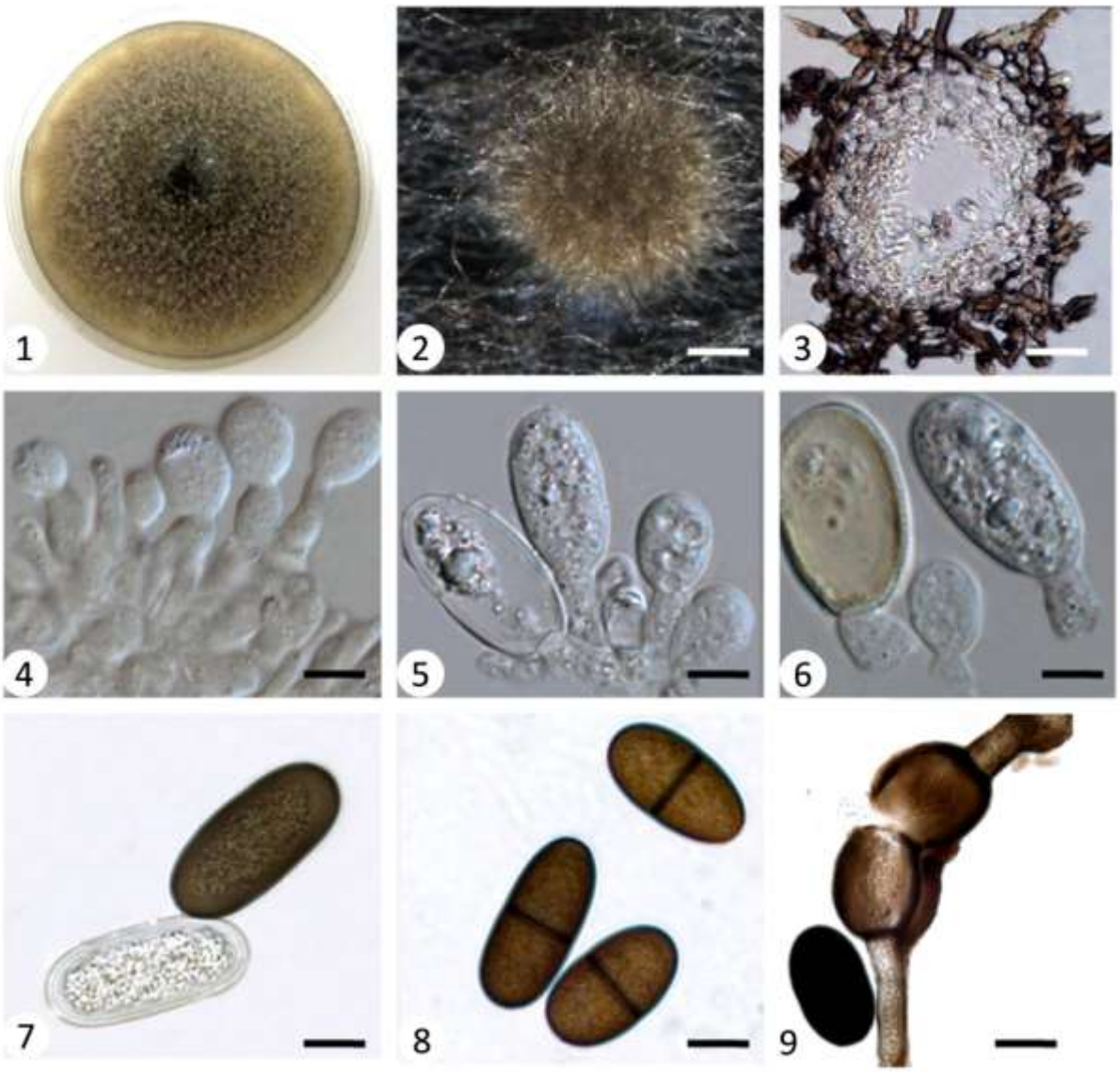


Figure 4. Micrographs of *Dothiorella dulcispinae*. **1.** Culture morphology on MEA; **2**. Pycnidium (scale bar = 100 μm); **C.** Longitudinal section through pycnidium (scale bar = 100 μm); **4-6.** Conidia and conidiogenous cells (scale bar = 10 μm), **7-8.** Conidia (scale bar = 10 μm); **9.** Chlamydospore (scale bar = 10 μm).

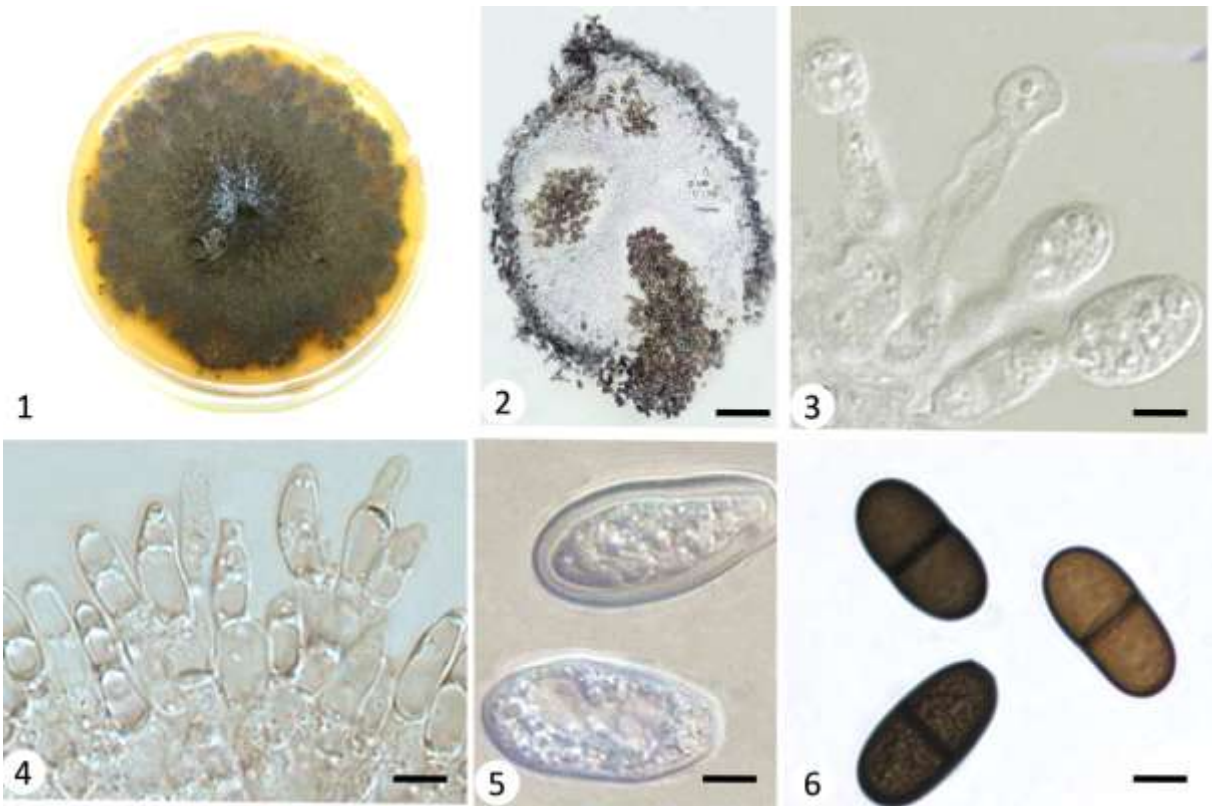


Figure 5. Micrographs of *Dothiorella brevicollis*. **1.** Culture morphology on MEA; **2.** Longitudinal section through pycnidium (scale bar = 100 µm); **3-4.** Conidiogenous cells (scale bar = 10 µm); **5.** Young Conidia (scale bar = 5 µm); **6.** Conidia at various stages of maturity (scale bar = 10 µm).

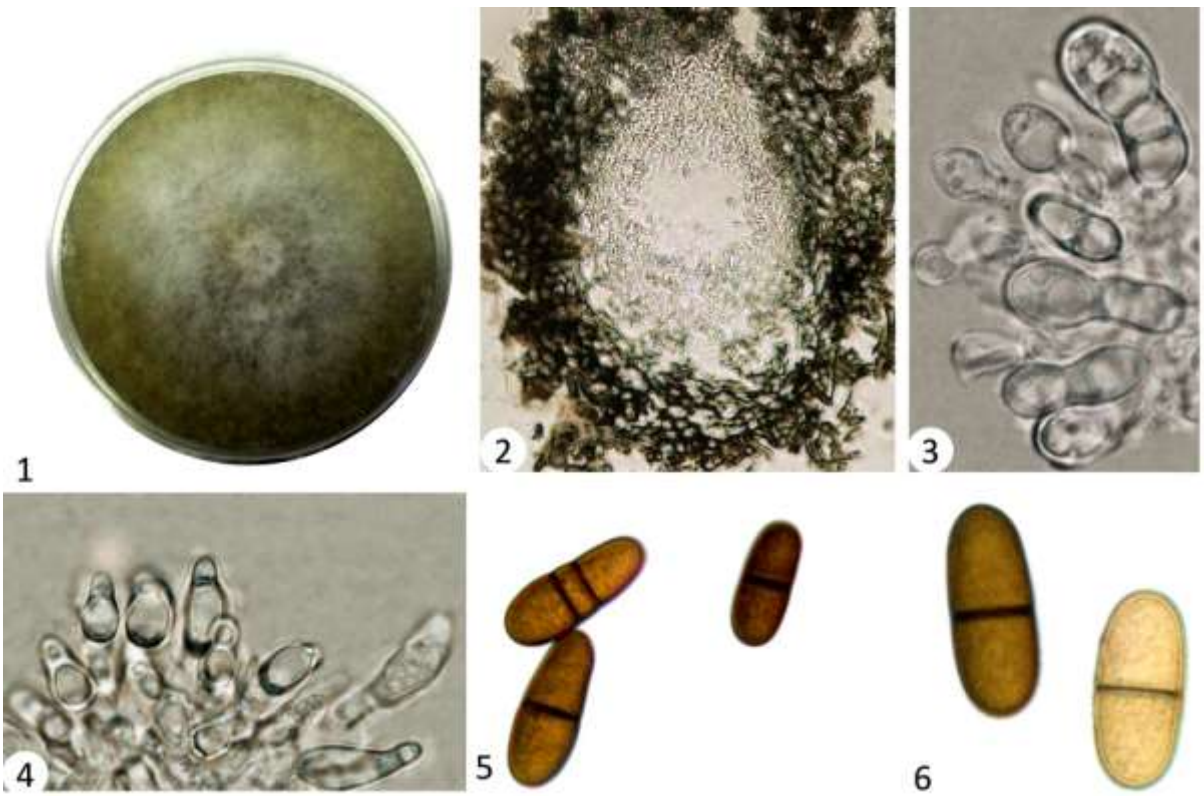


Figure 6. Micrographs of *Spencermartinsia pretoriensis*. **1.** Culture morphology on MEA; **2.** Longitudinal section through pycnidium (scale bar = 100 μm), **3-4.** Conidiogenous cells (scale bar = 20 μm), **5-6.** Conidia (scale bar = 10 μm).

Chapter 2

Greater Botryosphaeriaceae diversity in healthy than associated diseased *Acacia karroo* tree tissues

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ABSTRACT

Botryosphaeriaceae are common endophytes of trees. Some species are also known to be pathogens. It is, therefore, assumed that endophytic Botryosphaeriaceae are often involved in general die-back diseases. Here we test this assumption in severe branch die-back observed on *Acacia karroo* trees in the Pretoria area of South Africa. The presence of the Botryosphaeriaceae was compared between healthy and diseased tissue on the same trees. Eight Botryosphaeriaceae species were isolated from die-back and healthy branches. Of these, six species, namely *Tiarosporella urbis-rosarum, Diplodia allocellula, Phaeobotryosphaeria variabilis, Dothiorella brevicollis* and *Neofusicoccum vitifusiforme* were obtained from healthy tissues, and only two species, *Dothiorella dulcispinae* and *Spencermartinsia pretoriensis,* were exclusively found in die-back branches. *Spencermartinsia viticola* was found in both tissue types and this fungus was also the most commonly isolated species from both healthy and die-back samples. Results of pathogenicity trials showed highly variable results for the isolated species and that the two species associated only with die-back symptoms, were weakly pathogenic. These results suggest that the Botryosphaeriaceae found in these trees were not directly associated with the die-back symptoms, despite their diversity and common occurrence in these tissues. The situation is different in other tree systems where dominant species, often with wide host ranges, have been shown to be involved in die-back diseases. This indicates the importance of characterizing the unique aspects of each tree disease system.

INTRODUCTION

Species residing in the fungal family Botryosphaeriaceae include latent pathogens that occur asymptomatically as endophytes for extended periods, but cause disease under stress conditions (Slippers and Wingfield 2007; Smith et al. 1996; Van Niekerk et al. 2011; Denman et al. 2000). Symptoms of these diseases include die-back followed by resin exudation, blackish discoloration of the heartwood and pith, fruit rot, leaf blight, premature leaf drop, gummosis and in severe cases tree death (Slippers et al. 2007; Slippers and Wingfield 2007). Some species of the Botryosphaeriaceae have wide host ranges and they also occur on all continents other than Antarctica (Slippers and Wingfield 2007; Taylor et al. 2009).

The pathogenicity of some Botryosphaeriaceae species has been well established, but the true role of most described species in disease is poorly studied. Many Botryosphaeriaceae have been isolated from die-back symptoms, others only from asymptomatic tissues and some have been found in both tissue types (Slippers and Wingfield 2007). When tested, many species have been shown to be pathogenic (Slippers and Wingfield 2007). For instance, species from die-back symptoms that have been shown to be aggressive pathogens in artificial inoculation trials include *Diplodia africana* on *Juniperus phoenicea* (Linaldeddu et al. 2012), *Neofusicoccum parvum* on *Eucalyptus globulus* (Iturritxa et al. 2011) and *Syzygium paniculatum* (Ploetz et al. 2009), *Lasiodiplodia theobromae, L. pseudotheobromae* and *L. egyptiacae* on *Mangifera indica* (Ismail et al. 2012) and *Botryosphaeria dothidea, N. luteum, N. mediterraneum* and *N. parvum* on *Ficus microcarpa* (Mayorquin et al. 2012). However, *L. gonubiensis* has been isolated from asymptomatic tissues of the native tree *S. cordatum* (Pavlic et al. 2004), but was also shown to cause lesions in pathogenicity trials (Pavlic et al. 2007). Several species have been isolated from both healthy and die-back tissues. *Lasiodiplodia theobromae* were isolated from necrotic branches of *Vaccinium* species (Wright and Harmon 2009), vine die-back (Taylor et al. 2005; Van Niekerk et al. 2004) and healthy tissues of *Terminalia catappa* (Begoude et al. 2010) and *Eucalyptus* spp. (Pérez et al. 2010), while *L.*

margaritacea were isolated from both healthy and die-back symptoms on native *Adansonia gregorii* (Pavlic et al. 2008; Sakalidis et al. 2011). All of these species were shown to be pathogenic in pathogenicity tests. The pathogenicity of some other Botryosphaeriaceae isolated from die-back symptoms, such as *N. protearum* from die-back of native *Protea* spp. (Denman et al. 2003), *Spencermartinsia viticola* and *Dothiorella iberica* on grapevine in New South Wales and South Australia (Luque et al. 2005; Pitt et al. 2010) still remain unknown in plant pathogenicity tests. This matter is further complicated because many studies fail to clearly indicate whether isolates have been obtained as endophytes or from diseased tissue.

Acacia karroo or sweet thorn (Fabales: Mimosoideae) is the most widespread native *Acacia* in southern Africa (Timberlake et al. 1999) and plays an important role in increasing soil fertility through nitrogen fixation with rhizobia nodules (Barnes et al. 1996). Die-back symptoms on branches of *A. karroo* are common in South Africa but they have become quite severe in the Pretoria area (Gauteng Province). Larval tunnels of an unidentified cerambycid beetle were sometimes observed in these dieback symptoms, and especially in the necrotic parts of the branch samples (Figure 1). Cerambycid beetles have been reported from *Acacia* species in various parts of the world (Eisa and Roth 2009; Elliott and De Little 1985; Watt 1983). The larvae of cerambycid beetles (Coleoptera: Cerambycidae) are xylophagous and create a network of tunnels while feeding in different tissues of healthy, dead or decaying woody tissues of plants (Haack and Slansky 1987). Some cerambycid beetles can directly kill the trees or branches because their feeding in the cambium layers destroys the vascular tissues (Rad 2006; Hawkeswood 2011). These larvae could thus be involved in causing some of these symptoms or cause stress to the tree, but were not associated with them frequently enough to be the main causal agent.

Botryosphaeriaceae species are known to be associated with die-back symptoms on *A. karroo* and other *Acacia* trees in South Africa (Jami et al. 2012; Van der Walt 2008), and could thus be associated with the increased die-back of *A. karroo* in the Pretoria area. The aim of this study was to determine whether these species of Botryosphaeriaceae are

also present and associated with the striking branch die-back symptoms in Pretoria, which have not been sampled previously. Due to the endophytic nature of the Botryosphaeriaceae, species of these fungi would most likely be associated with branches. Species occurring in die-back branches were thus compared with those found in asymptomatic tissues to establish a better understanding of the diversity of species existing as endophytes on these trees, and their potential relationship with those involved in the die-back symptoms.

MATERIALS AND METHODS

Collection of samples

Samples were collected from 40 *A. karroo* trees having branches with die-back at different locations in the greater Pretoria area, Gauteng Province. The die-back begins where leaves on branch tips begin to wilt, turn brown and die, but they remain attached to the plant resulting in "flagging" symptoms (Figure 1). Other than wilting, no symptoms were observed on the leaves. Lesions were formed in the woody tissue, with dead tissue extending internally within branches and often associated with gum production on the outside of the branches. These lesions were clearly the cause of the die-back symptoms as the wilting only occurred to the terminal ends of these lesions and wilting due to problems at the roots were unlikely.

A single branch showing die-back with internal lesions and an asymptomatic branch were collected from each tree. The branch samples were placed in paper bags and transported to the laboratory. For endophyte isolations, a selected portion of each branch was cut into 0.5 cm and twelve pieces were randomly selected from each branch. From the die-back branches, the portions selected were taken from the border zone between healthy and discolored wood. All of these amounted to 480 pieces in total from die-back and 480

from healthy branches, which were surface-disinfested in 10 % hydrogen peroxide for two minutes, and rinsed three times in sterile water. Representative samples from all branches were placed onto 2 % malt extract agar (four pieces per plate) (Biolab, Midrand, South Africa). Pieces from diseased tissue were thus also placed onto agar selective for *Phytophthora* (NARPH) (Shearer and Dillon 1995) and between two slices of carrot for the isolation of *Ceratocystis* species (Moller and Devay 1968).

The plates and carrot pieces were incubated at 24 °C for seven days and the fungal growth from each wood sample showing morphology characteristic of the Botryosphaeriaceae was transferred from the primary isolations to new MEA plates. After 4-5 days, all those isolates showing typical fast growing, white to black cultures with fluffy aerial hyphae were transferred to 15% WA (water agar) in order to make single hyphal tip sub-cultures. These isolates are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

DNA sequence analyses

Isolates (Table 1) were initially grouped based on culture morphology (fast growing, white to black cultures with aerial hyphae). DNA was extracted from the mycelium of 5-day-old single hyphal-tip cultures (Lee and Taylor 1990) representing three cultures for each morphological group. Sequence data were obtained for the internal transcribed spacer region of the ribosomal RNA (rRNA) operon using primers ITS-1 (Gardes and Bruns 1993) and ITS-4 (White et al. 1990), the β-tubulin gene using primers Bt2a and Bt2b (Glass and Donaldson 1995), the translation elongation factor 1-α (TEF-1α) gene using primers EF1-728F and EF1-986R (Carbone and Kohn 1999) and the large subunit rDNA (LSU) gene region using primers LR0 and LR5 (Vilgalys and Hester 1990).

The PCR reaction mixture, PCR conditions and visualization were as described by Jami et al. (2012). The amplified PCR fragments were purified with Sephadex (Sigma,

Steinheim, Germany) and sequenced with the BigDye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, California, USA) in both directions, with the same primers used for the PCR reactions. PCR products were sequenced using an ABI 3730 48 capillary sequencer (Applied Biosystems).

Sequences of the isolates were edited using Vector NTI 11 (Lu and Moriyama 2004). DNA sequences for relevant Botryosphaeriaceae species previously published were retrieved from GenBank (http://www.ncbi.nlm.gov). The resulting data matrices for each gene region were rooted with *Pseudofusicoccum stromaticum* following the example of Phillips et al. (2008). The data matrices were aligned online using MAFFT (http://align.bmr.kyushuu.ac.jp/mafft/online/server/) version 6 (Katoh et al. 2005) and checked manually for alignment errors.

Phylogenetic analyses of sequence data for Maximum Parsimony (MP) and Maximum Likelihood (ML) were made using PAUP* v.4.0b10 (Swofford 2001). Maximum parsimony (MP) genealogies for single genes were constructed with the heuristic search option (100 random taxa additions, tree bisection and reconstruction or TBR in PAUP). The uninformative aligned regions within each dataset were removed from the analyses, gaps were treated as fifth character and all characters were unordered and of equal weight. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the tree(s) obtained was evaluated by 1000 bootstrap replications. Congruence between the different datasets was tested using the Partition Homogeneity Test (PHT) in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Farris et al. 1995; Huelsenbeck et al. 1996), with the uninformative characters removed before analysis. Other measures such as tree length (TL), consistency index (CI), rescaled consistency index (RC), and the retention index (RI) (Hillis and Huelsenbeck 1992) were recorded.

For ML analyses, the best nucleotide substitution models for each dataset were found separately with Modeltest 3.7 (Posada and Buckley 2004). All three datasets best

fitted the GTR model with the ITS, TEF-1α, β-tubulin and combined datasets having the following parameters: for ITS G=0.337, I=0.468; for TEF-1α G=0.819, I=0.759; for β-tubulin G=0.246, I=0; for LSU G=0.310, I=0 and for combined datasets G= 0.318, I=0.436. The analyses were performed in PAUP 4.0b10 and confidence levels were determined with 1000 bootstrap replications.

Pathogenicity tests

One-year-old *A. karroo* seedlings with stems ranging in height from 70–110 cm and 0.7–1.5 cm in diameter, and growing in a 1:2:4 mixture of river sand, red top soil and pine bark potting soil in 5-L plastic bags, were maintained in a greenhouse at 26°C and watered daily. Two to three isolates of each Botryosphaeriaceae species were randomly selected and each isolate was inoculated into 10 trees with one additional tree in each replication inoculated as a control. In total, 23 isolates were inoculated into 230 plants, totaling to 253 plants including the plants inoculated as negative controls.

For inoculation, a section of bark was removed from the main stems of the seedlings 15 cm above the soil level with a 6 mm sterilized cork-borer to expose the cambium. A six mm plug of agar, covered with mycelium of the test fungus, was placed with the mycelium surface facing inwards onto the wound, while clean agar discs were used in control inoculations. The inoculated wounds were sealed with Parafilm to minimize contamination and to prevent desiccation of the inoculum. Lesion lengths were measured six weeks after inoculation. Variation in the extent of the lesions was analyzed through a one-way analysis of variance (Karadzic 2003) with the general linear model procedure from SAS (version 9.3).

To re-isolate the inoculated fungi in order to confirm Koch’s postulates, one plant was chosen from those inoculated for each isolate (in total 23 plants) and from the controls of each species (in total 8 plants). These were identified based on the conidial morphology. A small sample of tissue was cut from the lesions, including the inoculation points, surface

disinfested with 10 % hydrogen peroxide for two minutes, and rinsed three times in sterile water. The tissue samples were plated onto 2 % malt extract agar and incubated at 24 °C for seven days.

RESULTS

Collection of samples

In total 164 Botryosphaeriaceae isolates were isolated from *A. karroo* branches. Of these 84 isolates were from the 480 healthy wood pieces and 80 isolates were from the equivalent number of wood pieces displaying die-back symptoms. At least 50% of sampled trees yielded isolates and these were from across the geographical range sampled. No isolates of *Ceratocystis* or *Phytophthora* were obtained in this study.

DNA sequence analyses

The datasets for the ITS, TEF-1α and β-tubulin sequences were analyzed individually and in combination. The ITS sequence dataset contained 546 characters (4 parsimony-uninformative, 148 parsimony-informative, 370 constant characters) with CI = 0.602, RI = 0.902, RC = 0.543, HI = 0.398 and TL = 321. The TEF-1α dataset contained 362 characters (3 parsimony-uninformative, 224 parsimony-informative, 135 constant characters) with CI = 0.651, RI = 0. 919, RC = 0.550, HI = 0.383 and TL = 986. The β-tubulin dataset contained 471 characters (0 parsimony-uninformative, 142 parsimony-informative, 329 constant characters) with CI = 0.698, RI = 0.901, RC = 0.629, HI = 0.302, and TL=304. The LSU dataset contained 845 characters (7 parsimony-uninformative, 72 parsimony-informative and 766 constant characters) with CI = 0.642, RI = 0.875, RC = 0.587, HI = 0.328 and TL = 138. The tree statistics for the combined dataset (TreeBase

Accession No. S12358) were CI = 0.487, RI = 0.854, RC = 0.416, HI = 0.513, TL = 2148, and the partition homogeneity test (PHT) on the datasets produced a P-value of 0.01.

The topology of the trees obtained using the ML and MP analyses were similar for the individual gene regions, as well as in the combined analysis with regards to the clades representing species isolated in this study. However, clades representing genera occasionally collapsed in individual analyses. Isolates resided in five genera and eight species that were identified as follows: *Spencermartinsia pretoriensis*, *S. viticola*, *Dothiorella dulcispinae*, *Do. brevicollis*, *Diplodia allocellula*, *Phaeobotryosphaeria variabilis*, *Tiarosporella urbis-rosarum* and *Neofusicoccum vitifusiforme* (Figure 2).

Of the eight Botryosphaeriaceae species, *S. viticola* was most common and dominant in both healthy and die-back tissue. Of total isolates, 77.3% represented *S. viticola*, 2.3% *Do. brevicollis*, 9.5% *D. allocellula*, 3.5% *P. variabilis*, 3.5% *T. urbis-rosarum* and 3.5% *N. vitifusiforme* (Table 1). The isolates of *Spencermartinsia pretoriensis* (CMW36480 and CMW36481), *Dothiorella dulcispinae* (CMW36460, CMW36461 and CMW36462) and *S. viticola* (CMW37931, CMW37932 and CMW37933) were representative of isolates from branches with die-back (2.5% *S. pretoriensis*, 10% *Do. dulcispinae* and 87.5% *S. viticola*) (Table 1). Representative isolates from healthy branches included *S. viticola* (CMW37928, CMW37929 and CMW37930), *Do. brevicollis* (CMW36464 and CMW 36463), *Diplodia allocellula* (CMW36468, CMW36469 and CMW36470), *Phaeobotryosphaeria variabilis* (CMW36482, CMW36483), *Tiarosporella urbis-rosarum* (CMW36479, CMW36465 and CMW36466) and *Neofusicoccum vitifusiforme* (CMW37934, CMW37935). Some isolates were obtained from the same tree such as those of *Do. brevicollis* (CMW 36463) and *S. viticola* (CMW37928), *S. viticola* (CMW37931) and *S. pretoriensis* (CMW36480), and *Do. dulcispinae* (CMW36462) and *N. vitifusiforme* (CMW37935), respectively (Table 1). The rest of the isolates were obtained from different trees.

Pathogenicity tests

All 23 isolates of the eight species produced lesions in the cambium of inoculated branches after six weeks (Figure 3), and the average lesions length were significantly ($P < 0.05$) larger than those observed for the controls. Statistical analyses showed that lesion sizes varied little between the ten inoculated trees for each isolate, but did vary considerably between the isolates used for some species, namely *Do. brevicollis, T. urbis-rosarum* and *S. viticola*. The longest lesions were produced by isolates of *P. variabilis* (average lesion length 30.5 mm), one isolate of *Do. brevicollis* (average lesion length 17 mm), one isolate of *S. viticola* (average lesion length 12 mm) and one isolate of *T. urbis-rosarum* (average lesion length 11 mm). All these isolates were obtained from healthy tissues, even though the pathogenicity test showed that they were the most pathogenic isolates. Isolates obtained from die-back tissues represented some of the isolates producing the shortest lesions, namely isolates of *S. pretoriensis* (average lesion length 5 mm) and *Do. dulcispinae* (average lesion length 8.5 mm). Two isolates of *S. viticola* (average lesion length 5 mm), of which one originated from die-back and another from healthy tissues, and two isolates of *T. urbis-rosarum* (average lesion length 4.5 mm) that were both from healthy tissues (Figure 4) were among those isolates that produced the smallest lesions. All eight Botryosphaeriaceae species were re-isolated from lesions and no Botryosphaeriaceae were isolated from the controls.

DISCUSSION

Eight species of Botryosphaeriaceae were isolated from branches of 40 *A. karroo* trees, either healthy or displaying symptoms of die-back. Most of these species, including *Tiarosporella urbis-rosarum, Diplodia allocellula, P. variabilis, Dothiorella brevicollis, Neofusicoccum vitifusiforme* and *Spencermartinsia viticola*, were obtained from healthy tissues. In contrast, only three species, namely *Dothiorella dulcispinae, S. pretoriensis* and

S. viticola, were isolated from die-back tissue. Thus only *S. viticola* occurred on both healthy and diseased tissue types and only two species, *Do. dulcispinae* and *S. pretoriensis*, were found exclusively in die-back tissue. A maximum of two species co-occurred in a single tree, including *Do. brevicollis*, *S. viticola, S. pretoriensis*, *Do. dulcispinae* and *N. vitifusiforme*.

All the isolated species of Botryosphaeriaceae produced lesions on inoculated *A. karroo* branches. Surprisingly, the two species found only in branches with die-back, namely *S. pretoriensis* and *Do. dulcispinae,* were among those producing the smallest lesions. These two species also represented only 12.5% of the total number of isolates from diseased tissue while 87.5% of isolates were those of *S. viticola*. These results suggest that the Botryosphaeriaceae isolated in this study play little if any role in the development of die-back symptoms on *A. karroo*.

Spencermartinsia viticola was isolated from both die-back and healthy tissue, and this species was also the most dominant species isolated. *S. viticola* was described for the first time from decline of *Vitis vinifera* in Spain as a saprophyte (Luque et al. 2005). However, since then it has been reported from diseases on many other plants such as warts on trunks of *Populus cathayana* in China (Zhang et al. 2009), canker of citrus in California (Adesemoye and Eskalen 2011) and from healthy tissues of *Acacia mellifera* in South Africa (Van der Walt 2008). The results of our study confirm that this species can produce lesions on *A. karroo* in pathogenicity tests, but it was amongst those producing fairly short lesions and this species thus does not appear to be a particularly aggressive pathogen on *A. karroo*.

In a previous study, *Phaeobotryosphaeria variabilis* was isolated from die-back branches on *A. karroo*, but its pathogenicity was not considered (Van der Walt 2008). In the present study, we found *P. variabilis* only in healthy tissues, but it produced the longest lesions of all the species in the pathogenicity trial. This species thus appears to have the capacity to cause disease on *A. karroo*, although it was apparently not involved with the

die-back symptoms studied here.

Isolates of some species differed significantly in their aggressiveness in pathogenicity tests. For example, some isolates of *T. urbis-rosarum*, *S. viticola* and *Do. brevicollis* produced long lesions in the inoculation tests while the other isolates of these species were amongst those that produced small lesions. This could be due to genetic differences in the isolates themselves or differences in susceptibility of genotypes of seedlings, which is not unusual as it is known that isolates of species can differ considerably in their pathogenicity (Mayer 2006; Müller et al. 2001). Little variation was observed in our study between the trees inoculated with the particular isolates, and the variation observed thus appears linked to differences in virulence of the various isolates. This implies that due to these differences in virulence the most virulent isolates may not have been used for the pathogenicity tests, and that more virulent isolates for each species could occur in branches, contributing to die-back.

It is possible that the damage caused by the feeding cerambycid larvae or other biological agents places the branches under stress allowing endophytic, opportunistic Botryosphaeriaceae to have the opportunity to cause disease symptoms (Slippers and Wingfield 2007). Further studies will be necessary to determine if there is any direct association between the Botryosphaeriaceae species occurring naturally as endophytes in *A. karroo* and such other biological agents, or whether the presence of the Botryosphaeriaceae and possible die-back symptoms are only co-incidental and opportunistic. Such further studies would, for example, include determining whether these fungi are associated with the larvae, targeting development of lesions linked to earlier stages of beetle larval infestation, and considering possible transmission mechanisms by the beetles.

The results of this study emphasize the extensive diversity of the Botryosphaeriaceae community that can exist on trees in a limited area. Furthermore, these are all potential pathogens. Our study also elucidates the necessity to study each tree

system separately to determine the ecological roles of the Botryosphaeriaceae found in the tree species, which may differ to those in other tree systems. Expanding in depth sampling in particular areas, such as on more than one host, together with extensive pathogenicity studies will be very helpful to further characterize the potential role these fungi can play in disease of such native communities.

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Table 1. Representative isolates from *Acacia karroo* used in the phylogenetic analyses.

Isolate No.	Identity	Host	Location	Symptoms	Collector	GenBank			
						ITS	EF1-α	LSU	β-tubulin
CMW37928	*Spencermartinsia viticola*	Tree no.6	Pretoria, SA	Healthy	F. Jami & M. Gryzenhout	JX283730	JX283741	JX456000	JX283717
CMW37929	*S. viticola*	Tree no.22	Pretoria, SA	Healthy	"	JX283731	JX283744	JX456001	JX283720
CMW37930	*S. viticola*	Tree no.19	Pretoria, SA	Healthy	"	JX283732	JX283745	JX456002	JX283721
CMW37931	*S. viticola*	Tree no.34	Pretoria, SA	Die-back	"	JX283733	JX283742	JX456003	JX283716
CMW37932	*S. viticola*	Tree no.33	Pretoria, SA	Die-back	"	JX283734	JX283743	JX456004	JX283718
CMW37933	*S. viticola*	Tree no.1	Pretoria, SA	Die-back	"	JX283735	JX283745	JX45599	JX283719
CMW36463	*Dothiorella brevicollis*	Tree no.6	Pretoria, SA	Healthy	"	JQ239403	JQ239390	JQ239416	JQ239371
CMW36464	*Do. brevicollis*	Tree no.38	Pretoria, SA	Die-back	"	JQ239404	JQ239391	JQ239417	JQ239372
CMW36460	*Dothiorella dulcispinae*	Tree no.39	Pretoria, SA	Die-back	"	JQ239400	JQ239387	JQ239413	JQ239373
CMW36461	*Do. dulcispinae*	Tree no.12	Pretoria, SA	Die-back	"	JQ239401	JQ239388	JQ239414	JQ239374
CMW36462	*Do. dulcispinae*	Tree no.14	Pretoria, SA	Healthy	"	JQ239402	JQ239389	JQ239415	JQ239375
CMW36480	*Spencermartinsia*	Tree no.34	Pretoria, SA	Die-back	"	JQ239405	JQ239392	JQ239418	JQ239376

	pretoriensis								
CMW36481	*S. pretoriensis*	Tree no.36	Pretoria, SA	Die-back	"	JQ239406	JQ239393	JQ239419	JQ239377
CMW37934	*Neofusicoccum vitifusiforme*	Tree no.35	Pretoria, SA	Healthy	"	JX283728	JX283746	JX456005	JX283722
CMW37935	*N. vitifusiforme*	Tree no.14	Pretoria, SA	Healthy	"	JX283729	JX283747	JX456006	JX283723
CMW36482	*Phaeobotryosphaeria variabilis*	Tree no.21	Pretoria, SA	Healthy	"	JX283726	JX283738	JX456007	JX283714
CMW36483	*P. variabilis*	Tree no.23	Pretoria, SA	Healthy	"	JX283727	JX283739	JX456008	JX283715
CMW36468	*Diplodia allocellula*	Tree no.7	Pretoria, SA	Healthy	"	JQ239397	JQ239384	JQ239410	JQ239378
CMW36469	*D. allocellula*	Tree no.8	Pretoria, SA	Healthy	"	JQ239398	JQ239385	JQ239411	JQ239379
CMW36470	*D. allocellula*	Tree no.9	Pretoria, SA	Healthy	"	JQ239399	JQ239386	JQ239412	JQ239380
CMW36465	*Tiarosporella urbis-rosarum*	Tree no.4	Pretoria, SA	Healthy	"	JX283736	JX283748	JX45597	JX283724
CMW36466	*T. urbis-rosarum*	Tree no.5	Pretoria, SA	Healthy	"	JX283737	JX283749	JX45598	JX283725
CMW36479	*T. urbis-rosarum*	Tree no.3	Pretoria, SA	Healthy	"	JQ239409	JQ239396	JQ239422	JQ239383

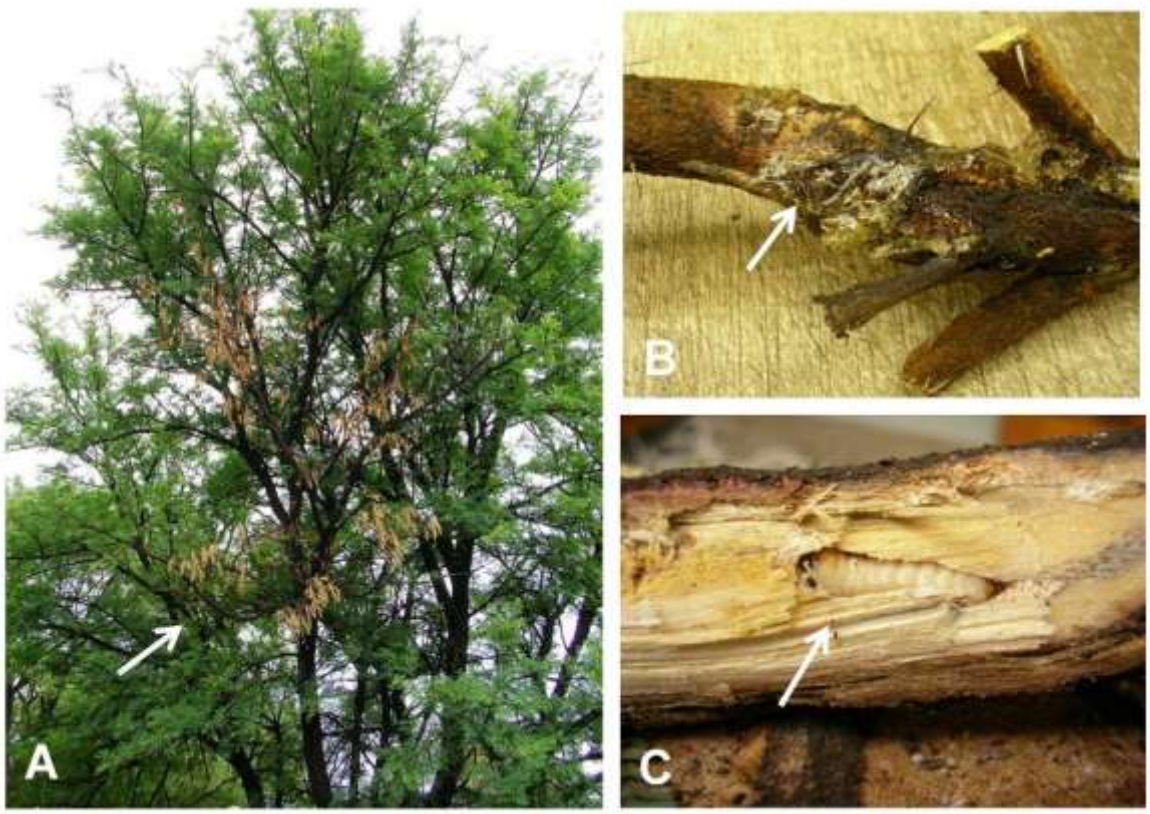


Figure 1. (A) *Acacia karroo* tree with die-back branches (arrow). (B) Gummosis (arrow) on die-back branch. (C) Cerambycid larva that are occasionally found within a die-back branch.

Figure 2. Maximum Likelihood (ML) tree of the combined data set of ITS ribosomal DNA, TEF-1α, β-tubulin and LSU gene region sequences. Bootstrap values for ML (roman) and Maximum Parsimony (italic) above 60 % are given at the nodes. The tree was rooted to *Pseudofusicoccum stromaticum* (CBS117448 and CBS117449). H=Isolates from healthy branches, D=Isolates from die-back branches.

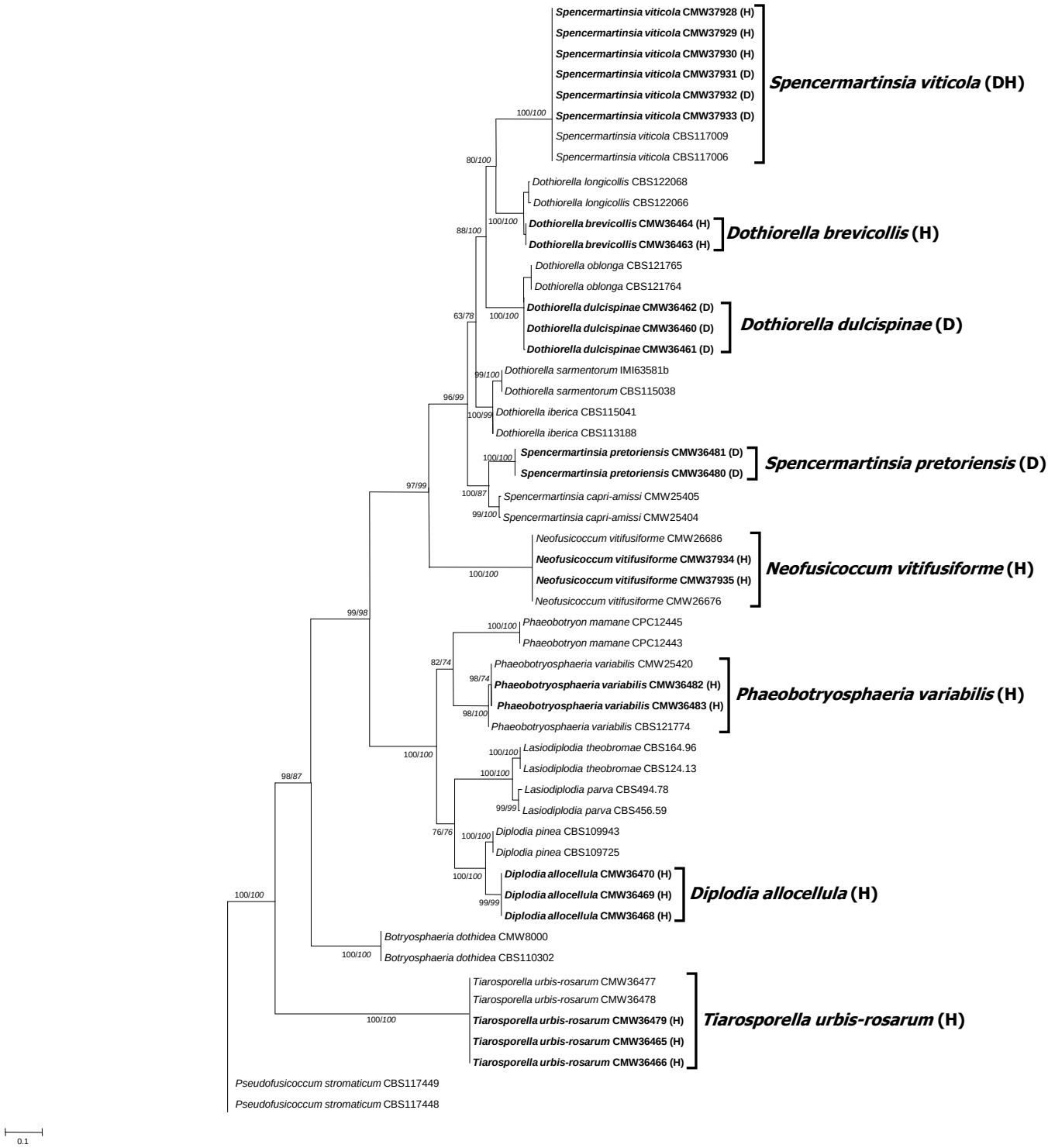
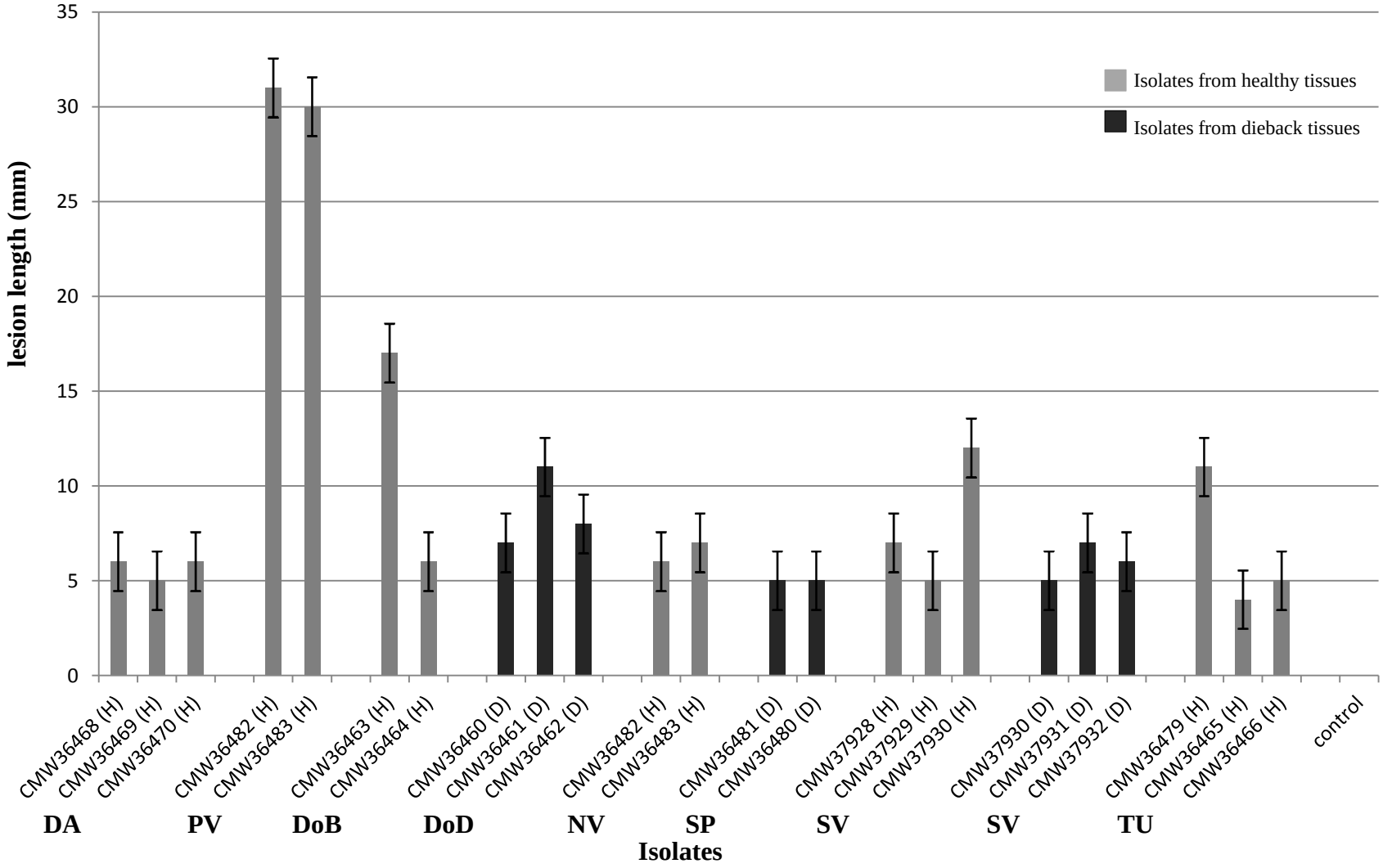

Spencermartinsia viticola CMW37928 (H)
Spencermartinsia viticola CMW37929 (H)
Spencermartinsia viticola CMW37930 (H)
Spencermartinsia viticola CMW37931 (D)
Spencermartinsia viticola CMW37932 (D)
Spencermartinsia viticola CMW37933 (D)
Spencermartinsia viticola CBS117009
Spencermartinsia viticola CBS117006
Spencermartinsia viticola (DH)
Dothiorella longicollis CBS122068
Dothiorella longicollis CBS122066
Dothiorella brevicollis CMW36464 (H)
Dothiorella brevicollis CMW36463 (H)
Dothiorella brevicollis (H)
Dothiorella oblonga CBS121765
Dothiorella oblonga CBS121764
Dothiorella dulcispinae CMW36462 (D)
Dothiorella dulcispinae CMW36460 (D)
Dothiorella dulcispinae CMW36461 (D)
Dothiorella dulcispinae (D)
Dothiorella sarmentorum IMI63581b
Dothiorella sarmentorum CBS115038
Dothiorella iberica CBS115041
Dothiorella iberica CBS113188
Spencermartinsia pretoriensis CMW36481 (D)
Spencermartinsia pretoriensis CMW36480 (D)
Spencermartinsia pretoriensis (D)
Spencermartinsia capri-amissi CMW25405
Spencermartinsia capri-amissi CMW25404
Neofusicoccum vitifusiforme CMW26686
Neofusicoccum vitifusiforme CMW37934 (H)
Neofusicoccum vitifusiforme CMW37935 (H)
Neofusicoccum vitifusiforme CMW26676
Neofusicoccum vitifusiforme (H)
Phaeobotryon mamane CPC12445
Phaeobotryon mamane CPC12443
Phaeobotryosphaeria variabilis CMW25420
Phaeobotryosphaeria variabilis CMW36482 (H)
Phaeobotryosphaeria variabilis CMW36483 (H)
Phaeobotryosphaeria variabilis CBS121774
Phaeobotryosphaeria variabilis (H)
Lasiodiplodia theobromae CBS164.96
Lasiodiplodia theobromae CBS124.13
Lasiodiplodia parva CBS494.78
Lasiodiplodia parva CBS456.59
Diplodia pinea CBS109943
Diplodia pinea CBS109725
Diplodia allocellula CMW36470 (H)
Diplodia allocellula CMW36469 (H)
Diplodia allocellula CMW36468 (H)
Diplodia allocellula (H)
Botryosphaeria dothidea CMW8000
Botryosphaeria dothidea CBS110302
Tiarosporella urbis-rosarum CMW36477
Tiarosporella urbis-rosarum CMW36478
Tiarosporella urbis-rosarum CMW36479 (H)
Tiarosporella urbis-rosarum CMW36465 (H)
Tiarosporella urbis-rosarum CMW36466 (H)
Tiarosporella urbis-rosarum (H)
Pseudofusicoccum stromaticum CBS117449
Pseudofusicoccum stromaticum CBS117448
100/100
80/100
100/100
88/100
63/78
100/100
99/100
96/99
100/99
100/100
97/99
100/87
99/100
100/100
99/98
100/100
82/74
98/74
98/100
100/100
98/87
100/100
100/100
99/99
76/76
100/100
100/100
100/100
99/99
100/100
100/100
100/100
Pseudofusicoccum stromaticum (H)
0.1

Figure 3. (A) Stem of seedling with a distinct lesion resulting from inoculation with *Phaeobotryosphaeria variabilis*. (B) Seedling inoculated as a control with no evidence of lesion development.

Figure 4. Average length (mm) of lesions produced on *Acacia karroo* seedlings inoculated with 19 isolates of eight Botryosphaeriaceae species (isolates were obtained from healthy and diseased tissue) and a negative control after 5 weeks in a greenhouse. All isolates produced lesions significantly greater ($P < 0.05$) than those of the controls. **H**= Isolates from healthy branches, **D**= Isolates from die-back branches. **DA**= *Diplodia allocellula*, **PV**= *Phaeobotryosphaeria variabilis*, **DoB**= *Dothiorella brevicollis*, **DoD**= *Do. dulcispinae*, **NV**= *Neofusicoccum vitifusiforme*, **SP**= *Spencermartinsia pretoriensis*, **SV**= *S. viticola*, **TU**= *Tiarosporella urbis-rosarum*


lesion length (mm)
35
30
25
20
15
10
5
0
Isolates from healthy tissues
Isolates from dieback tissues
CMW36468 (H)
CMW36469 (H)
CMW36470 (H)
CMW36482 (H)
CMW36483 (H)
CMW36463 (H)
CMW36464 (H)
CMW36460 (D)
CMW36461 (D)
CMW36462 (D)
CMW36482 (H)
CMW36483 (H)
CMW36481 (D)
CMW36480 (D)
CMW37928 (H)
CMW37929 (H)
CMW37930 (H)
CMW37930 (D)
CMW37931 (D)
CMW37932 (D)
CMW36479 (H)
CMW36465 (H)
CMW36466 (H)
control
DA
PV
DoB
DoD
NV
SP
SV
SV
TU
Isolates

Chapter 3

The pattern of Botryosphaeriaceae on four unrelated native South African hosts

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ABSTRACT

The Botryosphaeriaceae represents an important and diverse family of latent fungal pathogens of woody plants. While some species appear to have wide host ranges, others are reported only from single hosts. It is, however, not clear whether apparently narrow host ranges reflect specificity or if this is an artifact of sampling. In this study, we address this question by sampling native South African trees from four different families, including *Acacia karroo* (Leguminosae), *Celtis africana* (Cannabaceae), *Searsia lancea* (Anacardiaceae) and *Gymnosporia buxifolia* (Celastraceae). As part of this process, two new species of the Botryosphaeriaceae, namely *Tiarosporella africana* sp. nov. and *Aplosporella javeedii* sp. nov., emerged from sequence comparisons based on the ITS rDNA, TEF-1α, β-tubulin and LSU rDNA gene regions. An additional five known species were identified including *Neofusicoccum parvum*, *N. kwambonambiense*, *Spencermartinsia viticola*, *Diplodia pseudoseriata* and *Botryosphaeria dothidea*. Despite extensive sampling of these trees, some species known from these hosts were not detected. These included *B. dothidea*, which is known to have a broad host range and that was found only on *A. karroo*. This could have resulted from the fact that it is a rare species in the region. Collectively, the results suggest that some intrinsic host factors, possibly combined with local environmental conditions, affect the distribution and co-infectivity of various hosts by the Botryosphaeriaceae. This would be in contrast to a general ability of a species in the Botryosphaeriaceae to infect a broad range of plants. It might also explain why some Botryosphaeriaceae with apparently broad host ranges are found on different suites of hosts in different areas of the world.

INTRODUCTION

Fungi residing in the Botryosphaeriaceae (Ascomycota: Botryosphaeriales) have been characterised from a wide variety of trees. They commonly occur as endophytes in asymptomatic plant tissues (Smith et al. 1996b), but some species are also important pathogens. The shift in habit from endophyte to being virulent pathogens typically occurs when trees are subjected to stress (Slippers and Wingfield 2007). Some Botryosphaeriaceae infect several different hosts, which may or may not be related to each other. Other species are known from only a single host. While there appear to be some distinct patters of host association for those species that infect conifers as opposed to angiosperms (De Wet et al. 2008), relatively little is known regarding the epidemiology and host ranges of these intriguing fungi.

Species of Botryosphaeriaceae occur widely in South Africa and they have been found on virtually every tree species that has been sampled for them. Hosts include native trees such as *Terminalia catappa* (Myrtales: Combretaceae) (Begoude et al. 2010), *Pterocarpus angolensis* (Fabales: Leguminosae) (Mehl et al. 2011), *Syzygium cordatum* (Myrtales: Myrtaceae) (Pavlic et al. 2007), *Acacia melifera* (Fabales: Leguminosae) (Slippers et al. 2013), *A. karroo* (Jami et al. 2012), and woody species of *Leucadendron*, *Leucospermum* and *Protea* (Proteales: Proteaceae) (Denman et al. 2003). Non-native hosts of the Botryosphaeriaceae in South Africa include *Pinus* spp. (Pinales: Pinaceae), *Eucalyptus* spp. (Myrtales: Myrtaceae), *Prunus* spp. (Rosales: Rosaceae), and *Vitis vinifera* (Vitales: Vitaceae) (Damm et al. 2007a; Van Niekerk et al. 2004; Smith et al. 1996a). Despite relatively intensive sampling over many years, numerous native woody hosts in South Africa have not been sampled for the presence of Botryosphaeriaceae.

Some species of Botryosphaeriaceae have broad host ranges, occurring on both native and non-native hosts in a sampled area. For example, *Neofusicoccum vitifusiforme* (Van Niekerk & Crous) Crous, Slippers & A.J.L. Phillips, *N. australe* (Slippers, Crous & M.J.

Wingf.) Crous, Slippers & A.J.L. Phillips, *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *N. luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *N. kwambonambiense* Pavlic, Slippers & M.J. Wingf., *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Diplodia seriata* De Not., *Spencermartinsia viticola* (A.J.L. Phillips & J. Luque) A.J.L. Phillips, A. Alves & Crous and *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not., have been found on various native and non-native trees in South Africa (Damm et al. 2007a; Van Niekerk et al. 2004; Smith et al. 1996a; Slippers et al. 2007; Denman et al. 2003; Pavlic et al. 2007, 2009a; Pillay et al. 2013). Some Botryosphaeriaceae can also infect a variety of native hosts and examples include *Dothiorella dulcispinae* Jami, Gryzenh., Slippers & M.J. Wingf., *Phaeobotryosphaeria variabilis* F.J.J. van der Walt, Slippers & G.J. Marais, and *Spencermartinsia rosulata* F.J.J. van der Walt, Slippers & G.J. Marais, that infect different *Acacia* species (Slippers et al. 2013; Jami et al. 2012), *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous from *Pt. angolensis*, *T. catappa* and *S. cordatum* (Begoude et al. 2010; Pillay et al. 2013; Mehl et al. 2011), and *N. protearum* (Denman & Crous) Crous, Slippers & A.J.L. Phillips that infects *Leucadendron laureolum* × *Ldn. salignum* and *Protea* spp. (Denman et al. 2003). In contrast, some species have thus far been found only on a single host plant, for example *Tiarosporella urbis-rosarum* Jami, Gryzenh., Slippers & M.J. Wingf., *Diplodia allocellula* Jami, Gryzenh., Slippers & M.J. Wingf., *Dothiorella brevicollis* Jami, Gryzenh., Slippers & M.J. Wingf., *Do. oblonga* F.J.J. van der Walt, Slippers & G.J. Marais, *Spencermartinsia pretoriensis* Jami, Gryzenh., Slippers & M.J. Wingf., *S. capri-amissi*, *N. viticlavatum* Van Niekerk & Crous) Crous, Slippers & A.J.L. Phillips and *L. pyriformis* F.J.J. van der Walt, Slippers & G.J. Marais (Jami et al. 2013; Van Niekerk et al. 2004; Slippers et al. 2013). This pattern of association could be attributed to a sampling effect. For example, sampling has not been particularly intensive for most tree species and sampling has also tended to focus on particular areas. It is thus not clear whether species known from a limited number of hosts are host specific, or if they simply have not been sampled from other hosts.

Acacia karroo has been subjected to intense surveys for Botryosphaeriaceae across various geographical areas in southern Africa (Jami et al. 2012, 2013; Slippers et al. 2013). A large diversity of Botryosphaeriaceae has been found during these studies, including *T. urbis-*

rosarum, D. allocellula, P. variabilis, Do. brevicollis, Do. dulcispinae, N. vitifusiforme, S. viticola, S. pretoriensis, S. rosulata, N. australe, N. parvum, N. kwambonambiense, B. dothidea and *L. theobromae*. Some of these species are known from hosts other than *A. karroo*, while others have been reported only from this tree. As in other systems, the question arises as to whether this reflects the level of host specificity or if it is due to a sampling bias.

The aim of this study was to determine patterns of overlap of the Botryosphaeriaceae occurring on *A. karroo* and three unrelated and commonly occurring hosts that grow in areas surrounding it. These hosts included *Celtis africana* (Rosales: Cannabaceae), *Searsia lancea* (Sapindales: Anacardiaceae), and *Gymnosporia buxifolia* (Celastrales: Celastraceae). Sampling was made at a particular point in time and at a single location to exclude the effect of temporal and geographical diversity. We also considered the level of diversity of Botryosphaeriaceae in different tissues on these hosts. It was thus anticipated that the results would provide a rudimentary estimation of the patterns of diversity for Botryosphaeriaceae in South Africa that might be expected across different hosts.

MATERIALS AND METHODS

Collection of samples and isolations

Healthy plant material from *A. karroo* and three commonly occurring and surrounding tree species, namely *C. africana, S. lancea* and *G. buxifolia* were collected in October 2011 (spring). Ten healthy and co-occurring trees of each species were randomly chosen for sampling. Three healthy branches including leaves were collected from each tree, placed in paper bags, and transferred to the laboratory to be processed for isolations. Samples were obtained from a nature reserve area in Pretoria, South Africa.

For each sample, 12 pieces (0.5 cm in length) of tissue were taken from each branch and 12 pieces were cut from the simple leaves. The samples were surface disinfested in 10% hydrogen peroxide for two minutes, rinsed three times in sterile distilled water and cultured on 2% malt extract agar (MEA) (Biolab, S.A.). Single hyphal-tips of isolates displaying a cultural morphology typical of the Botryosphaeriaceae, such as rapid growth and white to black mycelium with aerial hyphae, were transferred to fresh plates until pure cultures had been obtained. Single hyphal-tip cultures of these isolates are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, and duplicate isolates of the new species were deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS), The Netherlands.

DNA sequence analyses

Isolates utilised in this study were grouped based on culture morphology. DNA was extracted (Lee and Taylor 1990) from fungal mycelium of 5-day-old single hyphal-tip cultures of three to five representatives for each morphological group. Four gene regions were used for comparison based phylogenetic analyses to determine the identities of the unknown isolates. These included the internal transcribed spacer region of the ribosomal RNA (rRNA) operon amplified with primers ITS-1F (Gardes and Bruns 1993) and ITS-4 (White et al. 1990), the translation elongation factor 1-α (EF1-α) gene amplified with primers EF1-728F and EF1-986R (Carbone and Kohn 1999), the β-tubulin gene using primers Bt2a and Bt2b (Glass and Donaldson 1995) and the large subunit rDNA (LSU) gene region using primers LR0 and LR5 (Vilgalys and Hester 1990).

The conditions and procedures for PCR, sequencing and phylogenetic analyses were the same as those described in Jami et al. (2012). The phylogenetic analyses for all the datasets were performed using Maximum Likelihood (ML), Maximum parsimony (MP) and Bayesian analyses. For ML analyses, the best nucleotide substitution models for each dataset were found separately with Modeltest 3.7 (Posada and Buckley 2004). The model for GTR + G

(G=0.2390, I=0.0) were chosen for the combined datasets of ITS, LSU, TEF-1α, β-tubulin. The ML analyses were performed in PAUP 4.0b10 and confidence levels were determined with 1000 bootstrap replications. For MP analyses, single genes were constructed with the heuristic search option (100 random taxa additions), tree bisection and reconstruction (TBR) in PAUP. The uninformative aligned regions within each dataset were removed from the analyses, gaps were treated as fifth character and all characters were unordered and of equal weight. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the tree(s) obtained was evaluated by 1000 bootstrap replications.Congruence between the different datasets was tested using the Partition Homogeneity Test (PHT) in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Farris et al. 1995; Huelsenbeck et al. 1996), with the uninformative characters removed before analysis. Other measures such as tree length (TL), consistency index (CI), rescaled consistency index (RC), and the retention index (RI) (Hillis and Huelsenbeck 1992) were recorded. Bayesian analyses using the Markov Chain Monte Carlo (MCMC) method were performed to ascertain the topology of trees obtained with ML. The MCMC analyses, with four chains, started from random tree topology and lasted 3,000,000 generations. Trees were saved every 100th generation. The burn-in number was graphically estimated (3000) from the likelihood scores and trees outside this point were discarded in the analyses. The consensus trees were constructed in MEGA version 4 and posterior probabilities were assigned to branches after a 60% majority rule.

Morphological characteristics

To induce sporulation, cultures were inoculated onto sterilized twigs of *A. karroo* placed on the surface of 2% MEA (Biolab), and these were incubated at 25 °C under near-UV light (Jami et al. 2012). Fifty released conidia, and 30 pycnidia and conidiogenous cells were measured for the isolates chosen to represent holotypes for each putative new species, and the ranges and averages of these were computed. Measurements and digital images were made with an HRc Axiocam digital camera and accompanying Axiovision 3.1 software (Carl Zeiss

Ltd., Munich, Germany). Dried cultures representing type specimens were deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

Colony morphology and colour were determined for cultures grown on MEA at 5–35°C, at 5°C intervals, in the dark. For these, 6 mm diam. mycelial plugs were taken from the edges of actively growing 4-day-old single conidial cultures, and transferred to the centres of 90 mm diam. Petri dishes containing MEA. Three replicate plates were used for each isolate per temperature. Two measurements perpendicular to each other were taken of the colony diameter daily until the mycelium of the fastest growing isolates had covered the plates and averages were computed. Colony colours were assigned using the designations of Rayner (1970).

Statistical analyses of species diversity

To determine the variability and overlap of the Botryosphaeriaceae species from the four hosts, data generated from the isolations were subjected to statistical analyses to determine whether the variation was significant or not. In addition, the variability and overlap in diversity and species between tissue types (branches and leaves) for each host and in total were determined. A one-way ANOVA with the general linear model procedure was used with JMP (version 10, SAS Institute Inc. 2012).

RESULTS

Collection of samples and isolates

A total of 191 isolates were obtained from the four host trees, with 119 from branches and 72 from leaves. These included 82 isolates from *A. karroo* (50% of sampled trees), 72

from *C. africana* (40% of sampled trees), three from *S. lancea* (10% of sampled trees) and 34 isolates from *G. buxifolia* (50% of sampled trees). Isolates from *A. karroo* included 42.9% of total isolates, while those from *S. lancea* included only 1.5% of the total collection.

DNA sequence analyses

The sequence datasets for the ITS, TEF-1α, β-tubulin and LSU rDNA regions were analyzed individually and in combination. The ITS sequence dataset contained 552 characters (excluding 366 and including 186 characters) with RI = 0.972, RC = 0.809, HI = 0.167 and TL = 301.8. The TEF-1α dataset contained 287 characters (excluding 60 and including 227 characters) with RI = 0.891, RC = 0.550, HI = 0.383 and TL = 523. The β-tubulin dataset contained 366 characters (excluding 239 and including 127 characters) with RI = 0.965, RC = 0.825, HI = 0.302, and TL=185.1. The LSU dataset contained 848 characters (excluding 460 and including 484 characters) with RI = 0.983, RC = 0.906, HI = 0.078 and TL = 548.8. The tree statistics for the combined dataset were RI = 0.854, RC = 0.416, HI = 0.513, TL = 2148 (TreeBase Accession No. S12358), and the partition homogeneity test (PHT) on the datasets gave a P-value of 0.01.

The topology of the trees emerging from the ML, MP and MrBayes analyses were similar for the individual gene regions, as well as in the combined analyses, with regards to the clades representing species isolated in this study. Seven clades were identified in all the analyses and these represented *S. viticola, B. dothidea, N. parvum, N. kwambonambiense, D. pseudoseriata* and two unidentified groups within the clades accommodating *Aplosporella* and *Tiarosporella,* respectively (Figure 1). The distinct groupings of two new species in *Aplosporella* and *Tiarosporella* were based on fixed sequence variants linked to the two groups and identified in the datasets (Tables 2 & 3).

From *A. karroo,* three species were identified, namely *B. dothidea* (CMW38114, CMW38115, CMW38116), *D. pseudoseriata* (CMW38137, CMW38138) and *S. viticola*

(CMW38079). Four species, namely *S. viticola* (CMW38082), *N. kwambonambiense* (CMW38426), *Tiarosporella* sp. nov. (CMW38423, CMW38424, CMW38425, CMW38428) and *Aplosporella* sp. nov. (CMW38165, CMW38166, CMW38167) were isolated from *C. africana*. This is in contrast to *S. viticola* (CMW38080) and *N. parvum* (CMW38161) that were obtained from *G. buxifolia.* Only the *Aplosporella* sp. nov. (CMW38168 CMW38169, CMW38170) was identified from *S. lancea. Spencermartinsia viticola* was common among *A. karroo, C. africana* and *G. buxifolia*, but was not found on *S. lancea.* The *Aplosporella* sp. nov. overlapped on *S. lancea* and *C. africana* (Table 1, Figure 2).

Morphological characteristics

The isolates in the group corresponding to *Tiarosporella* in the DNA sequence comparisons were fast-growing with white, raised aerial mycelium around the edges of the culture, with grey centres viewed from the top and bottom of the plate. These cultures produced large hyaline conidia with appendages of different sizes. Isolates in the other six groups had dark grey or olivaceous colonies with aerial hyphae and dematiaceous conidia. *Aplosporella* isolates were slow growing, and had grey-olivaceous mycelium with light, irregular edges and mostly aseptate conidia that were narrow at the centres. Other known *Aplosporella* spp. have ellipsoidal to sub-cylindrical conidia. The substantial overlap in these morphological characters allowed only limited comparisons with characteristics published for the species.

Statistical analyses of species diversity

There were no statistically significant differences between species composition (not considering frequency of individual species) on the different hosts ($P > 0.05$). Among the fungal species, *S. viticola* was the only species that had a host association that was significantly different from the other species ($P < 0.05$) in terms of frequency of occurrence. It was dominant on three of the hosts with 79.3% of isolates from *A. karroo*, 55.6% of isolates

from *C. africana*, and 88.2% of the isolates from *G. buxifolia*. There was no significant difference between the diversity of species found from leaves and branches ($P > 0.05$). There was also no significant difference between the frequency of species found on leaves and branches ($P > 0.05$) (Figure 3). Of the seven isolated species, *S. viticola* was the most commonly isolated from both leaves and branches. *N. parvum, N. kwambonambiense*, *Aplosporella* sp. nov. and *Tiarosporella* sp. nov. were found only on branches, while *D. pseudoseriata* and *B. dothidea* were exclusively isolated from leaves (Figure 4).

TAXONOMY

The phylogenetic analyses revealed two new taxa and these taxa were supported by morphological studies. These species are described below:

Aplosporella javeedii Jami, Gryzenh., Slippers & M.J. Wingf. sp. nov.- MB803637 (Figure 5)

Etymology: The name is derived from the Persian name "Javeed Jami", meaning "long lived".

No teleomorph observed.

Pycnidia formed on MEA in 2 weeks, solitary, globose, grey-olivaceous (23````i), unilocular, immersed to semi-immersed, average 850 × 820 µm, wall 6-10 cell layers thick, outer layers composed of dark-brown textura angularis, becoming thin-walled and hyaline towards the inner region.

Conidiogenous cells formed from the cells lining the inner walls of the pycnidia, holoblastic, determinate, simple, ellipsoidal and slightly tapered towards the apex, hyaline. *Conidia* aseptate, initially hyaline, becoming dark brown, smooth-walled, broadly ellipsoidal to sub-cylindrical, with rounded ends, (18.3–)21.2–24.6(–26.7) × (6.9–)8.1–9.6(–10.1) µm.

Colonies on MEA after 5 days in the dark: olivaceous to grey-olivaceous (23````i), similar in reverse; aerial mycelium appressed, floccose, white to smoke-grey. Colonies flat with undulate edge. Growth at 5-35°C. Growth rate 10 mm per day at an optimal temperature of 25°C; covering the agar surface in a 90 mm diam. Petri dish after 9 days in the dark.

Specimens examined: South Africa, Gauteng Province, Pretoria, November 2011, F. Jami & M. Gryzenhout, from healthy wood section of *Celtis africana*, holotype PREM60865, ex-type culture CMW38165 = CBS133954.

Additional specimens: South Africa, Gauteng Province, Pretoria, November 2011, F. Jami & M. Gryzenhout, from healthy branch of *Celtis africana*, paratype (living cultures CMW38166, CMW38167 = CBS135852 = PREM60880) and *Searsia lancea*, paratype (living cultures CMW38168 = CBS135853 = PREM60881, CMW38169, CMW38170).

Tiarosporella africana Jami, Gryzenh., Slippers & M.J. Wingf. sp. nov.-MB803638 (Figure 6)

Etymology: The name refers to Africa and the continent from which this species was collected.

No teleomorph observed.

Pycnidia formed on *Acacia karroo* twigs on MEA in 2-3 weeks under ultra-violet (UV), solitary, globose, dark black (29`````m), unilocular, immersed, average 1100 × 300 µm, wall 5-7 cell layers thick, outer layers composed of dark-brown textura angularis, becoming thin-walled and hyaline towards the inner region.

Conidiogenous cells formed from the cells lining the inner walls of the pycnidia, holoblastic, determinate, simple, ellipsoidal and slightly tapered towards the apex, hyaline. *Conidia* aerogenous, solitary, hyaline, smooth, thin-walled, straight, fusiform with truncate base and obtuse apex, (15.6–)19.5–31.8(–35.5) × (7.4–)8.6–11.6(–12.2) µm. During development, conidia are in a gelatinous sheath which may remain as an apical, hyaline, cone-like appendage that are (23.8–)24.5–45.4(–49.9) × (11.5–)12.8–22.2(–25.11) µm.

Colonies on MEA with appressed mycelial mats, pycnidia emerging after 2-3 weeks under near-ultraviolet light on *A. karroo* twigs. Mycelium grey, becoming dark grey from the center, white and fluffy at the edges, reverse dark grey to black. Growth at 5-35°C. Growth rate 22.5 mm per day at an optimal temperature of 30°C; covering the agar surface in a 90 mm diam. Petri dish after 4 days in the dark.

Specimens examined: South Africa, Gauteng Province, Pretoria, November 2011, F. Jami & M. Gryzenhout, from healthy wood section of *Celtis africana*, holotype PREM60866 resulting from inoculations of living isolate to *A. karroo* twigs, living ex-type cultures CMW38423 = CBS 133854.

Additional specimens: South Africa, Gauteng Province, Pretoria, November 2011, F. Jami & M. Gryzenhout, from healthy branch of *Celtis africana*, paratype (living cultures CMW38424 = CBS135850 = PREM60882, CMW38425 = CBS135851 = PREM60882, CMW38428).

DISCUSSION

Seven Botryosphaeriaceae species were identified from the four tree species growing in close proximity to each other. These fungi included species known in South Africa (*N. parvum, N. kwambonambiense, S. viticola, D. pseudoseriata, B. dothidea*) and the two new taxa *Tiarosporella africana* and *Aplosporella javeedii.* Five of these species occurred on only a single host but *A. javeedii* was found on two and *S. viticola* occurred on three of the tree species sampled. Results of this study, based on the single location with only four hosts sampled, represent high levels of biodiversity for the Botryosphaeriaceae.

Botryosphaeria dothidea, N. parvum, N. kwambonambiense, T. africana and *D. pseudoseriata* were found only on one host in this study. This could be interpreted as host specificity, as has been postulated for other endophytes (Zhou and Hyde 2001; Cohen 2006, 2004; Porras-Alfaro and Bayman 2011). Some Botryosphaeriaceae species are also thought to have some level of host preference, such as *D. pinea, D. scrobiculata* and *D. cupressi* that are found predominantly on certain conifers (Alves et al. 2006; De Wet et al. 2008). However, we do not expect that this pattern reflects host specificity in these cases, because all these fungi are known from previous studies to have broad host ranges. In particular, *B. dothidea*, *L. theobromae* and *N. parvum* are known to have extremely broad host ranges (Slippers and Wingfield 2007; Sakalidis et al. 2013; Punithalingam 1976). In South Africa, *B. dothidea,* has been reported previously from *Acacia* spp., *Eucalyptus* spp., *Podocarpus* spp., *Syzygium* spp. and *Heteropyxis natalensis* (Smith et al. 2001; Pavlic et al. 2007; Slippers et al. 2013). Likewise, *N. parvum* has been found on *S. cordatum, Eucalyptus* spp., and *T. catappa*, (Slippers et al. 2004; Begoude et al. 2010; Pavlic et al. 2007). Also, *L. theobromae* has been identified from *Vitis vinifera, S. cordatum, T. catappa* and *Pt. angolensis* in South Africa (Mehl et al. 2011; Van Niekerk et al. 2004; Begoude et al. 2010; Pavlic et al. 2007). Given that sampling was relatively intensive at this single location, the data suggest that the occurrence of species in this study might reflect factors influencing distribution other than host specificity, such as environmental factors, and sampling effect. To determine true host ranges of these fungi, considerably more intensive and wider sampling will need to be done.

The number of Botryosphaeriaceae species infecting the different tree hosts varied considerably in this study. Most of the trees sampled were infected by multiple (up to four) species of Botryosphaeriaceae. For example, *C. africana* had the most diverse assemblage of these fungi while *S. lancea* had the lowest level of diversity. Several factors could affect these patterns of endophyte infection on a particular plant host, including biotic (e.g. plant defences, competition, etc.) and abiotic factors (e.g. local climate affecting growth, sporulation, etc.). None of these factors have, however, been studied in detail for the Botryosphaeriaceae on tree hosts.

In terms of understanding host defences, *S. lancea* could offer an interesting opportunity for further studies. The abundance of Botryosphaeriaceae found on the other hosts, compared to this host might suggest some characteristic of *S. lancea* that makes it less favourable for infection by these fungi. Future studies should consider the Botryosphaeriaceae on this tree in other areas of South Africa and also studies of biochemical characteristics of this tree that might explain the low number of Botryosphaeriaceae in this tree as compared to, for instance, *A. karroo*.

The same result has been observed in a study on *Eucalyptus gomphocephala* with eight surrounding native hosts by Taylor et al. (2009). Eight Botryosphaeriaceae species were found from all nine native hosts. While only *N. australe* was common on all hosts, the pattern of species diversity was varied on different hosts. The investigation was conducted by Sakalidis et al. (2011a), on *Adansonia gregorii* and native surrounding trees in three sites in Australia. In that study, 11 Botryosphaeriaceae species were found from both *A. gregorii* and surrounding hosts which of three species did not observe on *A. gregorii*. Interestingly *L. pseudotheobromae* was only found on *A. gregorii* not on the others (Sakalidis et al. 2011a). Although Botryosphaeriaceae are cosmopolitan with a wide hosts range but the impact of these extending hosts are unknown.

This study revealed a number of new hosts for some of the Botryosphaeriaceae. For example, we isolated *S. viticola* on two new native hosts, namely *C. africana* and *G. buxifolia*. This fungus was previously known from *Prunus* spp., *Vitis vinifera*, *A. karroo* and *A. mellifera* in South Africa (Slippers et al. 2013; Jami et al. 2013; Damm et al. 2007a; Van Niekerk et al. 2004). *Spencermartinsia viticola* was originally found from grapevine in Spain (Luque et al. 2005), but has since been reported from other areas on this host (Úrbez-Torres et al. 2007) and from the other hosts such as *Populus cathayana* (Zhang et al. 2009), and citrus (Adesemoye and Eskalen 2011). There is a clear association of this fungus with *V. vinifera* although this is clearly not fixed. The question thus arises as to where the fungus might be native and whether it has moved from commercially propagated to native plants or vice versa.

Neofusicoccum kwambonambiense represents another example of a species in the Botryosphaeriaceae that was isolated from *C. africana* for the first time in this study. This fungus was previously reported from *Syzygium cordatum*, *Eucalyptus grandis* and *A. karroo* in South Africa (Pavlic et al. 2009a; Pillay et al. 2013), from *E. dunnii* and *Corymbia torelliana* in Australia (Sakalidis et al. 2011b), and also from *V. vinifera* in Uruguay (Abreo et al. 2013). Such expansion of the known host range following expanded sampling appears to be a common pattern of recent studies on the Botryosphaeriaceae, and these are changing perceptions of host association drastically. For example, *N. eucalyptorum* was initially thought to be specific to *Eucalyptus* spp. in South Africa and Australia (Slippers et al., 2004), but was later found on other hosts in Uruguay (Pérez et al. 2009). These findings suggest that extensive and global sampling will be necessary to fully understand the host associations and distribution of the Botryosphaeriaceae. For the present, caution would be advisable when drawing conclusions regarding host association and distribution of these fungi.

Some endophytes are known to be tissue specific (de Abreu et al. 2010; Ganley and Newcombe 2006; Fisher et al. 1993). However, results of this study provided no evidence that the Botryosphaeriaceae sampled are specific to either leaves or woody tissue, although the frequency of occurrence of some species such as S. *viticola* varied on tissue types. In the present study, *N. kwambonambiense* was found only on branch tissue of *C. africana*, and it has

been isolated on branches of the other hosts, including *S. cordatum, Eucalyptus dunnii* and *Corymbia torelliana* (Pavlic et al. 2009b; Sakalidis et al. 2011b). In those studies, the samples were only taken from branches. Therefore, we cannot say that *N. kwambonambiense* is exclusive to branches. Similar to our study, Wunderlich et al. (2011) also found no indication of tissue specificity for Botryosphaeriaceae species on *V. vinifera*. To fully explore the issue of variation in relative infection frequency of different species in different tissues, a metagenetics approach using either multi-species primers for the specific detection of Botryosphaeriaceous species (Ridgway et al. 2011) or next generation sequencing might be needed to overcome potential sampling bias.

A new species of *Tiarosporella* was described in this study from a native South African host. Several *Tiarosporella* spp. have been reported from different hosts in the U.K, U.S.A, India, Yugoslavia and South Africa (Sutton and Marasas 1976; Karadzic 2003), but those were identified based only on morphology. Sequence data of only four species, namely *T. tritici*, *T. graminis* var. *karroo*, *T. madreeya* (Crous et al. 2006) and *T. urbis-rosarum* (Jami et al. 2012) are available in GenBank, all of which have been isolated from different hosts in South Africa (from Poaceae, Zygophyllaceae, Asteraceae and Leguminosae) (Sutton and Marasas 1976; Jami et al. 2012). It is not clear whether this current restriction of sequences for the genus from isolates from southern African is due to the lack of sampling in some other regions of the world. While some areas have been fairly well sampled, this group could also have been overlooked during isolation, because of its atypical culture morphology for Botryosphaeriaceae. For example, hyphae of *Tiarosporella* typically grow faster than the other Botryosphaeriaceae, but take longer to become grey after isolation. These atypical morphological characteristics and the fact that DNA sequence comparisons have not been conducted for species recorded outside South Africa might suggest problems regarding the identification of some collections of these fungi.

Recent studies have identified a number of unique *Aplosporella* spp. from different hosts and areas in South Africa. Of the four recently identified *Aplosporella* species, only *A. yalgorensis* was identified outside Africa from *Acacia cochlearis* and *Eucalyptus*

gomphocephala in Australia (Taylor et al. 2009). The other three species have all been described from southern Africa, with *A. prunicola* identified from *Prunus* in South Africa (Damm et al. 2007b), *A. africana* from *A. mellifera* in Namibia and *A. papillata* from *A. tortillas* and *A. erioloba* in South Africa (Slippers et al. 2013). The present study adds a fourth species, *A. javeedii*, and two new host records namely *C. africana* and *S. lancea*. Given fairly extensive sampling in other regions of the world, it would appear that southern Africa represents a centre of diversity for this group in the Botryosphaeriaceae.

The results of this study revealed the diversity of Botryosphaeriaceae on three previously unsampled plant families. They confirm the view that these fungi occur on most, if not all, woody plants. The data emerging from this and previous studies also suggest that many of these Botryosphaeriaceae are not host specific over the range of their distribution. Yet, the discovery of two new Botryosphaeriaceae species from a region that was previously intensively sampled for other hosts, suggest that host diversity does contribute to the diversity of Botryosphaeriaceae in an area. Thus, despite not being host specific, their host ranges might be limited to or more common on a certain suite of hosts in a particular area. The data, in particular from *S. lancea*, suggest that host factors could play a role in determining the diversity of Botryosphaeriaceae infection, even in the presence of species that have a general ability to infect many different hosts. Unravelling the limits of the host ranges of these different species, most representing plant pathogens, and how local environments influence them, remains one of the intriguing questions for this group of fungi.

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Table 1. Representative isolates of this study used in the phylogenetic analyses.

Isolate No.	Identity	Host	Tissue	Location	Collector	GenBank			
						ITS	EF1-α	β-tubulin	LSU
CMW38165 CBS 133954	***Aplosporella javeedii****	*Celtis africana*	Branches	Pretoria, SA	F. Jami & M. Gryzenhout	KC769938	KC769846	KC769903	KC769979
CMW38166	*A. javeedii**	*Celtis africana*	Branches	Pretoria, SA	"	KC769939	KC769847	KC769904	KC769980
CMW38167 CBS 135852	*A. javeedii**	*Celtis africana*	Branches	Pretoria, SA	"	KC769940	KC769848	KC769905	KC769981
CMW38168 CBS 135853	*A. javeedii**	*Searsia lancea*	Branches	Pretoria, SA	"	KC769941	KC769849	KC769906	KC769982
CMW38169	*A. javeedii**	*Searsia lancea*	Branches	Pretoria, SA	"	KC769942	KC769850	KC769907	KC769983
CMW38170	*A. javeedii**	*Searsia lancea*	Branches	Pretoria, SA	"	KC769943	KC769851	KC769908	KC769984
CMW38114	*Botryosphaeria dothidea*	*Acacia karroo*	Leaves	Pretoria, SA	"	KC769944	KC769856	KC769898	-
CMW38115	*B. dothidea*	*Acacia karroo*	Leaves	Pretoria, SA	"	KC769945	KC769857	KC769899	-
CMW38116	*B. dothidea*	*Acacia karroo*	Leaves	Pretoria, SA	"	KC769946	KC769858	KC769900	-
CMW38137	*Diplodia pseudoseriata*	*Acacia karroo*	Leaves	Pretoria, SA	"	KC769954	KC769863	KC769896	-
CMW38138	*D. pseudoseriata*	*Acacia karroo*	Leaves	Pretoria, SA	"	KC769955	KC769864	KC769897	-
CMW38131	*Neofusicoccum*	*Acacia karroo*	Branches	Pretoria, SA	"	KC769949	KC769862	KC769902	KC769988

	kwambonambiense								
CMW38426	*N. kwambonambiense*	*Celtis africana*	Branches	Pretoria, SA	"	KC769948	KC769861	KF512019	KC769989
CMW 38161	*N. parvum*	*Gymnosporia buxifolia*	Branches	Pretoria, SA	"	KC769947	KC769859	KC769901	-
CMW 38079	*Spencermartinsia viticola*	*Acacia karroo*	Branches	Pretoria, SA	"	KC769952	KC769866	KC769895	KC769987
CMW 38081	*S. viticola*	*Gymnosporia buxifolia*	Branches	Pretoria, SA	"	KC769951	KC769865	KC769894	KC769986
CMW 38082	*S. viticola*	*Celtis africana*	Branches	Pretoria, SA	"	KC769950	KC769867	KC769893	KC769985
CMW38423	***Tiarosporella africana****	*Celtis africana*	Branches	Pretoria, SA	"	KC769956	KC769852	KC769909	KC76999
CBS 133854									
CMW38424	*T. africana**	*Celtis africana*	Branches	Pretoria, SA	"	KC769957	KC769853	KC769910	KC76999
CBS 135850									
CMW38425	*T. africana**	*Celtis africana*	Branches	Pretoria, SA	"	KC769958	KC769854	KC769911	KC76999
CBS 135851									
CMW38428	*T. africana**	*Celtis africana*	Branches	Pretoria, SA	"	KC769959	KC769855	KC769912	KC76999

Culture collections: CMW- FABI, University of Pretoria, South Africa; CBS- Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. Isolate accession numbers in bold signify holotype cultures. Isolates for new described species are indicated with an asterisk (*) and ex-type isolates are indicated in bold type.

Table 2. Polymorphic nucleotides from sequence data of the ITS, TEF-1α and LSU showing the relationships between *Aplosporella papillata* and *Aplosporella javeedii*. Polymorphisms unique to *A. javeedii* are highlighted.

Identity	Isolate No.	ITS														
		111	**162**	**166**	**169**	**168**	**169**	**176**	**177**	**181**	**441**	**447**	**473**	**482**	**495**	**553**
Aplosporella papillata	CBS121780	G	T	C	C	C	G	G	A	G	G	A	G	C	G	C
A. papillata	CBS121782	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. javeedii	CBS 133954	A	G	T	G	T	T	A	G	A	A	G	A	T	C	T
A. javeedii	CMW38166	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. javeedii	CBS 135852	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. javeedii	CBS 135853	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. javeedii	CMW38169	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. javeedii	CMW38170	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

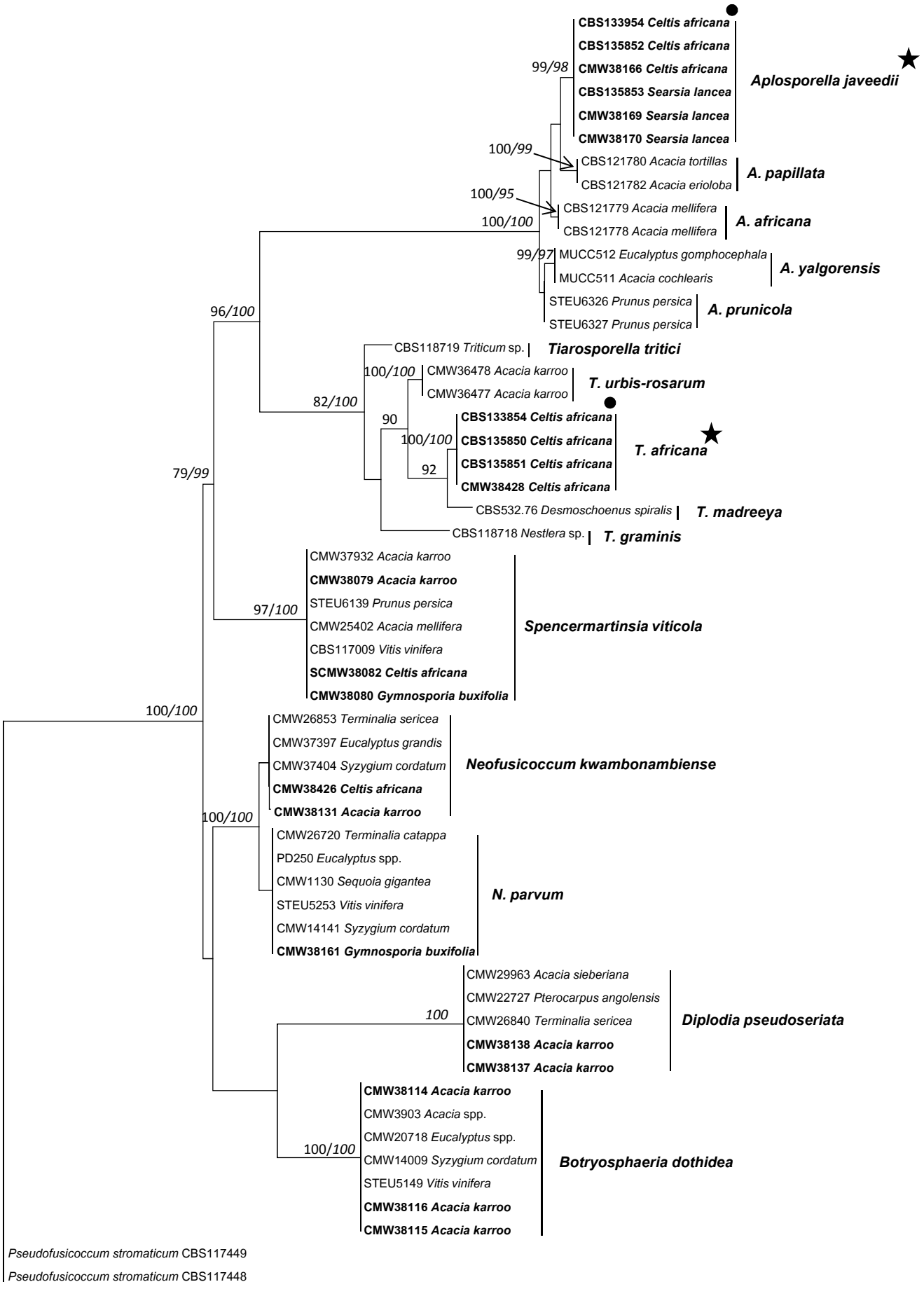
Identity	Isolate No.	TEF-1α						LSU	
		89	**142**	**143**	**148**	**171**	**303**	**352**	**373**
Aplosporella papillata	CBS121780	A	G	C	A	C	C	C	T
A. papillata	CBS121782	.	.	.	.	.	.	.	.
A. javeedii	CBS 133954	G	C	G	C	G	G	T	C
A. javeedii	CMW38166	.	.	.	.	.	.	.	.
A. javeedii	CBS 135852	.	.	.	.	.	.	.	.
A. javeedii	CBS 135853	.	.	.	.	.	.	.	.
A. javeedii	CMW38169	.	.	.	.	.	.	.	.
A. javeedii	CMW38170	.	.	.	.	.	.	.	.

Table 3. Polymorphic nucleotides from sequence data of the LSU showing the relationships between *Tiarosporella madreeya* and *Tiarosporella africana*. Polymorphisms unique to *T. africana* are highlighted.

Identity	Isolate No.	LSU										
		137	**412**	**425**	**435**	**441**	**442**	**466**	**468**	**493**	**630**	**633**
Tiarosporella madreeya	CBS532.76	T	C	C	A	T	C	T	G	T	G	A
T. africana	CBS 133854	C	T	T	G	C	T	C	T	A	T	C
T. africana	CBS 135850	.	.	.	.	.	.	.	.	.	.	.
T. africana	CBS 135851	.	.	.	.	.	.	.	.	.	.	.
T. africana	CMW38428	.	.	.	.	.	.	.	.	.	.	.

Figure 1. Maximum Likelihood (ML) tree of the combined data set of ITS ribosomal DNA, TEF-1α, β-tubulin and LSU gene region sequences. Bootstrap values for ML (roman) and MrBaysen (italic) above 60 % are given at the nodes. The tree was rooted to *Pseudofusicoccum stromaticum* (CBS117448 and CBS117449). Isolates of this study are indicated as bold.

***** Newly described species in this study. • indicates for ex-type isolates.


CBS133954 Celtis africana
CBS135852 Celtis africana
CMW38166 Celtis africana
CBS135853 Searsia lancea
CMW38169 Searsia lancea
CMW38170 Searsia lancea
99/98
Aplosporella javeedii
100/99
CBS121780 Acacia tortillas
CBS121782 Acacia erioloba
A. papillata
100/95
CBS121779 Acacia mellifera
100/100
CBS121778 Acacia mellifera
A. africana
99/97
MUCC512 Eucalyptus gomphocephala
MUCC511 Acacia cochlearis
A. yalgorensis
STEU6326 Prunus persica
STEU6327 Prunus persica
A. prunicola
96/100
CBS118719 Triticum sp.
Tiarosporella tritici
100/100
CMW36478 Acacia karroo
CMW36477 Acacia karroo
T. urbis-rosarum
82/100
90
CBS133854 Celtis africana
100/100
CBS135850 Celtis africana
CBS135851 Celtis africana
92
CMW38428 Celtis africana
T. africana
79/99
CBS532.76 Desmoschoenus spiralis
T. madreeya
CBS118718 Nestlera sp.
T. graminis
CMW37932 Acacia karroo
CMW38079 Acacia karroo
STEU6139 Prunus persica
97/100
CMW25402 Acacia mellifera
CBS117009 Vitis vinifera
SCMW38082 Celtis africana
CMW38080 Gymnosporia buxifolia
Spencermartinsia viticola
100/100
CMW26853 Terminalia sericea
CMW37397 Eucalyptus grandis
CMW37404 Syzygium cordatum
CMW38426 Celtis africana
CMW38131 Acacia karroo
Neofusicoccum kwambonambiense
100/100
CMW26720 Terminalia catappa
PD250 Eucalyptus spp.
CMW1130 Sequoia gigantea
STEU5253 Vitis vinifera
CMW14141 Syzygium cordatum
CMW38161 Gymnosporia buxifolia
N. parvum
CMW29963 Acacia sieberiana
CMW22727 Pterocarpus angolensis
100
CMW26840 Terminalia sericea
CMW38138 Acacia karroo
CMW38137 Acacia karroo
Diplodia pseudoseriata
CMW38114 Acacia karroo
CMW3903 Acacia spp.
CMW20718 Eucalyptus spp.
100/100
CMW14009 Syzygium cordatum
STEU5149 Vitis vinifera
CMW38116 Acacia karroo
CMW38115 Acacia karroo
Botryosphaeria dothidea
Pseudofusicoccum stromaticum CBS117449
Pseudofusicoccum stromaticum CBS117448
0.02

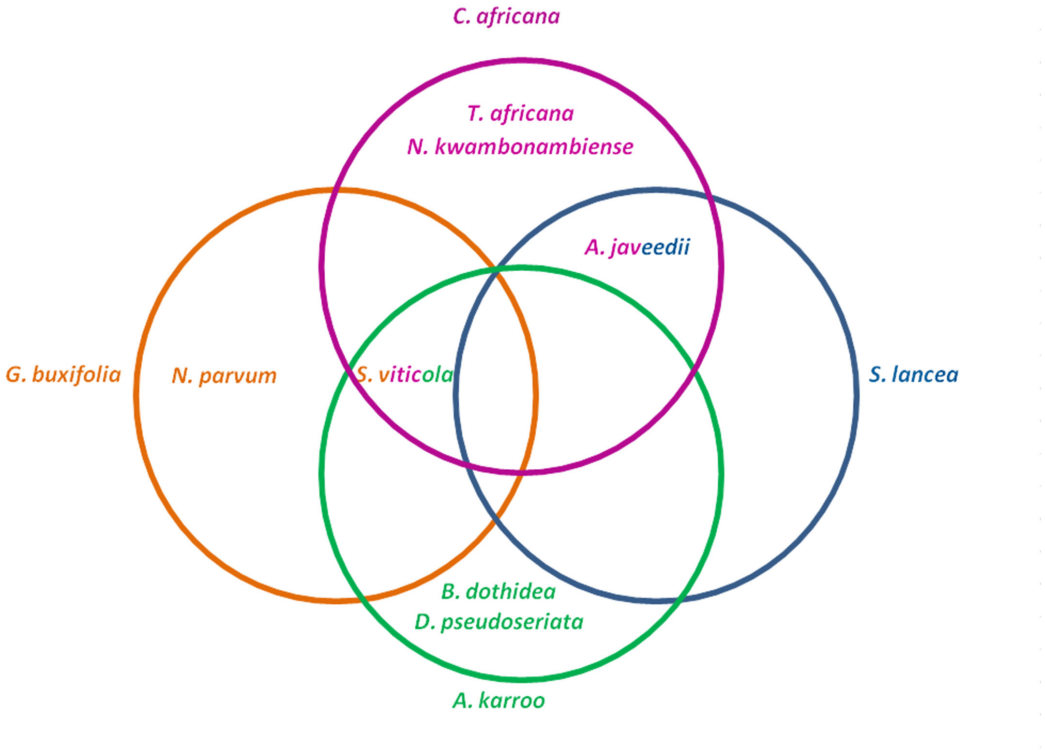


Figure 2. The pattern of overlapping Botryosphaeriaceae species among *Acacia karroo, Celtis africana, Searsia lancea* and *Gymnosporium buxifolia*

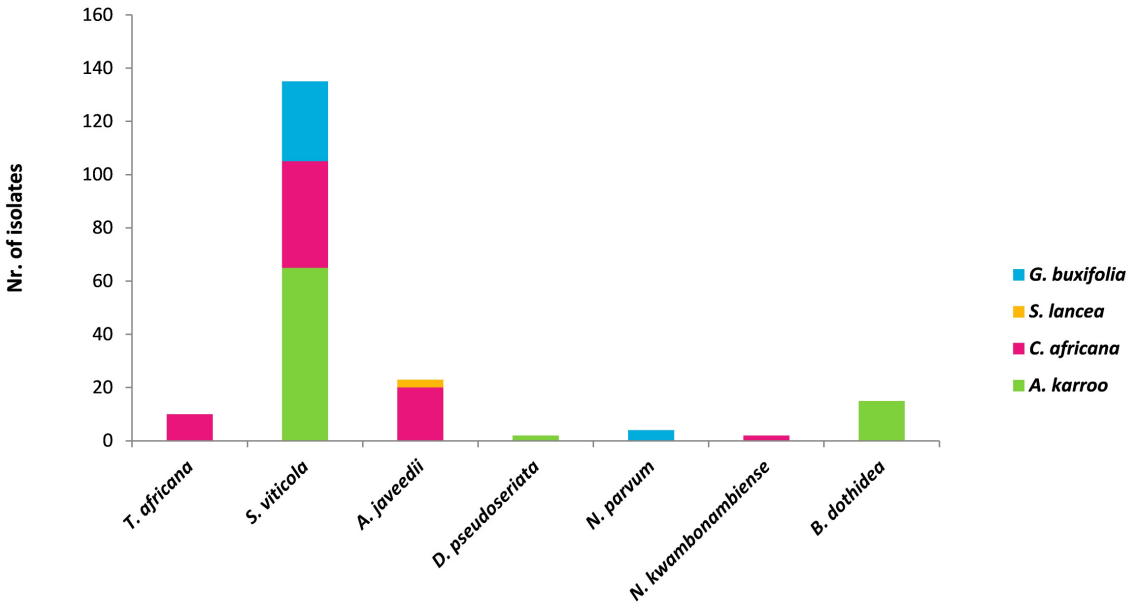


Figure 3. Diversity of Botryosphaeriaceae species on four hosts, namely *Acacia karroo, Celtis africana, Searsia lancea* and *Gymnosporium buxifolia*

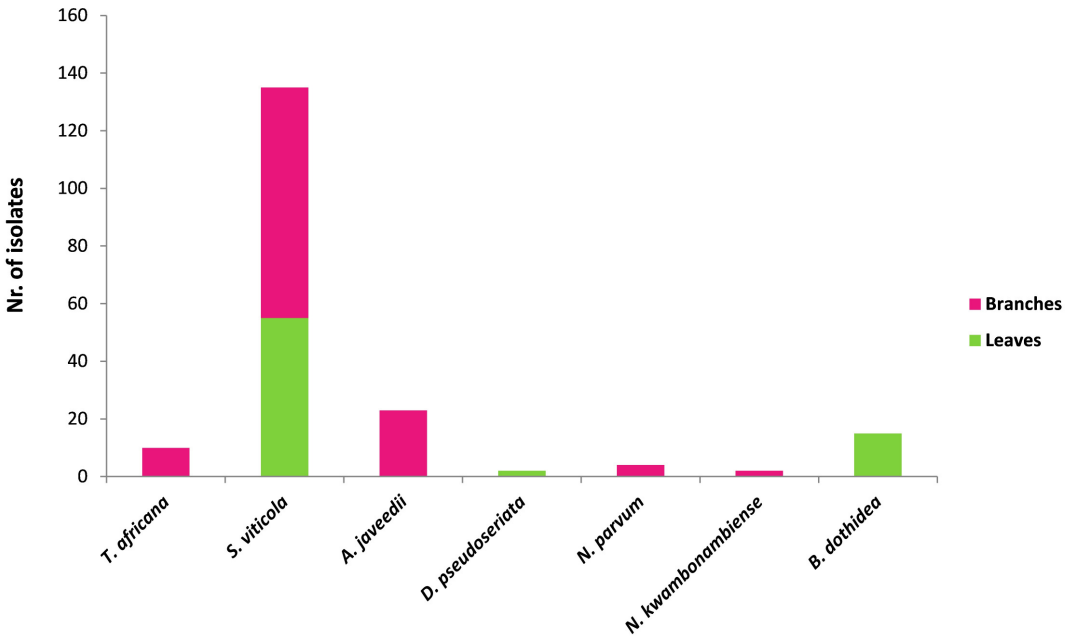


Figure 4. Diversity of Botryosphaeriaceae species on leaves and branches of *Acacia karroo, Celtis africana, Searsia lancea* and *Gymnosporium buxifolia*

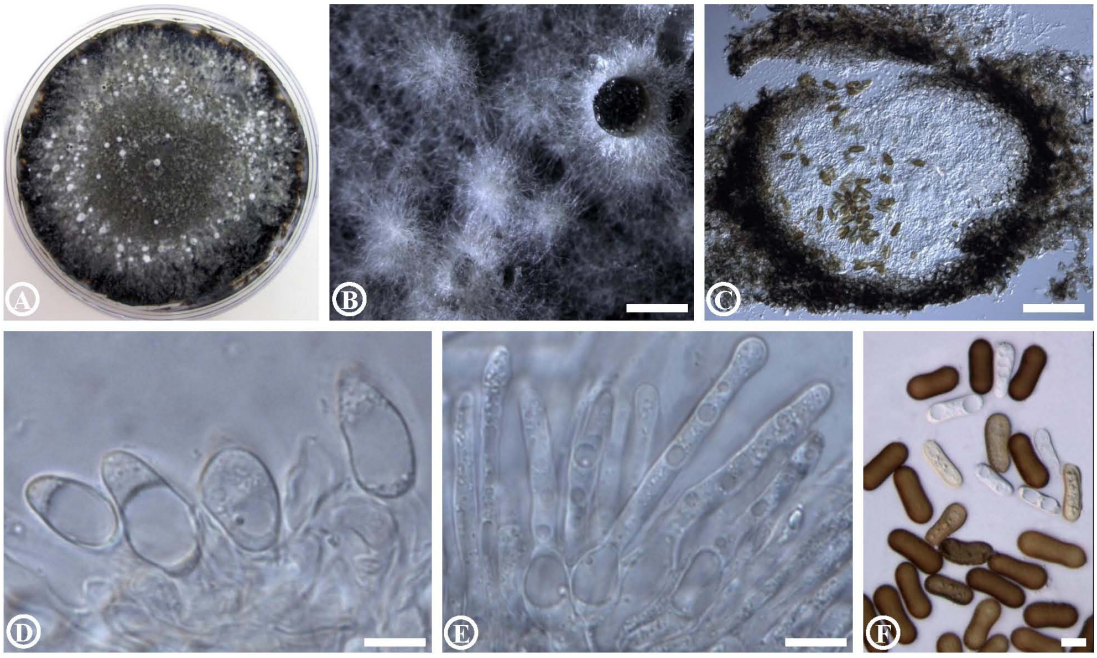


Figure 5. Micrographs of *Aplosporella javeedii* **(A)** Culture morphology on MEA in 25°C. **(B)** Pycnidia (scale bar = 1000 µm). **(C).** Longitudinal section through pycnidium (scale bar = 100 µm). **(D)** Conidiogenous cells (scale bar = 5 µm). **(E)** Paraphyses (scale bar = 10 µm). **(F)** Conidia (scale bar = 5 µm).

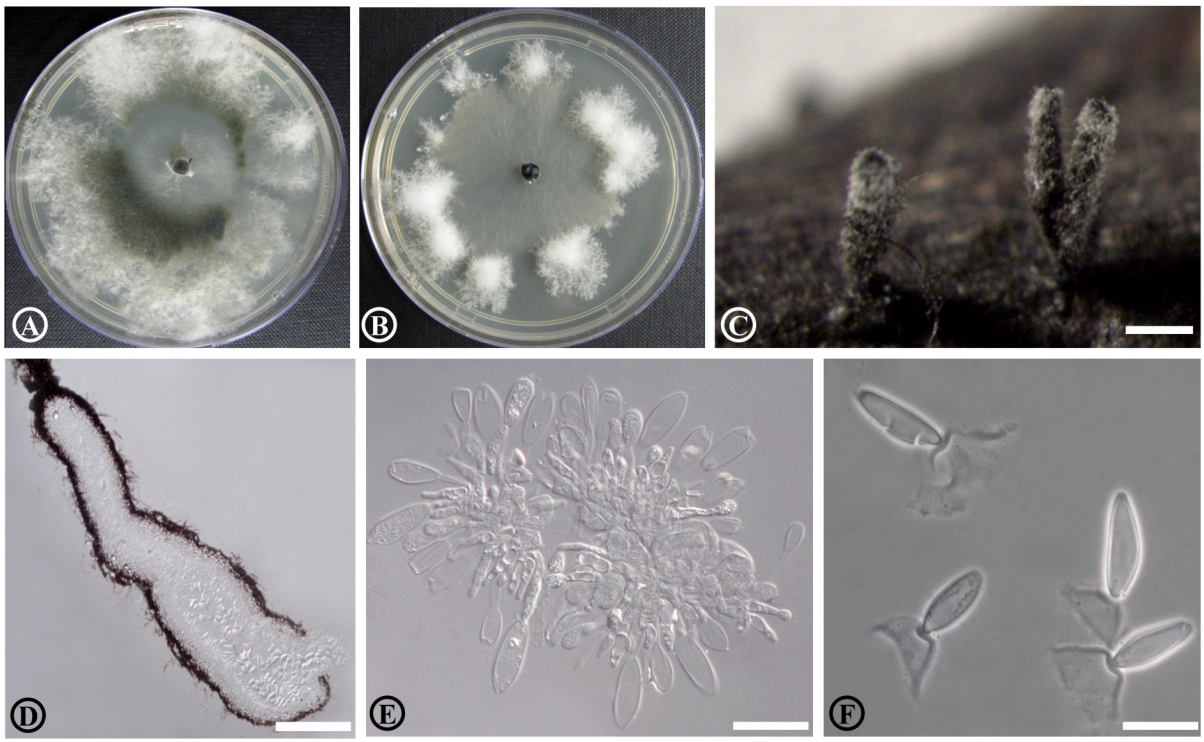


Figure 6. Micrographs of *Tiarosporella africana*. (**A)** Four days culture morphology on MEA in 30°C. (**B)** Four days culture morphology on MEA in 25°C. (**C)** Pycnidia (scale bar = 500 µm). (**D)** Longitudinal section through pycnidium (scale bar = 500 µm). (**E)** Conidiogenous cells and young conidia (scale bar = 20 µm). (**F)** Conidia (scale bar = 20 µm).

Chapter 4

Botryosphaeriaceae associated with *Acacia karroo* in South Africa: Temporal and spatial variation

ABSTRACT

The Botryosphaeriaceae are common and diverse members of fungal communities infecting both native and non-native plants. These fungi are increasingly being used as model organisms to understand patterns of global movement of latent pathogens as well as in causing disease. To interpret these patterns, it is necessary to understand whether these fungi are host or geographically restricted, and how this might vary over time and space. The aim of this study was, therefore, to consider the species diversity of the Botryosphaeriaceae associated with the native *Acacia karroo* across the South African landscape, and the variation of species at specific sites over time. In addition, the diversity of these fungi associated with different tissues of this host plant was considered. These questions were addressed by sampling healthy *A. karroo* from 23 sites across its distribution in South Africa. A more intensive hierarchical sampling was conducted in the Tshwane area over three years. Isolates were grouped based on morphology of cultures, and representative cultures were identified based on comparisons of sequence data for the ITS (Internal Transcribed Spacer) and TEF-1α (Translation elongation factor) loci. In total, 16 species of the Botryosphaeriaceae were identified, including seven species only from the Tshwane Metropolitan (Pretoria, Gauteng). There was a clear geographic structure, with some species occurring only in some parts of the country. However, variation over time in the Tshwane area was not significantly different. Species diversity was significantly different from different tissues, with no Botryosphaeriaceae identified from seeds and the highest number and diversity obtained from leaves. The results display a rich species diversity of Botryosphaeriaceae on a native host, even in the absence of obvious disease.

INTRODUCTION

Recent studies on native and non-native *Acacia* species have revealed a wide diversity of Botryosphaeriaceae in South Africa, a family of well-known fungal latent pathogens. This

includes five previously known (Jami et al. 2013a; Slippers et al. 2013) and 13 newly described species (Jami et al. 2012; Slippers et al. 2013). Of these, *Acacia karroo* is one of the most commonly occurring native tree species in South Africa with a wide distribution across large areas of the country (VanWyk and VanWyk 1997). Botryosphaeriaceae are known to infect *A. karroo* and 11 species have been found on this tree from different parts of the South Africa (Jami et al. 2012, 2013a; Slippers et al. 2013). A number of Botryosphaeriaceae have also been found on other species of *Acacia* such as the non-native *A. mearnsii* (Roux and Wingfield 1997), and native *A. tortillas*, *A. erioloba* and *A. mellifera* (Slippers et al. 2013). Studies such as these, and others considering native hosts in South Africa, provide a baseline of information for future studies on changes, new introductions, overlaps between native and non-native hosts and other related topics. Unfortunately, many previous studies have included few sampling locations, a limited number of tissue types and they tend not to be repeated at the same locations over time. This raises question whether those samples are representative of the communities of Botryosphaeriaceae and whether these would allow comparisons over time.

Apart from those on *A. karroo*, various other studies on the Botryosphaeriaceae in South Africa have identified 12 genera, including at least 48 species (including unpublished work by the authors), from different native and non-native hosts. These hosts include native woody plants such as *Syzygium cordatum* (Pavlic et al. 2007), *Pterocarpus angolensis*, *Terminalia catappa* (Combretaceae) (Mehl et al. 2011; Begoude et al. 2010), and species of *Leucadendron*, *Leucospermum* and *Protea* (Marincowitz et al. 2008; Denman et al. 2003). Forty of the Botryosphaeriaceae species occur on these native plants, of which 10 overlap with non-native trees such as *Pinus* spp., *Eucalyptus* spp. and fruit trees including peach, pear, almond, apple, plum, nectarine, apricot, and grapevine (Slippers et al. 2007; Damm et al. 2007; Smith et al. 2001; Swart and Wingfield 1991). There is clearly substantial overlap between the various hosts, but variable sampling efforts make it difficult to draw strong conclusions from them regarding host specificity.

The above-mentioned studies on Botryosphaeriaceae have shown a wide geographical distribution across South Africa with these fungi detected in all sampled areas. The results also suggest there could be unique patterns of diversity of Botryosphaeriaceae in different locations. Some species appear to be widely distributed such as *Botryosphaeria dothidea* and *Neofusicoccum parvum* (Denman et al. 2003; Pavlic et al. 2007; 2004; Smith et al. 2001; Slippers et al. 2004b; Jami et al. 2013b; Begoude et al. 2010; Van Niekerk et al. 2004), while others are only found in one or a small number of locations.

Tissue type has been shown to affect species diversity of endophytes (Shamoun and Sieber 2000; Gazis and Chaverri 2010; Gond et al. 2007; Kharwar et al. 2008; Yuan et al. 2009; Huang et al. 2008; Taylor et al. 1999; Mishra et al. 2012). For example *Pseudocercospora* sp., was isolated as an endophyte from leaves of *Tapirira guianensis*, while it was never isolated from the stems of this host (de Abreu et al. 2010). In contrast, *Stagonospora* sp., was found in stems of *T. guianensis*, while it was absent from the leaves of this host. However, no studies have been done on potential tissue specificity or preference of Botryosphaeriaceae species.

The aim of this study was to conduct a comprehensive survey of the endophytic Botryosphaeriaceae infecting *A. karroo* widely throughout South Africa. The data were then used to determine possible patterns of species diversity of the Botryosphaeriaceae on *A. karroo*. In the greater Tshwane Metropolitan area (Pretoria) the surveys were conducted over three years to assess the temporal changes in dominance of the obtained species. The diversity of these fungi was also compared among different tissues (leaves, branches, branchlets and seeds) of *A. karroo* in samples from the Tshwane area. This study focused only on healthy trees in order to avoid potential variation in infection levels over the study period. The pathogenicity of the isolated Botryosphaeriaceae was conducted to gain some insight into the potential of these species to cause disease.

MATERIALS AND METHODS

Isolates collection

Samples were collected from 318 healthy and randomly selected *A. karroo* trees in a countrywide survey of South Africa in 2010 (Table 1, Figure 2). One branch (~0.5–1 cm diameter) per tree was cut and all samples were placed in paper bags and taken to the laboratory for processing after storage at 4°C for one week. From each branch, 12 pieces (0.5 cm) were randomly selected, surface-disinfested in 10 % hydrogen peroxide for two minutes, and rinsed three times in sterile water. All branch samples were placed on 2 % malt extract agar (Biolab, Midrand, South Africa) in Petri dishes with four samples per plate. Petri dishes were incubated at 24 °C for seven days and fungal growth from each wood sample showing cultural morphology characteristics of the Botryosphaeriaceae (fast growing, white to black cultures with aerial hyphae) was transferred to fresh MEA plates. After 4-5 days, all isolates showing typical growth (fast growing, white to black cultures with fluffy aerial hyphae) were transferred to 15% water agar (WA) in order to make single hyphal tip sub-cultures. These isolates are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

To assess possible variation in Botryosphaeriaceae diversity over time one accessible region, namely the Tshwane Metropolitan area (Pretoria) with 40 sites suitable for sampling, was selected to conduct sampling over three years. Samples were collected from 2008-2010 in early winter from the healthy *A. karroo* trees at these 40 sites across diverse areas in and around the area. Trees were either planted in gardens or parks, or from natural regeneration within and outside the city border. One tree was randomly selected per site for sampling. Three healthy branches including leaves and thorns (in some cases with attached seeds) were collected from 40 trees in the first and second years and 20 trees for 20 randomly selected sites in the third year. The same locations were used, and where possible, the same trees were sampled in successive years.

DNA sequencing

Isolates were initially grouped based on culture morphology using the same criteria as reported by Jami et al. (2012). DNA was extracted from the mycelium of 5-day-old single hyphal-tip cultures (Lee and Taylor 1990) of three representatives for each morphological group. Sequence data were obtained for the internal transcribed spacer region of the ribosomal RNA (rRNA) operon using primers ITS-1 (Gardes and Bruns 1993) and ITS-4 (White et al. 1990) and the translation elongation factor 1-α (TEF-1α) gene using primers EF1-728F and EF1-986R (Carbone and Kohn 1999). The PCR reaction mixture, PCR conditions and visualization were as described by Jami et al. (2012). The amplified PCR fragments were purified with Sephadex (Sigma, Steinheim, Germany) and sequenced using BigDye terminator cycle sequencing kits (Perkin-Elmer Applied Biosystems, Foster City, California, USA) in both directions, with the same primers used for the PCR reactions. PCR products were sequenced using an ABI 3730 sequencer (Applied Biosystems).

Sequences of the isolates were edited using Vector NTI 11 (Lu and Moriyama 2004). DNA sequences for relevant Botryosphaeriaceae species previously published were retrieved from GenBank (http://www.ncbi.nlm.gov) and used in the analyses. The resulting data matrices for each gene region were rooted to *Pseudofusicoccum stromaticum* following the example of Phillips et al. (2008). The data matrices were aligned online using MAFFT version 6 (Katoh et al. 2005) and checked manually for alignment errors.

Phylogenetic analyses of sequence data for Maximum Parsimony (MP) and Maximum Likelihood (ML) were made using PAUP* v.4.0b10 (Swofford 2001). Maximum parsimony genealogies for single genes were constructed with the heuristic search option (100 random taxa additions, tree bisection and reconstruction or TBR) in PAUP. All characters were unordered and of equal weight and gaps were treated as missing data. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the tree(s) obtained was evaluated by 1000 bootstrap replications. Congruence between the different datasets was tested using the Partition Homogeneity Test (PHT) in PAUP

(Phylogenetic Analysis Using Parsimony) version 4.0b10 (Farris et al. 1995; Huelsenbeck et al. 1996), with the uninformative characters removed before analysis. Other measures such as tree length (TL), consistency index (CI), rescaled consistency index (RC), and the retention index (RI) (Hillis and Huelsenbeck 1992) were recorded. For ML analyses, the best nucleotide substitution models for each dataset were found separately with Modeltest 3.7 (Posada and Buckley 2004), and for the combined dataset. The ML analyses were performed in PAUP 4.0b10 and confidence levels were determined with 1000 bootstrap replications. The consensus trees were constructed in MEGA version 4 and posterior probabilities were assigned to branches after 60% majority rule.

Analyses of patterns of species diversity

The Kruskal-Wallis one-way analysis of variance was used in the BMDP Statistical Package program (BMDP, Los Angeles, CA) for the collections across three years from Tshwane. The variation in species diversity was compared between years and among the different tissue types (leaves, branches and branchlets) sampled. To determine the variation of monthly temperatures (minimum/maximum), monthly precipitation, humidity and sunlight hours with years as predictor variables, a one-way ANOVA was used in JMP version 6.0 (SAS Institute, Cary, NC, USA). For this purpose monthly temperature (minimum/maximum), precipitation, humidity and sunlight hour data (Table. 2), were obtained from the South African Weather Service (Pretoria station). Rarefactions were calculated via iNEXT online (http://glimmer.rstudio.com/tchsieh/inext/#overview) to determine the species richness obtained from the various collections and the number of the infected trees.

Pathogenicity tests

One-year-old *A. karroo* seedlings ranging in height from 70–110 cm and stems of 0.7–1.5 cm in diameter were maintained in a greenhouse at 26°C and watered once daily. The plants were grown in a 1:2:4 mixture of river sand, red top soil and pine bark potting soil in 5-

L plastic bags. Two to three isolates of each Botryosphaeriaceae species identified based on phylogenetic analyses were selected and each isolate was inoculated on 10 trees with one additional tree in each replication inoculated with sterile MEA medium as a control. Inoculations were made following the technique used by Jami et al. (2013a). In total, 23 isolates were inoculated onto 230 plants, with an additional 23 plants inoculated as negative controls. Lesion lengths were measured six weeks after inoculation. Variation in lesion length was analyzed using a one-way analysis of variance with the general linear model procedure in SAS, version 9.3.

To confirm Koch's postulates, one plant was chosen from those inoculated for each isolate (in total 23 plants) for re-isolation, as well as for 16 control plants. A small sample of tissue was cut from the lesions including the inoculation points, surface-disinfested with 10 % hydrogen peroxide for two minutes, and rinsed three times in sterile water. The tissue samples were plated onto 2% malt extract agar and incubated at 24°C for seven days after which isolates were identified based on morphological characters.

RESULTS

Isolates collection

In total, 404 Botryosphaeriaceae isolates were collected from *A. karroo* branches from 18 sites across South Africa. Of the sampled trees, 31% yielded isolates. With the exception of four sites, namely Kimberley, Prieska (in Northern Cape Province), Alexander Bay and Touws River (in Western Cape Province), isolates were obtained from all sites across the geographical range sampled (Table 3).

A total of 267 isolates of Botryosphaeriaceae were obtained over the three years sampling period in the Tshwane Metropolitan area. Of these, 110 isolates were obtained in 2008 (26 from leaves, 52 from stems, 32 from branches), 93 isolates in 2009 (44 from leaves, 21 from branchlets, 28 from branches), and 64 isolates in 2010 (60 from leaves and four from branches). These isolates were found from 55%, 45% and 50% of sampled trees over the three years, respectively. No Botryosphaeriaceae species were isolated from seeds during these three years.

DNA sequence analyses

The datasets for the ITS and TEF-1α sequences were analyzed individually and in combination. The ITS sequence dataset contained 522 characters (4 parsimony-uninformative, 148 parsimony-informative, 370 constant characters) with CI = 0.602, RI = 0.902, RC = 0.543, HI = 0.398 and TL = 321. The TEF-1α dataset had 362 characters (3 parsimony-uninformative, 224 parsimony-informative, 135 constant characters) with CI = 0.651, RI = 0. 919, RC = 0.550, HI = 0.383 and TL = 986. The tree statistics for the combined dataset (TreeBase Accession No. S12358) were CI = 0.487, RI = 0.854, RC = 0.416, HI = 0.513, TL = 2148, and the partition homogeneity test (PHT) on the datasets produced a P-value of 0.01. For ML analyses, the best nucleotide substitution models were found separately for the individual loci, as well as in the combined analysis, with Modeltest 3.7 (Posada and Buckley 2004). Both datasets best fitted the GTR model having the following parameters: for ITS G=0.337, I=0.468, TEF-1α G=0.811 and for combined datasets G= 0.663, I=0.553. The analyses were performed in PAUP 4.0b10 and confidence levels were determined with 1000 bootstrap replications.

The topology of the trees obtained using the ML and MP analyses were similar with regards to the clades representing species isolated for the individual gene regions, as well as in the combined analysis. Isolates resided in eight genera and 16 species groups that were identified as *Botryosphaeria dothidea, Diplodia allocellula, Dothiorella dulcispinae, Do.*

brevicollis, Lasiodiplodia theobromae, L. pseudotheobromae, L. gonubiensis, Neofusicoccum vitifusiforme, N. parvum, N. australe, N. kwambonambiense, N. protearum, Phaeobotryosphaeria variabilis, Spencermartinsia pretoriensis, S. viticola and *Tiarosporella urbis-rosarum* (Figure 1). *Botryosphaeria dothidea* and *S. viticola* were the most common species across the country and they represented 44% and 26% of the total collected isolates, respectively (Figure 2). The frequency of *B. dothidea* isolates was higher than for the other species in most areas where this species was found (Figure 2). The frequency of *S. viticola* was higher than the other species in the Tshwane area (Gauteng), Dundee (Kwazulu-Natal), Bloemfontein (Free State) and Aliwal North (Eastern Cape) areas (Figure 2).

Analyses of patterns of species diversity

The species isolated varied only slightly in composition over the three years sampled, with *N. parvum, N. vitifusiforme, S. viticola* and *L. theobromae* isolated in two or more years, and *B. dothidea, N. australe* and *N. kwambonambiense* isolated only in one of the three years (Figure 3). Statistical analyses showed that species diversity of Botryosphaeriaceae was not significantly different over three years ($P > 0.05$). *Spencermartinsia viticola* was dominant in all three years representing 46.9% (2008), 28.4% (2009) and 37.5% (2010) of the total number of isolates (Figures 3, 4).

The assemblage of species from the different tissue types differed over the three years of sampling (Figure 5). The species composition from branchlets was significantly different between the first and third year of sampling ($P < 0.05$), while species diversity obtained from branches were significantly different between the second and third year of sampling ($P < 0.05$). There were no significant differences in the species diversity found from leaves over the three years ($P > 0.05$). The more common species, *S. viticola, N. vitifusiforme, N. parvum* and *L. theobromae*, were isolated from all tissue types. However, the more rarely occurring species were only found in certain tissues, for instance *N. australe* was found only in leaves and branchlets, *B. dothidea* only in branchlets and *N. kwambonambiense* only in branch samples

(Figure 6). *Spencermartinsia viticola* was significantly more common in leaves and branchlets than in branch samples ($P < 0.05$).

Results of the ANOVA analysis comparing monthly means for maximum and minimum temperature, precipitation, humidity and sunlight hours showed no significant differences among the sampling years for these parameters as they relate to the occurrence of the Botryosphaeriaceae ($P > 0.05$). Rarefaction curves for all 23 sites including the Tshwane Metropolitan area showed that species diversity and the number of infected trees increased with increasing numbers of samples collected (Figures 7, 8). However, Tshwane Metropolitan (Pretoria) data showed different levels of increase in the number of samples and the number of isolated species.

Pathogenicity tests

All 45 isolates representing the 16 species produced lesions in the cambium of inoculated branches within six weeks. These lesions were significantly ($P < 0.05$) larger than those observed for the control inoculations (Figure 9). Statistical analyses showed that lesion size for the different fungi varied little between replicate trees inoculated per isolate, but varied considerably between isolates for certain species, including those of *B. dothidea*, *N. australe*, *Do. brevicollis*, *T. urbis-rosarum* and *S. viticola*. The longest lesions were produced by *P. variabilis* ($\bar{X}$ = 30.5 mm), *L. theobromae* ($\bar{X}$ = 27.7 mm) and *N. australe* ($\bar{X}$ = 22.7 mm). All 16 Botryosphaeriaceae species were re-isolated from lesions and no Botryosphaeriaceae were isolated from the controls (Figure 9). They were identified based on cultural and conidial morphology.

DISCUSSION

In this study, eight genera and 13 species of Botryosphaeriaceae were found on healthy *A. karroo* trees sampled broadly in South Africa. Thus, a total of 16 species residing in eight genera, including those previously recorded (2013a, 2012), are known from this tree. Of these genera, the most frequently encountered were *Botryosphaeria, Spencermartinsia* and *Neofusicoccum*, representing 44 % (n=184), 27 % (n=112) and 15 % (n=61), respectively, of the total number of isolates. *Diplodia* (1%), *Phaeobotryosphaeria* (2%), *Dothiorella* (2%), *Tiarosporella* (5%) and *Lasiodiplodia* (5%) are apparently less common genera. Apart from clear patterns of dominance in the community of Botryosphaeriaceae across South Africa, this study also revealed that this diversity varied across time and space (geographically and on individual trees).

Botryosphaeria dothidea was the most common species encountered in the country-wide survey of *A. karroo*, being isolated from 14 sites. *Botryosphaeria dothidea* is one of the most widespread species in the Botryosphaeriaceae globally (Slippers and Wingfield 2007) and its dominance in *A. karroo* is perhaps not surprising. In previous South African studies, *B. dothidea* has been isolated from nine woody plants occurring in different areas (KwaZulu-Natal, Mpumalanga and Eastern Cape) of the country (Smith et al. 2001; Pavlic et al. 2007; Roux and Wingfield 1997). Previous studies showed that *B. dothidea* infects trees more rapidly at higher temperatures (Michailides and Morgan 1992; Copes and Hendrix Jr 2004). However, the areas where it was most common are not those that are necessarily warmest in South Africa (Conradie 2012) and these results are somewhat confusing. Clearly further studies are needed to fully understand the temperature requirements of the fungus.

Botryosphaeria dothidea was first described from Europe, where it has been considered native given its high diversity, wide occurrence, and association with local hosts (Slippers and Wingfield 2007; Slippers et al. 2004a; Piskur et al. 2011). Subsequent to those studies, the fungus has been found in many different parts of the world (Slippers and

Wingfield 2007). Results of this study suggest that views regarding its origin are premature and that they must await further population level analyses. What is clear, however, is that *B. dothidea* is a very successful invader and colonist of both native and non-native trees, and in a wide range of environments including relatively undisturbed as well as intensively managed areas.

Spencermartinsia viticola was the second most frequent species of Botryosphaeriaceae on *A. karroo* in South Africa. This fungus has also commonly been isolated from *A. mellifera* in Pretoria (Gauteng) (Slippers et al. 2013) and *Vitis vinifera* in Stellenbosch (Western Cape) (Van Niekerk et al. 2004). Likewise, Jami et al. (2013a) found that *S. viticola* was the most common species among the eight isolated from *A. karroo* in the Tshwane Metropolitan (Pretoria). Its common occurrence on *A. karroo* in the present study supports previous findings that this tree appears to be a particularly common host, and possibly preferred, for this fungus. *Neofusicoccum australe* was isolated from *A. karroo* trees growing on four sites including a wide variety of climates, ranging from desert to warm temperate and humid climates. It has previously been reported from *S. cordatum* in the Eastern Cape (Pavlic et al. 2004), grape, pome and stone fruit trees in the Western Cape (Slippers et al. 2007; Van Niekerk et al. 2004; Damm et al. 2007) and *Eucalyptus* in Mtubatuba in northern KwaZulu-Natal (Pillay et al. 2013). *Neofusicoccum australe* was not found in the desert areas in any of of the previous South African investigations considering the Botryosphaeriaceae. It is thus interesting that it was found at one desert site in the present study. The remaining sites where it was found had temperate climates. This is in contrast of the studies in Australia where *N. australe* has been found to occur commonly in parts of Western Australia with a Mediterranean climate having hot and dry summers and cool wet winters (Burgess et al. 2006; Cunnington et al. 2007; Taylor et al. 2009).

Other species of the Botryosphaeriaceae isolated in this and previous studies from *A. karroo* were found at only a few sites. These were normally in close proximity to each other, or were from urban centers such as Pretoria. *Neofusicoccum parvum* was collected in Tshwane Metropolitan (Pretoria, Gauteng) and Aliwal-North (Western Cape), but it is also known from

T. catappa in Richardsbay (KwaZulu-Natal), *S. cordatum* in Kwambonambi (KwaZulu-Natal), *Prunus domestica* in Villiersdorp (Western Cape), and *V. vinifera* in Stellenbosch (Western Cape) (Van Niekerk et al. 2004; Slippers et al. 2007; Begoude et al. 2010; Pavlic et al. 2007). Based on these and other studies, Sakalidis et al. (2013) suggested that *N. parvum* might be native to South Africa. However, we did not isolate this species from *A. karroo* in the above-mentioned areas. It thus appears to be more rare on this tree than the hosts previously sampled.

Lasiodiplodia pseudotheobromae represents another example of a broadly distributed species, but which was rarely isolated in this study. This species was isolated from trees in the Tshwane Metropolitan (Pretoria) and Mokopane (Gauteng and Limpopo provinces) in the present survey. However, in previous studies it has been collected from *Pterocarpus* spp. (Mawewe Nature Reserve, Mpumalanga), *T. catappa* (Richardsbay, KwaZulu-Natal) and *S. cordatum* (Mtubatuba, KwaZulu-Natal) in South Africa (Pillay et al. 2013; Mehl et al. 2011; Begoude et al. 2010). Climate appears to provide an explanation regarding the distribution of *L. pseudotheobromae*, whereas this seems not to be the case for other rarely occurring species. This species is common in tropical parts of Africa and South America (Marques et al. 2013; Ismail et al. 2012; Castro Medina et al. 2013; Alves et al. 2008). While it is possible that *A. karroo* is not a primary host for this fungus, it is more likely that it does not occur in the drier and colder areas sampled in the study. The same would be true for *L. theobromae* (Punithalingam 1980; Alves et al. 2008) and *N. kwambonambiense*, both known from tropical areas of the world, but that were rare in this study (Sakalidis et al. 2013).

Apart from the examples treated above, a number of other species were described in this study from areas and tree hosts for the first time. For example *N. protearum* was previously known only from *Protea* spp. in the Western Cape (Denman et al. 2003; Marincowitz et al. 2008), while in this study it was found on *A. karroo* in Alliwal North (Eastern Cape) and Calitzdorp (Western Cape). *Neofusicoccum vitifusiforme* is reported in this study from *A. karroo* in Tshwane Metropolitan (Pretoria, Gauteng) and Dundee (KwaZulu-Natal), while it has previously been found on *V. vinifera* and *Prunus salicina* in the Western Cape and Modimolle (Limpopo) (Damm et al. 2007; Van Niekerk et al. 2004).

Phaeobotryosphaeria variabilis was found from Tshwane Metropolitan (Pretoria, Gauteng) and Cape Town (Western Cape) in this survey, and has previously been described from *A. hebeclade*, *A. mellifera* and *A. karroo* in Namibia and South Africa (Slippers et al. 2013; Jami et al. 2013a). This shows that they may be more widely distributed than originally thought, and that their known distribution is significantly influenced by the fact that they are rare and easily overlooked in small samples.

Increasing distribution data indicate possible endemism for some Botryosphaeriaceae in South Africa. Some species such as *S. pretoriensis* are thus far known only from a single area (Jami et al. 2012, 2013a). *Lasiodiplodia gonubiensis* was isolated only from the Eastern Cape, the same region it was originally described from *S. cordatum* (Pavlic et al. 2004). Whether these represent locally adapted, endemic species, or whether the results reflect depth of sampling for rare species, remains to be clarified. In the case of *L. gonubiensis*, which has been repeatedly sampled in this area but nowhere else, endemism appears to be an increasingly strong explanation.

The results of sampling over three years at one site (Tshwane Metropolitan area), revealed seven species of Botryosphaeriaceae, but no significant variation in species diversity between sampling years. *Spencermartinsia viticola*, *N. parvum* and *N. vitifusiforme* were present in all three sampling years, while *N. australe* and *B. dothidea* were found only in the first year, *N. kwambonambiense* only in the third year and *L. theobromae* in the second and third year. Climatic factors did not vary across the sites over this time period. Furthermore, potential spatial effects were minimized by sampling from the same locations and trees between years. Although much longer collection times will be needed to fully determine the effect of climate and location on variations in species diversity, the results suggests that time did not have a large effect. This might simplify future sampling efforts in areas with fairly stable weather conditions over time.

The diversity of Botryosphaeriaceae species varied among the tissue types (leaves, branchlets, branches) over the three years, but not in very consistent patterns. The only consistent pattern was that the species composition on branches and branchlets was significantly different over the three years. For example, in the third year, the species diversity was significantly higher between leaves, branches and branchlets, but this was not the case for the first and second year. Furthermore, specific species could not be linked to specific tissues and common species occurred on all tissues. Rare species were by their nature more restricted, but together with information from other studies also do not appear to be tissue specific. For example, *N. kwambonambiense* was found only on leaves in this study, but previously this species has been found on branches and fruits of *S. cordatum* (Pavlic et al. 2009) and branches of *E. grandis* (Pillay et al. 2013). Our data also contradict a previous study that reported that *B. dothidea* and *N. parvum* were only from branches of *Taxus chinensis* (Wu et al. 2013), while the present study yielded *B. dothidea* only on branchlets and *N. parvum* occurred on all tissue types sampled. Collectively, these data suggest the Botryosphaeriaceae are not tissue specific. Broader and denser sampling will result in higher diversity, but seemingly not vary significantly with respect to the most dominant fungi. Sampling could thus target any of these tissues. In terms of frequency and practicality, sampling from branches would be easiest.

It has been shown previously that endophytic fungal diversity can be influenced by plant tissue types (Kumar and Hyde 2004). Factors such as surface area and concentration of antimicrobial components are amongst the best explanations for this pattern (Chareprasert et al. 2006; Upadhyay et al. 2010). For example, *Lophodermium sensu lato* has been shown to occur only on needles of *Pinus monticola*, but to be absent from seeds (Ganley and Newcombe 2006), while *Rhodotorula minuta* was only detected within the cells of meristematic tissue and *Hormonema dematioides* only in the scale tissues of the buds of *Pinus sylvestris* (Pirttilä et al. 2003). For the Botryosphaeriaceae and other endophytic fungi, these questions can in future be more clearly answered through direct sequencing of fungal amplicons from plant tissues (Pillay 2012; Marsberg 2012).

In this survey, the species diversity was higher in the Tshwane Metropolitan area than elsewhere, even after correcting for sampling intensity. Interestingly, the isolation frequency per tree in the Tshwane Metropolitan area was also higher than the other sampled sites. It is not clear what drives this increased diversity but one possibility is that the higher plant diversity in the urban areas (introduced for horticulture) together with the higher frequency of trade (with Pretoria as one of the main urban centers in the country) compared to other sampled sites could influence the surrounding diversity in Botryosphaeriaceae. There could be other factors, such as the intense human activities, pollution, damage and other factors could influence this pattern of diversity. This would be a fruitful area to explore in future, especially with a view to understanding the potential influence of urban centers such as Pretoria as reservoirs or "biological bridges" for invasive fungi.

Comprehensive geographical surveys exist for numerous animal and plant species in South Africa. Although these distribution data are not complete in all cases, it is adequate to show patterns of occurrence, and it links to ecological function, diversity, areas of endemism and conservation priorities for these taxa. Such information is very limited for fungi, even for important groups of plant associated fungi such as endophytes or latent pathogens, because of the complexities to acquire relatively complete data. This study, together with a growing number of similar studies of fungi on native hosts in South Africa, will provide foundations on which a greater number of inventories and sampling efforts will grow.

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Table 1. Representative isolates used in phylogenetic analyses.

Isolate No.	Identity	Specimen	Location	Collector	GenBank No.	
					ITS	EF1-α
CMW39268	*Botryosphaeria dothidea*	Branches	Kuruman, Northern Cape, SA	F. Jami	KF270050	KF269999
CMW39267	*B. dothidea*	Branches	Graaff-Reinet, Eastern Cape, SA	F. Jami	KF270051	KF269998
CMW39269	*B. dothidea*	Branches	Bloemhof, North West, SA	F. Jami	KF270052	KF270000
CMW39270	*B. dothidea*	Branches	Bloemfontein, Free State, SA	M. Gryzenhout	KF270053	KF270001
CMW39271	*B. dothidea*	Branches	Queenstown, Eastern Cape, SA	F. Jami	KF270049	KF270002
CMW39272	*B. dothidea*	Branches	Mokopane, Limpopo, SA	F. Jami	KF270054	KF270003
CMW39273	*B. dothidea*	Branches	Augrabies, Northern Cape, SA	F. Jami	KF270055	KF270004
CMW39274	*B. dothidea*	Branches	Beaufort West, Eastern Cape, SA	F. Jami	KF270045	KF270005
CMW39275	*B. dothidea*	Branches	Upington, Northern Cape, SA	F. Jami	KF270056	KF270006
CMW39276	*B. dothidea*	Branches	Delareyville, North West, SA	F. Jami	KF270057	KF270007
CMW39277	*B. dothidea*	Branches	Hondeklipbaai, Northern Cape, SA	F. Jami	KF270046	KF270008
CMW39278	*B. dothidea*	Branches	Cape Town, Western Cape, SA	M.J. Wingfield	KF270047	KF270009
CMW39279	*B. dothidea*	Branches	Jansenville, Eastern Cape, SA	F. Jami	KF270048	KF270010
CMW39288	*Diplodia allocellula*	Branches	Mokopane, Limpopo, SA	F. Jami	KF270058	KF270019
CMW36468	*D. allocellula*	Branches	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	JQ239397	JQ239384
CMW36469	*D. allocellula*	Branches	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	JQ239398	JQ239385
CMW36463	*Dothiorella brevicollis*	Branches	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	JQ239403	JQ239390

CMW36464	*Do. brevicollis*	Branches	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	JQ239404	JQ239391
CMW39289	*Do. brevicollis*	Branches	Mokopane, Limpopo, SA	F. Jami, M. Gryzenhout	KF270036	KF270020
CMW36461	*Do. dulcispinae*	Branches	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	JQ239401	JQ239388
CMW36462	*Do. dulcispinae*	Branches	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	JQ239402	JQ239389
CMW36460	*Do. dulcispinae*	Branches	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	JQ239400	JQ239387
CMW39294	*Lasiodiplodia gonubiensis*	Branches	Haga Haga, Eastern Cape, SA	M.J. Wingfield	KF270063	KF270025
CMW39295	*L gonubiensis*	Branches	Haga Haga, Eastern Cape, SA	M.J. Wingfield	KF270064	KF270026
CMW39296	*L gonubiensis*	Branches	Haga Haga, Eastern Cape, SA	M.J. Wingfield	KF270065	KF270027
CMW39292	*L. pseudotheobromae*	Branches	Mokopane, Limpopo, SA	F. Jami	KF270059	KF270023
CMW39293	*L. pseudotheobromae*	Branches	Mokopane, Limpopo, SA	F. Jami	KF270060	KF270024
CMW38120	*L. theobromae*	Leaves	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	KC769935	KC769843
CMW39290	*L. theobromae*	Branches	Cape Town, Western Cape, SA	M.J. Wingfield	KF270062	KF270021
CMW39291	*L. theobromae*	Branches	Mokopane, Limpopo, SA	F. Jami	KF270061	KF270022
CMW39286	*Neofusicoccum australe*	Branches	Graaff-Reinet, Eastern Cape, SA	F. Jami	KF270037	KF270017
CMW38129	*N. australe*	Leaves	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	KC769930	KC769841

CMW39285	*N. australe*	Branches	Haga Haga, Eastern Cape, SA	M.J. Wingfield	KF270039	KF270016
CMW39287	*N. australe*	Branches	Hondeklipbaai, Northern Cape, SA	F. Jami	KF270038	KF270018
CMW38131	*N. kwambonambiense*	Leaves	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	KC769922	KC769833
CMW38132	*N. kwambonambiense*	Leaves	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	KC769923	KC769834
CMW38134	*N. parvum*	Leaves	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	KC769919	KC769830
CMW38135	*N. parvum*	Branchlets	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	KC769920	KC769831
CMW39283	*N. parvum*	Branches	Aliwal North, Eastern Cape, SA	F. Jami	KF270044	KF270014
CMW39280	*N. protearum*	Branches	Calitzdorp, Western Cape, SA	F. Jami	KF270041	KF270011
CMW39281	*N. protearum*	Branches	Calitzdorp, Western Cape, SA	F. Jami	KF270042	KF270012
CMW39282	*N. protearum*	Branches	Aliwal North, Eastern Cape, SA	F. Jami	KF270043	KF270013
CMW38123	*N. vitifusiforme*	Branches	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	KC769926	KC769838
CMW38125	*N. vitifusiforme*	Leaves	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	KC769927	KC769837
CMW38126	*N. vitifusiforme*	Leaves	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	KC769925	KC769836
CMW39284	*N. vitifusiforme*	Branches	Dundee, Kwazulu-Natal, SA	J.W.M. Mehl	KF270040	KF270015
CMW39297	*Phaebotryosphaeria variabilis*	Branches	Cape Town, Western Cape, SA	M.J. Wingfield	KF270066	KF270028

CMW39260	*Spencermartinsia viticola*	Branches	Delareyville, North West, SA	F. Jami	KF270031	KF269993
CMW38072	*S. viticola*	Leaves	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	KC769914	KC769822
CMW39261	*S. viticola*	Branches	Aliwal North, Eastern Cape, SA	F. Jami	KF270032	KF269994
CMW39262	*S. viticola*	Branches	Robertson, Western Cape, SA	F. Jami	KF270029	KF269995
CMW39263	*S. viticola*	Branches	Cape Town, Western Cape, SA	M.J. Wingfield	KF270030	Pending
CMW39264	*S. viticola*	Branches	Bloemfontein, Free State, SA	M. Gryzenhout	KF270033	Pending
CMW39265	*S. viticola*	Branches	Kuruman, Northern Cape, SA	F. Jami	KF270034	KF269996
CMW39266	*S. viticola*	Branches	Dundee, Kwazulu-Natal, SA	J.W.M. Mehl	KF270035	KF269997
CMW36477	*Tiarosporella urbis-rosarum*	Branches	Bloemfontein, Free State, SA	M. Gryzenhout	JQ239407	JQ239394
CMW36478	*T. urbis-rosarum*	Branches	Bloemfontein, Free State, SA	M. Gryzenhout	JQ239408	JQ239395
CMW36479	*T. urbis-rosarum*	Branches	Tshwane Metropolitan (Pretoria), SA	F. Jami, M. Gryzenhout	JQ239409	JQ239396

Table 2. Climate data obtained for the Tshwane Metropolitan (Pretoria, Gauteng) sites for the three years sampling.

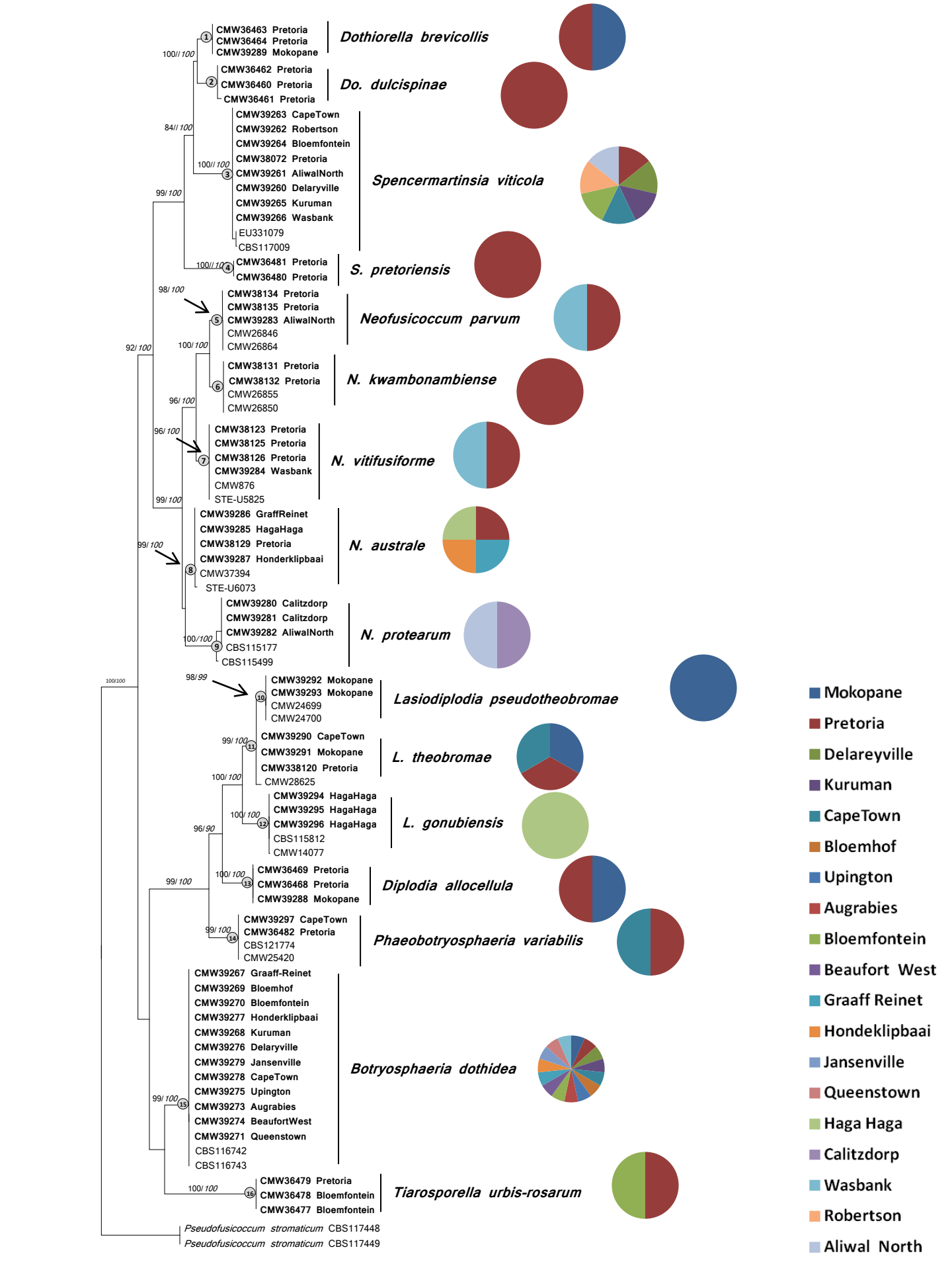
Climate factors	2008	2009	2010
Maximum temperature	23.9	24.51	24.5
Minimum temperature	11.25	11.62	11.54
Rainfall (mm)	765.6	588.4	801.1
Sunlight hours	97.7	99.3	100.4
Humidity (%)	69.58	70	71.58

Table 3. Number of Botryosphaeriaceae isolates from the sites across South Africa.

	**** BD***	***DA***	***DoB***	***DoD***	***LG***	***LT***	***LPt***	***NA***	***NK***	***NP***	***NPt***	***NV***	***PV***	***SP***	***SV***	***TU***
Aliwal North	-	-	-	-	-	-	-	-	-	5	1	-	-	-	10	-
Augrabies	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Beaufort West	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bloemfontein	10	-	-	-	-	-	-	-	-	-	-	-	-	-	22	21
Bloemhof	7	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-
Calitzdorp	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-
CapeTown	5	-	-	-	-	3	-	-	-	-	-	-	1	-	5	-
Delareyville	72	-	-	-	-	-	-	-	-	-	-	-	-	-	25	-
Graaff Reinet	4	-	-	-	-	-	-	3	-	-	-	-	-	-	-	-
Haga Haga	-	-	-	-	15	-	-	6	-	-	-	-	-	-	-	-
Hondeklipbaai	10	-	-	-	-	-	-	3	-	-	-	-	-	-	-	-
Jansenville	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kuruman	54	-	-	-	-	-	-	-	-	-	-	4	-	-	35	-
Dundee	12	-	-	-	-	-	-	-	-	-	-	10	-	-	-	-
Mokopane	6	1	1	-	-	2	3	-	-	-	-	-	-	-	-	-
Queenstown	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Robertson	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8	-
Upington	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pretoria	4	10	3	8	-	30	-	8	8	51	-	76	3	2	245	3

* **BD:** *Botryosphaeria dothidea*, **DA:** *Diplodia allocellula*, ***DoB:*** *Dothiorella brevicollis*, **DoD:** *Do. dulcispinae*, **LG:** *Lasiodiplodia gonubiensis*, **LT:** *L. theobromae*, **LPt:** *L. pseudotheobromae*, **NA:** *Neofusicoccum australe*, **NK:** *N. kwambonambiense*, **NP:** *N. parvum*, **NPr:** *N. protearum*, **NV:** *N. vitifusiforme*, **PV:** *Phaeobotryosphaeria variabilis*, **SP:** *Spencermartinsia pretoriensis.* , **SV:** *S. viticola*, **TU:** *Tiarosporella urbis-rosarum*

Figure 1. Maximum Likelihood (ML) tree of the combined data set of ITS ribosomal DNA and TEF-1α loci sequences. Bootstrap values for ML (Piano et al. 2005) and Maximum Parsimony (italic) above 60 % are given at the nodes. The tree was rooted to *Pseudofusicoccum stromaticum* (CBS117448 and CBS117449). Isolates sequenced in this study are presented in bold. Pie charts indicate the sites from which the species have been isolated.


CMW36463 Pretoria
CMW36464 Pretoria
CMW39289 Mokopane
Dothiorella brevicollis
CMW36462 Pretoria
CMW36460 Pretoria
CMW36461 Pretoria
Do. dulcispinae
CMW39263 CapeTown
CMW39262 Robertson
CMW39264 Bloemfontein
CMW38072 Pretoria
CMW39261 AliwalNorth
CMW39260 Delaryville
CMW39265 Kuruman
CMW39266 Wasbank
EU331079
CBS117009
Spencermartinsia viticola
CMW36481 Pretoria
CMW36480 Pretoria
S. pretoriensis
CMW38134 Pretoria
CMW38135 Pretoria
CMW39283 AliwalNorth
CMW26846
CMW26864
Neofusicoccum parvum
CMW38131 Pretoria
CMW38132 Pretoria
CMW26855
CMW26850
N. kwambonambiense
CMW38123 Pretoria
CMW38125 Pretoria
CMW38126 Pretoria
CMW39284 Wasbank
CMW876
STE-U5825
N. vitifusiforme
CMW39286 GraffReinet
CMW39285 HagaHaga
CMW38129 Pretoria
CMW39287 Honderklipbaai
CMW37394
STE-U6073
N. australe
CMW39280 Calitzdorp
CMW39281 Calitzdorp
CMW39282 AliwalNorth
CBS115177
CBS115499
N. protearum
CMW39292 Mokopane
CMW39293 Mokopane
CMW24699
CMW24700
Lasiodiplodia pseudotheobromae
CMW39290 CapeTown
CMW39291 Mokopane
CMW338120 Pretoria
CMW28625
L. theobromae
CMW39294 HagaHaga
CMW39295 HagaHaga
CMW39296 HagaHaga
CBS115812
CMW14077
L. gonubiensis
CMW36469 Pretoria
CMW36468 Pretoria
CMW39288 Mokopane
Diplodia allocellula
CMW39297 CapeTown
CMW36482 Pretoria
CBS121774
CMW25420
Phaeobotryosphaeria variabilis
CMW39267 Graaff-Reinet
CMW39269 Bloemhof
CMW39270 Bloemfontein
CMW39277 Honderklipbaai
CMW39268 Kuruman
CMW39276 Delaryville
CMW39279 Jansenville
CMW39278 CapeTown
CMW39275 Upington
CMW39273 Augrabies
CMW39274 BeaufortWest
CMW39271 Queenstown
CBS116742
CBS116743
Botryosphaeria dothidea
CMW36479 Pretoria
CMW36478 Bloemfontein
CMW36477 Bloemfontein
Tiarosporella urbis-rosarum
Pseudofusicoccum stromaticum CBS117448
Pseudofusicoccum stromaticum CBS117449
Mokopane
Pretoria
Delareyville
Kuruman
CapeTown
Bloemhof
Upington
Augrabies
Bloemfontein
Beaufort West
Graaff Reinet
Hondeklipbaai
Jansenville
Queenstown
Haga Haga
Calitzdorp
Wasbank
Robertson
Aliwal North

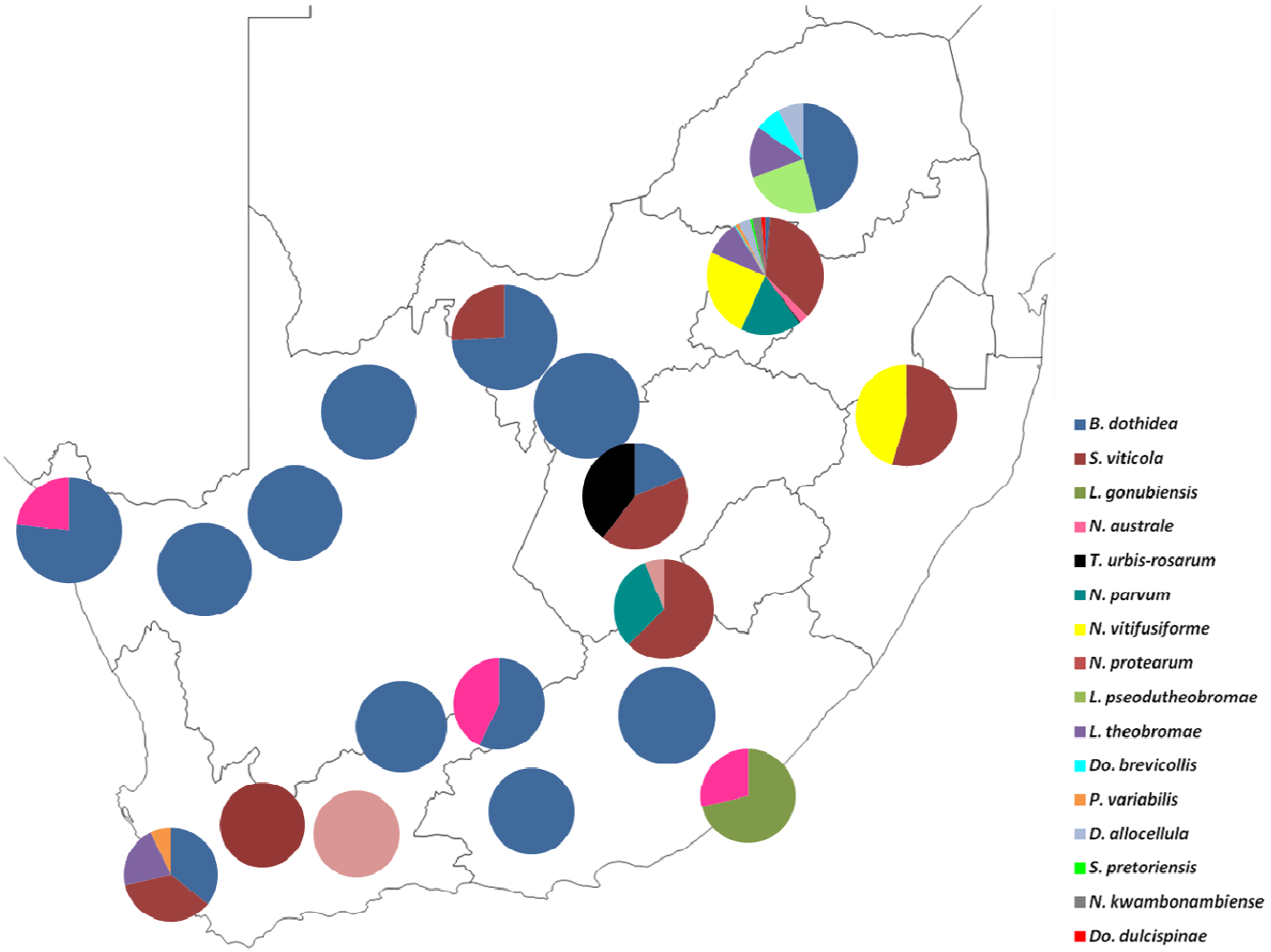


Figure 2. Species diversity of Botryosphaeriaceae in South Africa. Pie charts indicate the number of species and isolates percentage in each sampled site.

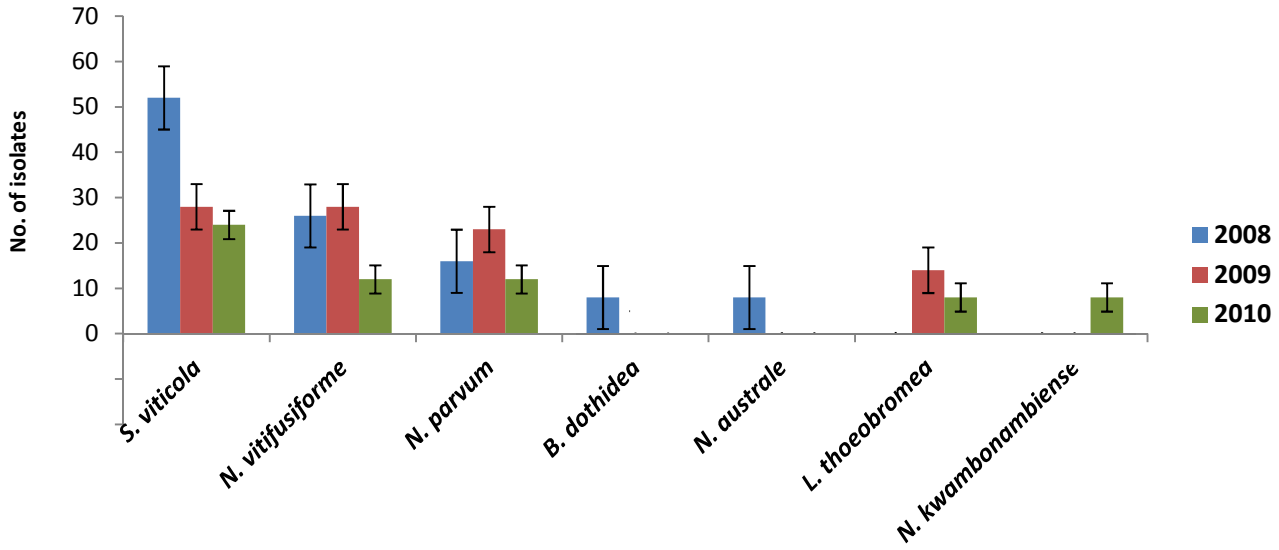


Figure 3. Species diversity of Botryosphaeriaceae on *Acacia karroo* for each year sampled in the greater Tshwane Metropolitan (Pretoria, Gauteng).

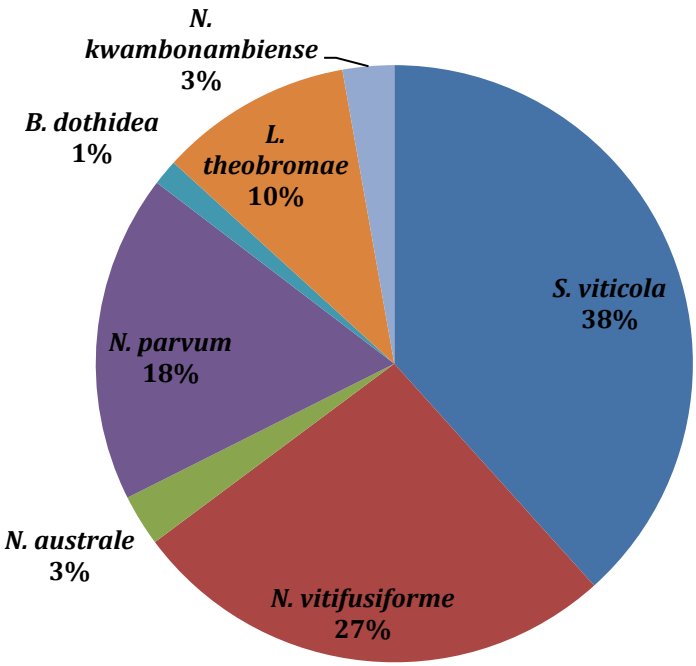


Figure 4. Total species and isolate number diversity of Botryosphaeriaceae on *Acacia karroo* in Tshwane areas between 2008-2010.

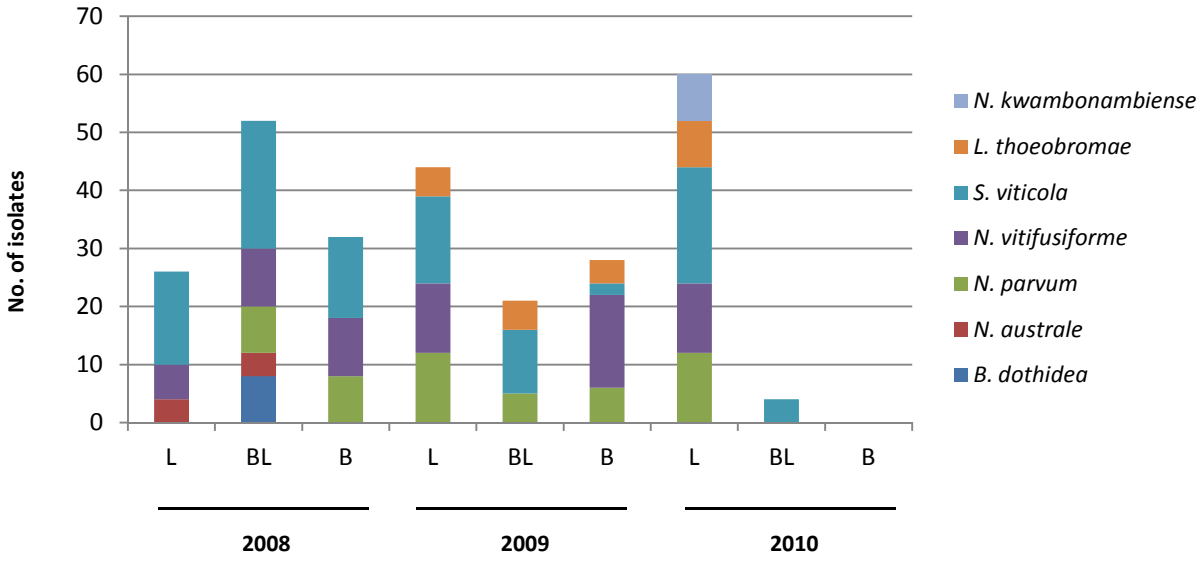


Figure 5. Species diversity of Botryosphaeriaceae on leaves (L), branchlets (BL) and branches (B) of *Acacia karroo* for 2008, 2009 and 2010.

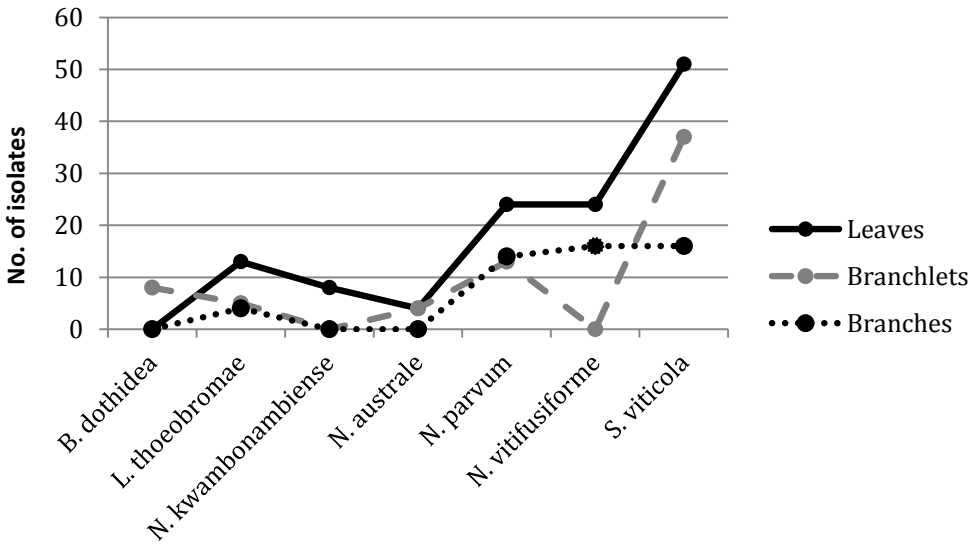


Figure 6. Species diversity of Botrysophaeriaceae on leaves, branchlets and branches of *Acacia karroo* in total of the three years sampling.

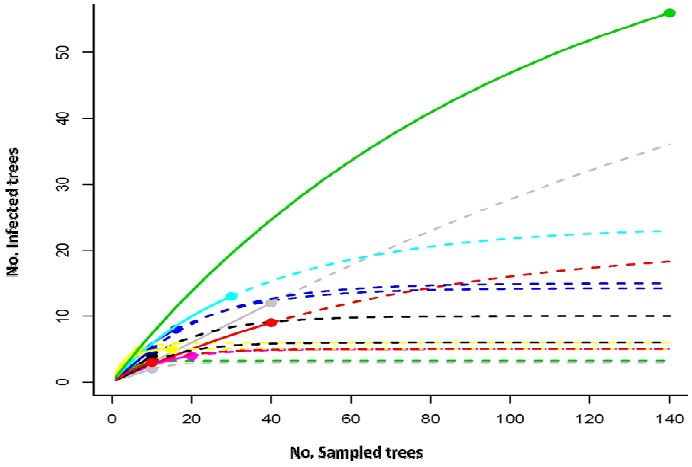


Figure 7. Rarefaction analysis for sampled trees and isolated species, demonstrating that the sampling intensity did not affect the species diversity as much as location did. Species diversity at sites such as Pretoria, Cape Town and Mokopane were generally higher than for the same number of samples at other sites.

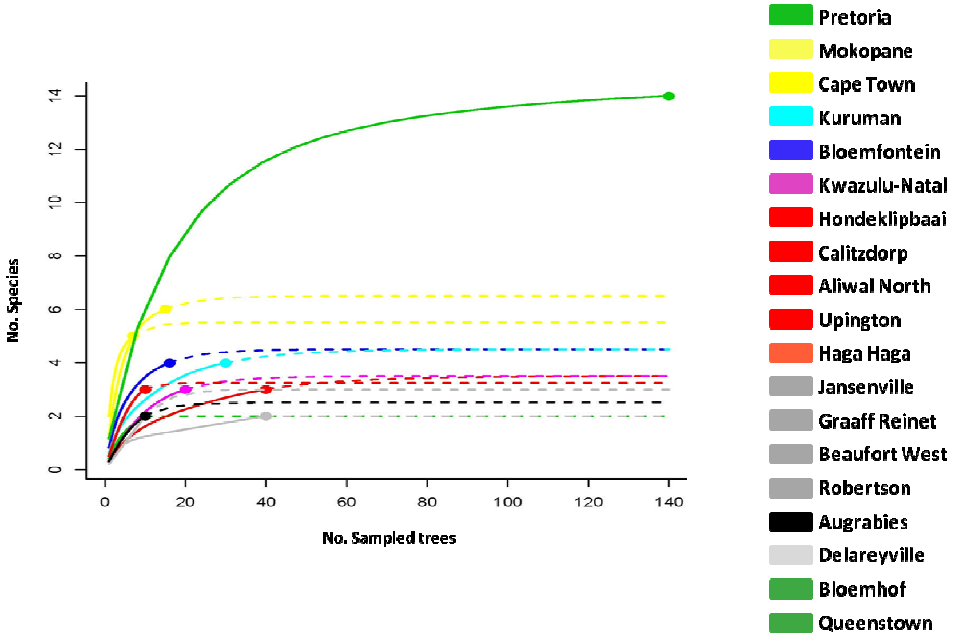


Figure 8. Rarefaction analysis for sampled trees and infected trees demonstrating that the sampling intensity did not affect the species diversity as much as location did. Trees at sites such as Pretoria more frequently yielded isolates for the same number of samples taken from other sites.

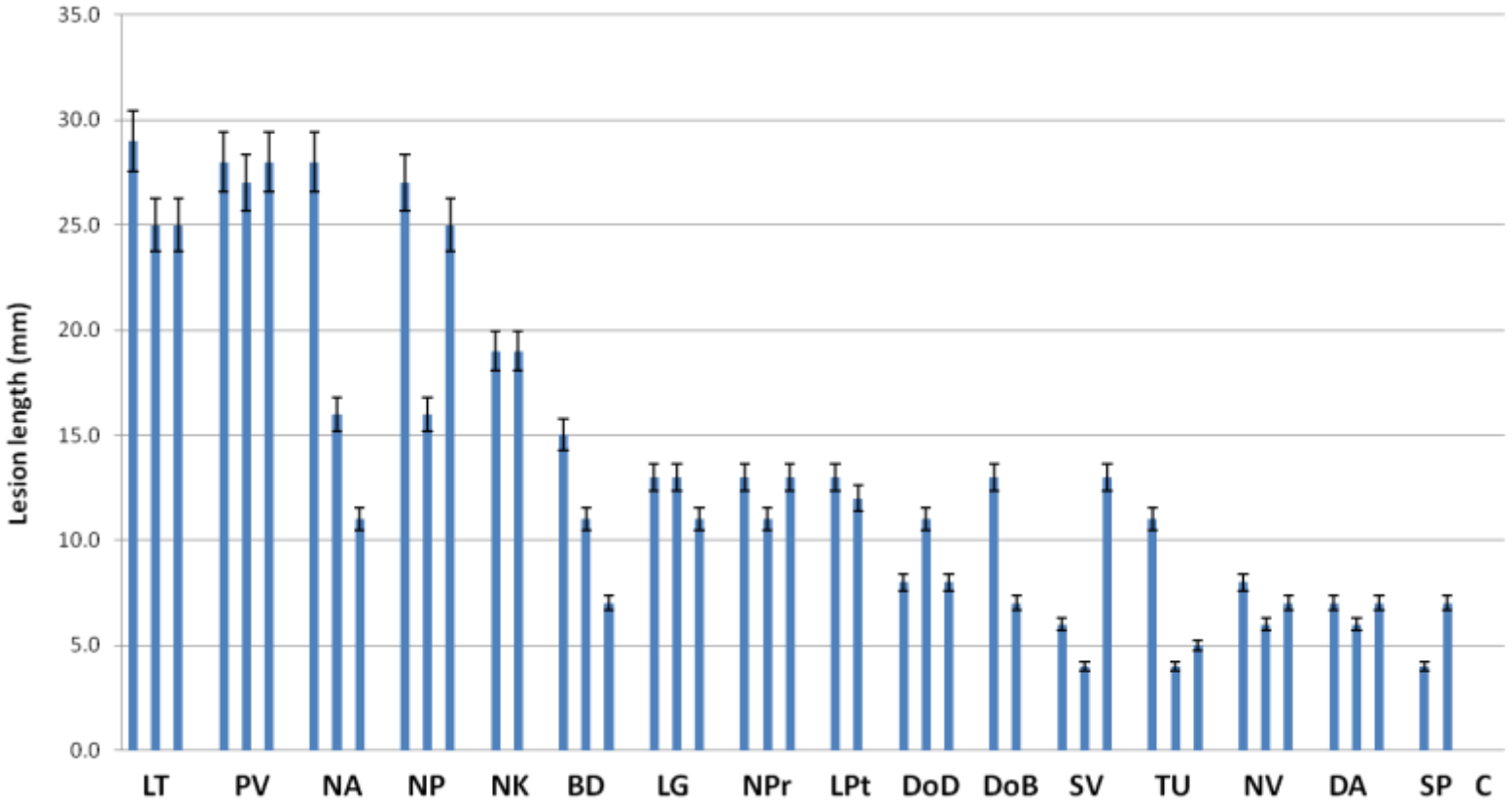


Figure 9. Mean lesion length (mm) for species of the Botryosphaeriaceae six weeks after inoculations on *Acacia karroo*. **LT:** *Lasiodiplodia theobromae*, **PV:** *Phaeobotryosphaeria variabilis*, **NA:** *Neofusicoccum australe*, **NP:** *N. parvum*, **NK:** *N. kwambonambiense*, **BD:** *Botryosphaeria dothidea*, **LG:** *L. gonubiensis*, **NPr:** *N. protearum*, **LPt:** *L. pseudotheobromae*, **DoD:** *Dothiorella dulcispinae*, **DoB:** *Do. brevicollis*, **SV:** *Spencermartinsia viticola*, **TU:** *Tiarosporella urbis-rosarum*, **NV:** *N. vitifusiforme*, **DA:** *Diplodia allocellula*, **SP:** *S. pretoriensis*, **C:** Control.

Chapter 5

Diversity and distribution of the Botryosphaeriaceae in South Africa and Namibia

1- INTRODUCTION

The Botryosphaeriaceae (Botryosphaeriales) occupy a wide range of niches and can be primary or opportunistic pathogens, endophytes or saprophytes. Botryosphaeriaceae species have a wide host range, including gymnosperms, monocotyledons and dicotyledons, and have been recovered from all continents sampled (Slippers and Wingfield 2007; De Wet et al. 2008). Endophytic Botryosphaeriaceae can be isolated commonly from a range of woody plants whereas non-woody hosts are normally not infected (Slippers and Wingfield 2007).

Numerous studies have been conducted on the Botryosphaeriaceae in South Africa and Namibia due to their importance as pathogens and prominence in plant tissues where endophytes are isolated from asymptomatic tissues. In fact, this family represents one of the best-studied groups of fungi in South Africa, with a number of other studies from other African countries adding related data and knowledge. This review aims to synthesize the information from various host and geographical reports of Botryosphaeriaceae from South Africa and Namibia. In order to place these findings in perspective, a review of the taxonomy, biology and pathology of the Botryosphaeriaceae is also included.

2. TAXONOMY AND SPECIES IDENTIFICATION

Botryosphaeria was described in 1863 with *Botryosphaeria dothidea* (= *Sphaeria dothidea*) as the type species (Slippers et al. 2004b; Cesati and Notaris 1863). Since then *Botryosphaeria* has been relegated to various different families and orders (Figure 1). Schoch et al. (2006) re-evaluated the systematic position of the Botryosphaeriaceae within the Dothideales and showed that the Botryosphaeriaceae grouped separately from the previously implied orders Pleosporales and Dothideales. Consequently, the Botryosphaeriales was introduced, incorporating only the Botryosphaeriaceae (Figure 1). Minnis et al. (2012) introduced the Planistromellaceae in the Botryosphaeriales, while Liu et al. (2012) pointed out that further division at the family level would most likely be justified.

Between 1863-1998, identification and classification of Botryosphaeriaceae relied heavily on morphological characters. Sexual state characters were commonly used, but sexual structures of these fungi (pseudothecia) structures are rare in nature, virtually unknown in culture and characters often overlap (Slippers et al. 2004b; Hanlin 1990). Characteristics of the asexual states were also widely used for taxonomic distinction of species in the Botryosphaeriaceae (Jacobs and Rehne 1998; Hanlin 1990). None of these morphological characteristics are, however, sufficiently informative for robust identification of species of this family, because of morphological variation within species and the limited fixed variation to be able to distinguish the myriad of species within the group (Crous et al. 2006; Pavlic et al. 2009).

Jacobs and Rehner (1998) were the first to use DNA sequence data to distinguish Botryosphaeriaceae species and to determine their phylogenetic relationships with each other. They combined anamorph morphology and DNA sequences of the nuclear rDNA internal transcribed spacers. That study provided connections between some asexual genera and *Botryosphaeria*, but was limited in representation of different genera. Denman et al. (2000) used asexual characters together with nuclear rDNA ITS sequence analyses and added further support to sub-divide *Botryosphaeria*-related taxa into two groups, namely those with *Diplodia*-like conidia (conidia mostly ovoid, pigmented, thick-walled) and *Fusicoccum*-like conidia (conidia mostly fusoid, hyaline, thin-walled). For some time after this change, the potential split of *Botryosphaeria* related taxa into dark, ellipsoid and hyaline, fusoid spored anamorphs was supported by the other studies (Zhou and Stanosz 2001; Slippers et al. 2004b; Alves et al. 2004), but as additional genera were characterised based on DNA sequence comparisons, it became clear that this distinction was not stable.

The early DNA sequence-based studies in the 1990's and early 2000's retained the view that the genera of the Botryosphaeriaceae were united under the sexual name *Botryosphaeria*. This was until a landmark study by Crous et al. (2006) that redefined the taxonomy of *Botryosphaeria sensu lato* based on DNA sequence data for the 28S rDNA large subunit region (LSU). The recognised phylogenetic clades in the Botryosphaeriaceae were supported by morphological differences in the asexual states. Hence, most genera were redefined and the names given were predominantly based on asexual names. The system of assigning asexual generic names to the new genera rather than continuing with the dual-name

system was followed to introduce a simplified, single name system for each new genus (Wingfield et al. 2012).

Increasingly, it became evident that a multigene approach was needed for species distinction and phylogenetic placement of genera in the Botryosphaeriaceae (Slippers et al. 2004b). Phillips et al. (2008) used a multi-gene approach (SSU, ITS, LSU, translation elongation factor 1-alpha and β-tubulin loci) to resolve the appropriate phylogenetic position of the dark-spored '*Botryosphaeria*' teleomorphs and related asexual species. Thus, *Diplodia* and *Lasiodiplodia* were recognised as separate genera and new dark-spored genera were described such as *Barriopsis* and *Spencermartinsia*. Other genera that were previously synonymised under *Botryosphaeria* were re-instated, including *Neodeightonia, Phaeobotryon* and *Phaeobotryosphaeria*. Subsequent studies added additional genera to the Botryosphaeriaceae, including *Aplosporella* (Damm et al. 2007b) and *Endomelanconiopsis* (Rojas et al. 2008). Currently, 29 genera have been linked through DNA sequence data to the Botryosphaeriaceae, most of which can also be defined based on morphological characteristics (Liu et al. 2012).

DNA sequence comparisons of several gene loci have been used to identify cryptic species of the Botryosphaeriaceae by applying a genealogical concordance phylogenetic species recognition concept (GCPSR) (De Wet et al. 2003; Pavlic et al. 2009; Slippers et al. 2004b). In addition to sequence data, additional molecular tools such as Simple Sequence Repeat (SSR) markers have been useful to provide support for distinction of cryptic species (Slippers et al. 2004a; Burgess et al. 2001). An extension of the GCPSR tool is the Genealogical Sorting Index (GSI), which has been used to clarify relationships between cryptic species based on DNA sequence comparisons (Cummings et al. 2008; Sakalidis et al. 2011b). For example, GCPSR has been applied for the *Neofusicoccum parvum*/*N. ribis* complex where five additional species have been identified using this tool (Pavlic et al. 2009; Begoude et al. 2010; Sakalidis et al. 2011b).

Molecular techniques other than DNA sequence comparisons have been employed to distinguish species of the Botryosphaeriaceae. These are usually applied to distinguish a defined group of species and not all known species. Such techniques include digestion of an SSR containing locus (*BotF15*) using the restriction enzyme *CfoI*, ribosomal DNA restriction

analyses (ARDRA), microsatellite-primed polymerase chain reaction (MSP-PCR) and repetitive-sequence-based polymerase chain reaction (rep-PCR) (Alves et al. 2005, 2007). These methods circumvent the need to sequence isolates, thus saving time and cost (Alves et al. 2007). However, they must be applied with care given the uncertainty that remains with regard to the diversity of Botryosphaeriaceae on large numbers of hosts and from many different geographical locations.

3. BIOLOGY AND PATHOLOGY

Species of Botryosphaeriaceae commonly reproduce sexually and asexually during their life cycles (Slippers and Wingfield 2007). This mixed reproductive strategy offers optimum evolutionary potential for the pathogen to exploit specific conditions by spreading well-adapted genotypes through asexual reproduction, or through recombination and generation of diversity to optimize opportunities for adaptation. Asexual reproduction, however, appears to be the most commonly encountered source of reproductive propagules for most species. It is also the predominant, if not only stage seen in culture. Whether these fungi are homothallic or heterothallic, or a mixture of these strategies is not known for most species.

Environmental conditions such as temperature and humidity influence conidial germination and infection (Michailides and Morgan 1992; Sutton and Arauz 1991; Amponsah et al. 2010). For example, *B. dothidea* develops on pistachio rapidly in the late spring and summer when maximum temperatures ranged from 21–40 ºC (Swart and Wingfield 1991). In contrast, *N. ribis* can perpetuate and proliferate rapidly in stems of *Melaleuca quinquenervia* under conditions with low temperatures, drought and defoliation (Rayachhetry et al. 1996). Furthermore, studies of Urbez-Torres et al. (2010) showed that conidia of Botryosphaeriaceae are able germinate over a wide temperature range.

The conidia and ascospores of the Botryosphaeriaceae are transmitted from one plant to another mostly by wind or rain splash (Úrbez-Torres et al. 2010; Epstein et al. 2008; Swart et al. 1987). Once on a host, these fungi can infect through wounds through natural openings

such as stomata. For example, *Neofusicoccum* spp. infect leaves of *Eucalyptus grandis* by germ tubes from conidia entering through the stomatal openings (Smith et al. 1996). Such infections have also been observed on leaves, rachis and shoots of pistachio (Michailides 1991). Once invasion has taken place, hyphae grow intra-cellularly in cortical parenchyma, callus parenchyma, xylem-ray parenchyma, vessels and tracheids, and the fungus can infect inter-cellularly in phloem fibers (Biggs and Britton 1988; Rayachhetry et al. 1996; Ploetz 2003). In late summer and early autumn, the xylem is blocked by hyphae and tyloses form in the vessels, causing restricted water flow, resulting in die-back and cankers on branches.

A small number of studies have suggested that the Botryosphaeriaceae species can be seed-borne. *Diplodia rosulata* has been isolated from healthy seeds of *Podocarpus falcatus* and *Prunus africana* (Gure et al. 2005), *Lasiodiplodia theobromae* from *Pinus elliottii* seeds (Cilliers et al. 1993) and *D. pinea* from *Pinus* spp. seeds (Burgess and Wingfield 2002). The seed-borne nature of these fungi had led to hypotheses that the global movement of the Botryosphaeriaceae around the world is facilitated by seeds. However, Bihon et al. (2011) showed that only 2.3% of *P. radiata* seeds were infected by *D. pinea* and importantly, that there is no transfer of *D. pinea* from seed to off-spring. In that study it was shown that seedlings grown in isolation were never infected, but seedlings grown in close proximity to infected adult trees were heavily infected. The transfer of infection is thus horizontal and not vertical (Bihon et al. 2011). Importation of infected seed during the course of the last 200 years into South Africa is thus believed to be the source of the high diversity of *D. pinea* in South Africa (Burgess et al. 2004b). A recent study of Bihon et al. (2012b), however, suggest that this high diversity could also have been influenced by a cryptic sexual cycle.

Diseases caused by the Botryosphaeriaceae are induced by stress conditions in the host. Stresses include those for example resulting from drought, frost damage, heavy snow, by hail or insect damage (Slippers and Wingfield 2007). Botryosphaeriaceae species have been linked to a wide range of symptoms, however, they are not specific to hosts or tissues or specific diseases. For example, *D. africana, N. parvum, L. theobromae, L. pseudotheobromae, L. egyptiacae, B. dothidea, N. luteum* and *N. mediterraneum* all produce die-back symptoms on a variety of woody plants (Linaldeddu et al. 2012; Iturritxa et al. 2011; Ploetz et al. 2009; Ismail et al. 2012; Mayorquin et al. 2012). *Botryosphaeria dothidea* is also known to cause post-harvest rots in avocado (Hartill 1991), fruit rot of olives (Phillips

et al. 2005) and other disease symptoms on fruits and nuts (Ko et al. 2011; Rao et al. 2011; Vasić et al. 2013; Zhuang et al. 2011). On pistachio, this pathogen causes a range of symptoms, from panicle shoot blight, wilting and drying of leaves and shrivelled fruit (Michailides 1991; Ma and Michailides 2002).

4- BOTRYOSPHAERIACEAE IN SOUTH AFRICA

The Botryosphaeriaceae is one of the best-studied fungal families in South Africa, occurring on both economically important crops and native plants. This is because woody plants often display high levels of colonization by these fungi as endophytes, irrespective of whether they are studied on agricultural crops, plantation forestry species or natural woody vegetation (Slippers and Wingfield 2007). Moreover, species of the Botryosphaeriaceae include important and dominant pathogens of native and non-native woody plants in South Africa, and elsewhere in the world. No Botryosphaeriaceae are known from grain crops in South Africa. Two species that were previously known as *Diplodia* spp. from maize are now known as *Stenocarpella maydis* and *S. macrospora* and based on DNA studies been relegated to the Diaporthaceae (Diaporthales) (Lamprecht et al. 2011; Crous et al. 2006).

Given the uncertainty regarding identifications of species in the Botryosphaeriaceae prior to DNA sequence comparisons, the views presented in the remaining part of this review are based predominantly on species for which DNA sequence comparisons have been conducted. This is particularly relevant where species are compared that are closely related, or where they are known to have been confused in the past. For example, many species were described under the name *B. dothidea* in the past (Crous et al. 2000; Slippers et al. 2004b). While recent studies have shown that this pathogen is present in South Africa, it is not the dominant species in most areas, and it is clear that numerous previous reports must have been referring to other species *Botryosphaeria* or *Neofusicoccum.*

Fifty-two species of Botryosphaeriaceae have been identified in previous studies in different parts of South Africa and Namibia from both native and non-native hosts (Table 1, Figure 2, 3). These include species clearly causing disease symptoms, those isolated from

disease symptoms, but not known to cause such symptoms, and also those isolated from asymptomatic tissues. *Neofusicoccum* was the most diverse genus identified in these studies, with 16 species being described from this genus. While *N. parvum* was found to infect the most diverse number of hosts, *B. dothidea* was the most common species in South Africa. In the following sections, we analyse these patterns of host association and distribution of Botryosphaeriaceae on native and non-native woody plants in South Africa in greater detail. Other than analyzing the patterns of diversity and distribution, an important objective is to consider knowledge gaps, particularly those relating to geographical distribution and host range.

4-1. Botryosphaeriaceae associated with diseases on forestry trees

The Botryosphaeriaceae pose an important threat for South African forestry. The commercial forestry area in South Africa consists of 1.3 million ha, of which 671 000 ha is planted to pine species (mainly *P. patula*, *P. elliottii*, *P. teada*), 516 407 ha to eucalypts (mostly *E. grandis* or derived hybrids and clones) and 100 606 ha to wattle (*Acacia mearnsii*) (Geldenhuys 1997; Http://www.forestry.co.za/statistical-data/ 2011). These plantations are important sources of structural timber, fuel wood, pulpwood and resin. Diseases associated with Botryosphaeriaceae are prominent in the South African forestry industry on all the genera planted and are responsible for significant economic losses.

Diplodia pinea is one of the most important die-back and canker pathogens on pine species worldwide, and this is especially true in South Africa (Swart and Wingfield 1991). The pathogen is particularly important due to its common occurrence, and latent, opportunistic nature where disease symptoms emerge after wounding and stress to the trees (Swart and Wingfield 1991). In infected areas, which include all of South Africa, this pathogen exists almost ubiquitously within healthy *Pinus* trees as an endophyte (Bihon et al. 2011). When the trees are weakened through wounding by hail, or due to drought or other forms of stress, die-back can be very rapid and dramatic. For example, *P. radiata* is a preferred forestry species, but is also one of the most susceptible *Pinus* species to *D. pinea*, and it is consequently no longer planted in large areas in South Africa where hail is common (Swart and Wingfield 1991).

Diplodia pinea together with its pine hosts has been introduced into South Africa. A number of studies have shown that *D. pinea* populations are highly diverse and must have been introduced many times (2012b; Bihon et al. 2012a; Burgess et al. 2004b; Smith et al. 2000). Bihon et al. (2010b) showed that this introduction is unlikely to have been predominantly by seed, and it must have been through seedling imports or other routes. Subsequent spread within the country is horizontal between established plants, and mostly via rain-splash and wind over relatively short distances (Úrbez-Torres et al. 2010; Epstein et al. 2008; Swart et al. 1987). A cryptic sister species, *D. scrobiculata*, also occurs in South Africa, but at a much lower frequency than *D. pinea* (Burgess et al. 2004a; Bihon et al. 2010).

Botryosphaeriaceae has been well studied on *Eucalyptus* spp. in many parts of the world, including South Africa (Maleme 2009; Pillay et al. 2013; Slippers et al. 2004c; Smith et al. 1996; Pavlic et al. 2007; Smith et al. 1994), Australia (Burgess et al. 2006; Slippers et al. 2004c; Taylor et al. 2009), Chile (Ahumada 2003), China (Chen et al. 2011), Colombia (Rodas et al. 2009), Congo (Roux et al. 2000), New Zealand (Billones-Baaijens et al. 2012), Spain (Iturritxa et al. 2011), Uganda (Nakabonge 2002), Uruguay (Pérez et al. 2008; Pérez et al. 2009) and Venezuela (Mohali et al. 2007). By 2009, at least 23 species of Botryosphaeriaceae had been recorded from *Eucalyptus* in different countries of the world (Slippers et al. 2009). Of these, eight species have been found on *Eucalyptus* spp. in South Africa. Many of these have been isolated from diseased plant tissue. For example, *N. australe*, *N. eucalyptorum, N. eucalypticola*, *N. parvum*, and *N. kwambonambiense* have been identified from die-back and stem cankers in South Africa (Maleme 2009; Slippers et al. 2004c; Smith et al. 1996). Other than *N. australe*, the remaining species are common on *Eucalyptus* in South Africa (Slippers et al. 2009; Pillay et al. 2013).

Several Botryosphaeriaceae species, including *L. theobromae, B. dothidea* and *Diplodia* spp., have been identified from both symptomatic and asymptomatic *A. mearnsii* in South Africa (Roux et al. 1997; Gibson 1975). More recent studies have also revealed the presence of a *Spencermartinsia* sp. (Van der Walt 2008), *N. australe, N. vitifusiforme* and *Phaeobotryosphaeria variabilis* on *A. mearnsii* in South Africa (Van der Linde et al. 2010). Pathogenicity trials have shown that *B. dothidea* and *N. australe* can be an important pathogens on *A. mearnsii* (Roux 1998; Van der Linde et al. 2010).

4-2. Botryosphaeriaceae associated with diseases on fruit trees

Botryosphaeriaceae are important pathogens of fruit trees. They cause disease symptoms on various plant tissues such as die-back and canker of branches and twigs, leaf necrosis, and post harvest decay. Various fruit trees in South Africa are affected, including peach, pear, almond, apple, plum, prune, nectarine and apricot and *Diplodia seriata*, *D. pinea*, *D. mutila*, *D. africana*, *S. viticola*, *N. australe*, *N. vitifusiforme*, *N. parvum*, *N. ribis*, *Aplosporella prunicola* and *L. plurivora* have been reported from die-back and canker of these fruit trees in the Western Cape Province (Slippers et al. 2007; Damm et al. 2007a). Among these species, *N. australe* and *D. seriata* are the most commonly encountered species on stone fruit in South Africa. All of the identified species have been shown to produce significant lesions in inoculation trials and are considered as potential pathogens in South Africa, however, *S. viticola* is considered less pathogenic than the other species on fruit trees in South Africa (Damm et al. 2007a). However, the ability of colonizing on wood may not always be directly related to the ability to cause the symptoms in the crown, as decline and die-back, and it should be more thoroughly explore in the future studies.

Botryosphaeriaceae diseases on grapevine have been well studied in South Africa (2004; Van Niekerk et al. 2010a; 2011a; Fourie and Halleen 2001; 2006). Species of Botryosphaeriaceae from grapevines include *B. dothidea*, *D. seriata*, *D. porosum*, *N. australe*, *N. luteum*, *N. parvum*, *N. ribis*, *L. crassispora*, *L. theobromae*, *N. vitifusiforme* and *N. viticlavatum*. Recent studies have shown that relative humidity, rainfall, temperature and wind play an important role in the dispersal of these fungi among grapevines in South Africa, thus contributing to disease development (van Niekerk et al. 2010b). Water stress and time of pruning also have an impact on the severity of Botryosphaeriaceae disease on grapevine (2011b; Van Niekerk et al. 2011a), for example late winter pruning wounds increase susceptibility to *N. australe* compared to early season pruning.

4-3. Botryosphaeriaceae on native hosts in South Africa

Canker and die-back symptoms typical of diseases caused by Botryosphaeriaceae have commonly been reported from native trees in South Africa. These have, however, received little attention until about a decade ago when the South African government funded

an initiative (Centre of Excellence in Tree Health Biotechnology) to specifically study the health of native trees (Steenkamp and Wingfield 2013). Subsequently a number of relatively intensive studies have been completed on Botryosphaeriaceae on native trees. In total, 41 species of Botryosphaeriaceae have been characterised from these studies and these have been from both symptomatic and asymptomatic tissues of the 22 native trees studied (Table 1). The role of many of these species in causing diseases is not known, but in those cases where pathogenicity studies have been conducted, most species appear to be pathogenic to a greater or lesser degree. Given this uncertainly, we consider all the species described from native hosts in this section as potential pathogens.

Seventeen species of the Botryosphaeriaceae have been reported from diseased tissue taken from native trees in South Africa. Botryosphaeriaceae associated with disease symptoms (canker and die-back) of native trees in South Africa include eight species (*B. dothidea, N. australe, N. parvum, N. ribis, N. luteum, N. mangiferae, L. theobromae* and *L. gonubiensis*) from *Syzygium cordatum* (Pavlic et al. 2007), two species (*D. pseudoseriata* and *L. pseudotheobromae*) from *Pterocarpus angolensis* (Mehl et al. 2011), three species (*L. pseudotheobromae, L. theobromae* and *N. parvum*) from *Terminalia catappa* (Begoude et al. 2010), three species (*Dothiorella dulcispinae, S. viticola* and *S. pretoriensis*) from *Acacia karroo* (Jami et al. 2013a), one species (*N. parvum*) from *Heteropyxis natalensis*, two species (*N. protearum* and *N. australe*) from both *Leucadendron* spp. and *Protea* spp. (Marincowitz et al. 2008; Denman et al. 2003), three species (*Neofusicoccum* sp.nov1., *N. parvum*, *N. kwambonambiense*) from *Afrocarpus falcatus* (Ndove 2013) and four species (*B. dothidea, Neofusicoccum* sp.nov2., *N. eucalypti*, *N. kwambonambiense*) from *Podocarpus* spp. (Ndove 2013). While all these species have been reported from diseased tissue, some might be associated as secondary pathogens or as saprophytic colonists of the dead material.

Inoculation studies have been conducted only on *S. cordatum, Pt. angolensis, T. catappa, A. karroo, Podocarpus henkelii* (Pavlic et al. 2007; Mehl et al. 2011; Jami et al. 2013a; Begoude et al. 2010; Ndove 2013). These studies showed that all the inoculated species including *B. dothidea, D. pseudoseriata, Do. dulcispinae, L. theobromae, L. gonubiensis, L. pseudotheobromae, Neofusicoccum* sp.nov1., *Neofusicoccum* sp.nov2., *N. australe, N. parvum, N. ribis, N. luteum, N. mangiferae, N. kwambonambiense, N. eucalypti, S. viticola* and *S. pretoriensis* were capable of causing lesions. Although they might thus be

considered as potential pathogens, their infection biology, particularly under natural conditions is not understood. Some give rise to very small lesions and may play very small or no role in disease development. The most aggressive species across these studies were *L. pseudotheobromae, L. theobromae, N. parvum* and *N. batangarum* on *T. catappa* (Begoude et al. 2010), *N. mangiferae* on *S. cordatum* (Pavlic et al. 2007), *L. pseudotheobromae* on *Pterocarpus* (Mehl et al. 2011), *Do. dulcispinae* on *A. karroo* (Jami et al. 2013a) and *Neofusicoccum* sp.nov2. on *Pt. henkelii* (Ndove 2013).

A total of 29 species of Botryosphaeriaceae have been isolated as endophytes on native plants in South Africa and Namibia. These species include seven species from the Proteaceae (e.g., *Leucadendron*, *Leucospermum* and *Protea*) (Denman et al. 2003; Marincowitz et al. 2008), 18 species from *Acacia* species (*Aplosporella africana, A. papillata, B. dothidea, Do. brevicollis, Do. oblonga, D. allocellula, D. pseudoseriata, Fusicoccum avasmontanum, L. pyriformis, L. theobromae, N. australe, N. parvum, N. kwambonambiense, N. vitifusiforme, Ph. variabilis, S. viticola, S. rosulata, Tiarosporella urbis-rosarum*) (Slippers et al. 2013; Jami et al. 2013a, 2012; 2013b), four species from *Celtis africana* (*A. javeedii, N. kwambonambiense, S. viticola, T. africana*), two species from *Gymnosporia buxifolia* (*N. parvum* and *S. viticola*), one species from *Searsia lancea* (*T. africana*) (Jami et al. 2013b), four species from *Pt. angolensis* (*F. atrovirens, L. theobromae, Pseudofusicoccum olivaceum* and *Ps. violaceum*) (Mehl et al. 2011) and nine species from *Podocarpus* spp. (*D. mutila, D. seriata, N. parvum, N. ursorum, S. viticola, N. eucalypti, L. gilanensis, N. crypto-australe* and *N. protearum*) (Ndove 2013). Many of these species are also known as pathogens, either on these same hosts or on other hosts. *Neofusicoccum parvum* for example is a common and well-known pathogen on numerous hosts, but is also a common endophyte in many of these studies.

There is clearly a large number of Botryosphaeriaceae associated with native plants. Whether they are all involved in causing the canker and die-back diseases observed on these plants is not clear. What is obvious, however, it is that a complex of species is typically involved in causing these diseases. In none of the studies mentioned was a single species exclusively associated with diseased tissue. Six species that appear to be amongst the most important pathogens in these systems include *B. dothidea, L. theobromae, N. australe, N. parvum, N. kwambonambiense* and *S. viticola*. These species were particularly common and

have been in found in both healthy and diseased tissues. The majority of the remaining 41 species of Botryosphaeriaceae associated with native trees were isolated from asymptomatic plant tissue.

5. HOST AND GEOGRAPHICAL STRUCTURE OF BOTRYOSPHAERIACEAE DIVERSITY IN SOUTH AFRICA

As should be clear from the studies mentioned above, the numbers of species of Botryosphaeriaceae from a variety of hosts in South Africa and Namibia is growing. Reports of these fungi normally focus on individual hosts, specific disease problems, or defined geographic areas. When considered collectively, these reports provide an ideal opportunity to consider patterns of host and geographic distribution for this group of fungi in southern Africa. Such analyses have been done for very few fungi, simply because records are typically not sufficiently complete.

5-1. Host association of the Botryosphaeriaceae

In total, 52 Botryosphaeriaceae species have been isolated from 54 hosts in South Africa and Namibia (Table 1; Figure 1). Many of these were newly described during the last 10 years. In the last decade, seven Botryosphaeriaceae species have been described as new taxa from non-native hosts and 26 from native hosts in South Africa and Namibia. These species includ *A. prunicola, D. africana, L. plurivora, N. eucalyptorum, N. crypto-australe, N. ursorum* and *N. viticlavatum* from non-native hosts, and *A. africana, A. javeedii, A. papillata, D. allocellula, Do. brevicollis, Do. dulcispinae, Do. oblonga, F. atrovirens, F. avasmontanum, L. gonubiensis, L. pyriformis, Neofusicoccum* sp. nov.1, *Neofusicoccum* sp. nov.2, *N. cordaticola, N. kwambonambiense, N. mangiferum, N. protearum, N. umdonicola, Ph. variabilis, Ps. olivaceum, Ps. violaceum, S. capri-amissi, S. rosulata, S. pretoriensis, T. africana* and *T. urbis-rosarum* from native trees (Figure 1, Table 1) (Maleme 2009; Van der Walt 2008; Pavlic et al. 2004; Denman et al. 2003; Jami et al. 2013b; Jami et al. 2012; Mehl et al. 2011; Pavlic et al. 2009). Among the newly described species, *N. kwambonambiense*

and *N. protearum*, occurred on the largest number of hosts, namely six and seven native trees, respectively. All the other species were reported from only one or two hosts (Table 1).

All results show that Botryosphaeriaceae species diversity on native hosts is higher (41 species) than non-native hosts (16 species) (discussed above). However, this could be due to a broader geographical sampling on native trees than non-native trees in South Africa and Namibia. Some of these species are only known from native or non-native trees while some of them have been found to infect both native and non-native hosts. Eleven of the Botryosphaeriaceae species (*B. dothidea, D. mutila, D. seriata, L. theobromae, N. australe, N. crypto-australe, N. kwambonambiense, N. parvum, N. ursorum, N. vitifusiforme* and *S. viticola*) have been isolated from both native and none-native trees in South Africa and Namibia (Figure 1, Table 1). Most of these species are those with an almost cosmopolitan distribution and very broad host ranges in general.

The number of hosts from which each Botryosphaeriaceae species has been reported varies significantly. Twenty-eight species have been found on a single host (Figure 4). Many of these species are, however, apparently rare and have been isolated only in low numbers. Their host records might thus be influenced by their rare occurrence, and not necessarily truly reflect their ability to infect other plants. The remaining species of Botryosphaeriaceae in South Africa and Namibia have been obtained from two or more hosts, of which seven species have been isolated from more than three hosts. *Neofusicoccum parvum* has been found from 23 hosts (9 natives, 14 non-natives) and shows the highest host diversity in South Africa and Namibia. A recent study by Sakalidis et al. (2013) reported this species from 90 hosts in many parts of the world. These authors speculated that it might be native to South Africa given its population diversity and common occurrence on native hosts in this region. *Neofusicoccum australe* and *B. dothidea* are the next most host commonly encountered species, and have been reported from 11 (3 natives, 8 non-natives) and 10 (5 natives, 5 non-natives) different hosts, respectively (Table 1, Figure 4). Interestingly, *N. parvum* and *N. australe* have been found on greater numbers of non-native than native plants in South Africa and Namibia (Table 1).

Of the 54 host plants considered in South Africa and Namibia, 24 were infected with only one species of Botryosphaeriaceae while the remaining trees were hosts to more than

one species. *Acacia karroo* with 18 Botryosphaeriaceae species showed the highest diversity of Botryosphaeriaceae in South Africa and Namibia (Slippers et al. 2013; Jami 2013). *Syzygium cordatum* (Pavlic et al. 2007; Pavlic et al. 2004), *A. mellifera* (Slippers et al. 2013), *P. henkelii* (Ndove 2013) and *Vitis vinifera* (Van Niekerk et al. 2004) have been recorded to be infected by 12, 11, 9 and 9 different Botryosphaeriaceae species, respectively. These data should also be viewed against the greater intensity and breadth of geographical sampling of these five hosts. The data however, illustrate the extent to which a host plant can be infected by multiple species of Botryosphaeriaceae. If sampled more intensively, the same pattern might be expected for other hosts.

5-2. Geographic distribution of Botryosphaeriaceae

The Botryosphaeriaceae occur throughout southern Africa, and while some species occur fairly broadly, others appear to have a limited geographic distribution. It is necessary to be cautious not to over interpret the results of studies thus far conducted, as obvious gaps clearly still exist. But the record of occurrence is becoming sufficiently complete to begin to understand some of the emerging patterns of diversity of Botryosphaeriaceae, and to consider how these might be related to climate or other factors shaping their distribution.

Botryosphaeria dothidea is one of the most widespread Botryosphaeriaceae species and has been found from 17 sites in different parts of the country (Figure 4). This species has been found across parts of South Africa and Namibia with climates that can be described as Mediterranean, to continental to sub-tropical and very dry and desert-like (Http://en.wikipedia.org/wiki/Climate_of_South_Africa 2007; Conradie 2012). This is perhaps not surprising given that recent studies have shown that *B. dothidea* can survive at a broad range of temperatures (Urbez-Torres et al. 2010; Copes and Hendrix Jr 2004; Michailides and Morgan 1992). Its pycnidia appear to be long-lived and produce conidia for at least 6 years. As with other species of Botryosphaeriaceae, rain and wind play an important role in spreading *B. dothidea*, which limits the distance of its distribution. The broad host range, however, would mean that it has a more or less continuous distribution of potential hosts that would make migration between regions possible (Ahimera et al. 2004; van Niekerk et al. 2010b).

Neofusicoccum parvum is the second most widespread species, being reported from 11 sites across South Africa (Figure. 4). This fungus has the widest global distribution and host range and it is one of the most pathogenic Botryosphaeriaceae on woody plants (Slippers and Wingfield 2007). *Neofusicoccum parvum* was absent from samples collected in the western part of South Africa. It is unlikely that this is host related, because the hosts sampled in that part of the country are infected by this fungus in other regions. This is also the driest part of the country (Conradie 2012) and it is possible that *N. parvum* requires higher levels of humidity or amounts of free water for sporulation, germination and survival of its spores. Humidity has been linked to germination of *N. parvum* spores (Thomidis et al. 2011; van Niekerk et al. 2010b; Úrbez Torres and Gubler 2011), but this is also true for *B. dothidea*, and it is probably true for all species. The differences in survival must, therefore, be due to more subtle interactions between this, or other, environmental factors affecting the fitness of the species in the area.

Neofusicoccum australe has been reported from 10 countries and 46 hosts and is particularly common in the southwest of Western Australia (Sakalidis et al. 2011a; Burgess et al. 2006; Taylor et al. 2009). This species is also one of the dominant Botryosphaeriaceae in South Africa (Slippers et al. 2009; Slippers et al. 2004c). It is the most widespread species in the country after *B. dothidea* and *N. parvum*, being reported from nine sites (Figure 3 and 4). This species has been found in both desert and temperate areas of South Africa. In parts of Western Australia, it is common in areas with a Mediterranean climate, having hot and dry summers and cool wet winters (Burgess et al. 2006; Cunnington et al. 2007; Taylor et al. 2009). Sakalidis et al. (2011a) showed that environmental factors are most likely driving the speciation of *N. australe* rather than host associations.

Lasiodiplodia theobromae has been shown to be an important species in South Africa (Figure 3, 4). This fungus has a wide host range both as endophyte and pathogen (Slippers and Wingfield 2007). It has a world-wide distribution (Punithalingam 1976) and has been reported on more than 500 hosts (Punithalingam 1980), although many of these host reports might refer to cryptic sister species of *L. theobromae*. Recent studies in South Africa have shown that *L. theobromae* occurs mostly in northern and western parts of the country. (Figure. 3), and not in central and eastern parts (Mehl et al. 2011; Begoude et al. 2010; Pavlic

et al. 2007; Van Niekerk et al. 2004; Jami 2013). These patterns are likely due to climate as *L. theobromae* is known to be confined, or most common, in tropical to sub-tropical climates.

The greatest species diversity for the Botryosphaeriaceae has been observed in the Western Cape Province with 17 different species occurring there. The lowest diversity is currently known from the Northern Cape Province (Figure. 3). This might be related to the number of studies in these areas, where most studies focus in the Western Cape or eastern to north-eastern coast of the country, and very few studies have been done in more central or north-western areas. It could also be associated with the greater plant diversity and density in the Western Cape, compared to for example the Northern Cape, as well as the harsher (drier) weather conditions. However, the recent studies only on *A. karroo* in South Africa (Jami et al. 2013a; Jami 2013) showed that the species diversity was higher in the Tshwane Metropolitan Area than elsewhere in South Africa, even after correcting for sampling intensity. It is not clear what drives this increased diversity but one possibility is that the higher plant diversity in the urban areas (introduced for horticulture) together with the higher frequency of trade (with Pretoria as one of the main urban centres in the country) compared to other sampled sites could influence the surrounding diversity in Botryosphaeriaceae. Other factors, such as the intense human activities, pollution and physical damage to name just a few could also influence this pattern of diversity.

A number of recent studies illustrate a high level of anthropogenic movement of Botryosphaeriaceae species around the world (Pavlic et al. 2009; Slippers 2003; Slippers and Wingfield 2007; Sakalidis et al. 2013). For example, Sakalidis et al. (2013) showed that *N. parvum* exist across the six continents and 29 countries from 90 hosts. This result strongly shows human mediated movement of *N. parvum* whether, it has been moved from native to non-native hosts or vice-versa. Similarly, *B. dothidea, D. pinea, L. theobromae* and *N. australe* reported commonly in South Africa, have more or less cosmopolitan distributions, and have most likely been moved by humans. The results of studies in South Africa show how widely such species can spread once introduced into a country, including into remote and fairly undeveloped regions such as the Northern Cape. It illustrates the fact that current quarantine measure are completely unable to account for the movement of latent pathogens such as the Botryosphaeriaceae.

6. CONCLUSIONS

The application of molecular tools and particularly DNA sequence based analyses has revolutionized the identification of species in the Botryosphaeriaceae. These data have led to a complete revision of the taxonomy of the group, and a necessity to re-evaluate previous reports of species, distributions, host associations and other factors relevant to understanding the processes influencing the biodiversity patterns. It has also led to the discovery of large diversity of species, and new insights into its distribution and host associations. This is especially true in South Africa where these fungi have been sampled as much, or more intensively than in many other parts of the world.

During the last two decades, focused studies on the Botryosphaeriaceae have revealed more than 33 new species and genera from South Africa, and the host and geographical reports of numerous other species. These fungi originated from various disease symptoms on important native and non-native trees in South Africa. A large number of species have also been isolated from healthy plant tissues. Whether isolated from healthy or diseased tissue, all species that have been tested in inoculation trials have been shown to result in lesions. However, this ability is very variable for different species.

Studies on the Botryosphaeriaceae in South Africa are beginning to reveal a number of interesting general patterns related to host and geographic distribution. Overall, they have shown that the Botryosphaeriaceae species diversity is greater on native hosts than none-native hosts in South Africa and Namibia. However, more intense sampling is needed, both on native and non-native host plants in some regions. Species distribution patterns have shown that diversity is greater in temperate than dry areas. But it remains unknown whether the greater diversity of Botryosphaeriaceae in some area is due to host factors or because sampling has been more intense there.

The worldwide distribution of Botryosphaeriaceae that are known to be serious pathogens, and their broad host and geographic distribution in South Africa, highlights an important issue relating to the quarantine of these fungi. Because they occur as endophytes, in virtually all woody plants, we have to accept that the billions of plants being shipped around the world annually are also bringing with them large numbers of Botryosphaeriaceae.

As illustrated in this review, these fungi are then able to spread and infect native plants, even in remote areas. The consequences of this movement are very poorly understood. But at least some of the indications of the involvement of these cosmopolitan species in climate related disease outbreaks (Piskur et al. 2011; Desprez-Loustau et al. 2007) should be reason for serious concern. Furthermore they justify continued investigations into the local and global patterns of host and geographic distribution of Botryosphaeriaceae diversity.

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Table1. Botryosphaeriaceae associated with southern African native and non-native trees

Host	Fungus	Symptoms/Plant tissue	Sampled area	Reference
Acacia erioloba	*Aplosporella papillata*	Not available	Northern Cape, SA	Slippers et al. 2013
A. erioloba	*Spencermartinsia Capri-amissi*	Not available	Gauteng, Northern Cape, SA	Slippers et al. 2013
A. hebeclada	*Phaeobotryosphaeria variabilis*	Not available	Windhoek, Namibia	Slippers et al. 2013
A. karroo	*Botryosphaeria dothidea*	Healthy branches & leaves	Gauteng , SA	Jami 2013
A. karroo	*Botryosphaeria dothidea*	Not available	Windhoek, Namibia	Slippers et al. 2013
A. karroo	*Diplodia allocellula*	Healthy branches	Gauteng, SA	Jami et al. 2012
A. karroo	*Diplodia pseudoseriata*	Healthy leaves	Gauteng, SA	Jami 2013
A. karroo	*Dothiorella brevicollis*	Healthy & die-back branches	Gauteng, SA	Jami et al. 2012, 2013a
A. karroo	*Dothiorella dulcispinae*	Die-back	Gauteng, SA	Jami et al. 2012, 2013a
A. karroo	*Lasiodiplodia gonubiensis*	Symptomless	Western Cape, SA	Jami F 2013
A. karroo	*Lasiodiplodia pseudotheobromae*	Symptomless	Limpopo, SA	Jami F 2013
A. karroo	*Lasiodiplodia theobromae*	Healthy leaves	Gauteng, SA	Jami F 2013
A. karroo	*Neofusicoccum australe*	Healthy leaves	Gauteng, SA	Jami F 2013
A. karroo	*Neofusicoccum kwambonambiense*	Healthy branches & leaves	Gauteng, SA	Jami 2013
A. karroo	*Neofusicoccum parvum*	Healthy leaves	Gauteng, SA	Jami F 2013
A. karroo	*Neofusicoccum protearum*	Symptomless	Western Cape, Eastern Cape, SA	Jami F 2013
A. karroo	*Neofusicoccum vitifusiforme*	Healthy branches & leaves	Gauteng, SA	Jami F 2013
A. karroo	*Phaeobotryosphaeria variabilis*	Healthy branches	Gauteng, SA	Jami et al. 2013a
A. karroo	*Spencermartinsia pretoriensis*	Die-back	Gauteng, SA	Jami et al. 2012, 2013a

A. karroo	*Spencermartinsia rosulata*	Not available	Northern Cape, SA, Windhoek, Namibia	Slippers et al. 2013
A. karroo	*Spencermartinsia viticola*	Healthy leaves, branches & die-back	Gauteng, SA	Jami et al. 2013a
A. karroo	*Tiarosporella urbis-rosarum*	Healthy branches	Gauteng, Free state, SA	Jami et al. 2012
A. mearnsii	*Aplosporella* sp.	Canker	KwaZulu-Natal, Southern Africa	Roux & Wingfield 1997
A. mearnsii	*Botryosphaeria dothidea*	Canker	KwaZulu-Natal, Southern Africa	Roux & Wingfield 1997
A. mearnsii	*Neofusicoccum vitifusiforme*	Not available	SA	Van der Linde 2009
A. mellifera	*Aplosporella africana*	Not available	Dordabis, Namibia	Slippers et al. 2013
A. mellifera	*Botryosphaeria dothidea*	Not available	Dordabis, Namibia	Slippers et al. 2013
A. mellifera	*Dothiorella dulcispinae*	Not available	Namibia	Slippers et al. 2013
A. mellifera	*Dothiorella oblonga*	Not available	Guateng Province, SA	Slippers et al. 2013
A. mellifera	*Fusicoccum avasmontanum*	Not available	Windhoek, Namibia	Slippers et al. 2013
A. mellifera	*Lasiodiplodia pseudotheobromae*	Not available	Rundu, Namibia	Slippers et al. 2013
A. mellifera	*Lasiodiplodia pyriformis*	Not available	Dordabis, Namibia	Slippers et al. 2013
A. mellifera	*Neofusicoccum* sp.nov2	Not available	Rundu, Namibia	Slippers et al. 2013
A. mellifera	*Phaeobotryosphaeria variabilis*	Not available	Northern Cape, SA	Slippers et al. 2013
A. mellifera	*Spencermartinsia rosulata*	Not available	Gauteng, Northern Cape, SA	Slippers et al. 2013
A. mellifera	*Spencermartinsia viticola*	Not available	Gauteng, SA	Slippers et al. 2013
A. tortillas	*Aplosporella papillata*	Not available	Northern Cape, SA	Slippers et al. 2013
A. tortillas	*Spencermartinsia rosulata*	Not available	Northern Cape, SA	Slippers et al. 2013
Afrocarpus falcatus	*Neofusicoccum kwambonambiense*	Die-back	Mpumalanga, SA	Ndove L 2013

A. falcatus	*Neofusicoccum parvum*	Die-back	Mpumalanga, SA	Ndove L 2013
A. falcatus	*Neofusicoccum* sp.nov1	Die-back	Gauteng, SA	Ndove L 2013
Celtis africana	*Aplosporella javeedii*	Healthy branches	Gauteng, SA	Jami et al. 2013b
C. africana	*Neofusicoccum kwambonambiense*	Healthy branches	Gauteng, SA	Jami et al. 2013b
C. africana	*Spencermartinsia viticola*	Healthy branches	Gauteng, SA	Jami et al. 2013b
C. africana	*Tiarosporella africana*	Healthy branches	Gauteng, SA	Jami et al. 2013b
Cupressus lusitanica	*Diplodia pinea*	Canker	Mpumalanga, SA	Linde et al. 1997
Eucalyptus citriodora	*Botryosphaeria dothidea*	Symptomless	Gauteng, SA	Maleme 2009
E. dorrigoensis	*Botryosphaeria dothidea*	Symptomless	Gauteng, SA	Maleme 2009
E. dorrigoensis	*Neofusicoccum parvum*	Symptomless	Gauteng, SA	Maleme 2009
E. grandis	*Neofusicoccum australe*	Symptomless	KwaZulu-Natal, SA	Pillay et al 2013
E. grandis	*Neofusicoccum eucalyptorum*	Canker & die-back	Mpumalanga, SA	Smith et al. 2001
E. grandis	*Neofusicoccum kwambonambiense*	Symptomless	KwaZulu-Natal, SA	Pillay et al 2013
E. grandis	*Neofusicoccum parvum*	Canker &die-back	Mpumalanga, SA	Smith et al. 2001; Slippers et al. 2004b
E. microcorvs	*Botryosphaeria dothidea*	Symptomless	Gauteng, SA	Maleme 2009
E. microcorvs	*Neofusicoccum parvum*	Symptomless	Gauteng, SA	Maleme 2009
E. nicholii	*Neofusicoccum parvum*	Symptomless	Gauteng, SA	Maleme 2009
E. nitens	*Neofusicoccum eucalyptorum*	Canker & die-back	Mpumalanga, SA	Smith et al. 2001
E. ovata	*Neofusicoccum parvum*	Symptomless	Gauteng, SA	Maleme 2009
E. robusta	*Neofusicoccum parvum*	Symptomless	Gauteng, SA	Maleme 2009

E. saligna	*Neofusicoccum parvum*	Symptomless	Gauteng, SA	Maleme 2009
E. scoparia	*Neofusicoccum parvum*	Symptomless	Gauteng, SA	Maleme 2009
E. smithii	*Neofusicoccum parvum*	Canker & die-back	Mpumalanga, SA	Smith et al. 2001; Slippers et al. 2004b
Eucalyptus sp.	*Neofusicoccum eucalypti*	Symptomless	Gauteng, SA	Maleme 2009
Eucalyptus sp.	*Neofusicoccum eucalyptorum*	Not available	SA	Slippers et al., 2004
Eucalyptus spp.	*Botryosphaeria dothidea*	Symptomless	Gauteng, SA	Maleme 2009
Eucalyptus spp.	*Neofusicoccum crypto-australe*	Symptomless	Gauteng, SA	Maleme 2009
Eucalyptus spp.	*Neofusicoccum parvum*	Symptomless	Gauteng, SA	Maleme 2009
Eucalyptus spp.	*Neofusicoccum ursorum*	Symptomless	Gauteng, SA	Maleme 2009
E. tereticornis	*Neofusicoccum parvum*	Symptomless	Gauteng, SA	Maleme 2009
Gymnosporia buxifolia	*Neofusicoccum parvum*	Healthy branches	Gauteng, SA	Jami et al. 2013b
G. buxifolia	*Spencermartinsia viticola*	Healthy branches	Gauteng, SA	Jami et al. 2013b
Heteropyxis natalensis	*Neofusicoccum parvum*	Canker & die-back	KwaZulu-Natal, SA	Denman et al. 2003
H. natalensis	*Neofusicoccum protearum*	Canker & die-back	Western Cape, SA	Denman et al. 2003
Leucadendron laureolum X L. Salignum	*Neofusicoccum protearum*	Canker & die-back	Western Cape, SA	Denman et al. 2003
Malus domestica	*Neofusicoccum australe*	Diseased tissues	Western Cape, SA	Slippers et al. 2007
Malus sp.	*Diplodia seriata*	Diseased tissues	Western Cape, SA	Slippers et al. 2007
Nestlera sp.	*Tiarosporella graminis*	Disease tissue	Free State, A	Crous et al 2006
Pinus patula	*Diplodia pinea*	Diseased tissues/ Symptomless	KwaZulu-Natal, Western Cape, SA	Swart et al. 1985; Smith et al. 1996b
P. patula	*Diplodia scrobiculata*	Symptomless/ Die-back	KwaZulu-Natal, Mpumalanga,	Bihon et al. 2010a

			SA	
P. radiata	*Diplodia pinea*	Diseased tissues/ Symptomless	Eastern Cape, Western Cape, SA	Swart et al. 1985; Smith et al. 1996b
Podocarpus elongatus	*Botryosphaeria dothidea*	Die-back	Gauteng, SA	Ndove L 2013
Po. elongatus	*Neofusicoccum eucalypti*	Die-back	Gauteng, SA	Ndove L 2013
Po. henkelii	*Botryosphaeria dothidea*	Die-back	Gauteng, SA	Ndove L 2013
Po. henkelii	*Diplodia mutila*	Symptomless	Western Cape, SA	Ndove L 2013
Po. henkelii	*Neofusicoccum crypto-australe*	Die-back	Gauteng, SA	Ndove L 2013
Po. henkelii	*Neofusicoccum eucalypti*	Die-back	Gauteng, SA	Ndove L 2013
Po. henkelii	*Neofusicoccum kwambonambiense*	Die-back	Mpumalanga, SA	Ndove L 2013
Po. henkelii	*Neofusicoccum parvum*	Symptomless	Gauteng, SA	Ndove L 2013
Po. henkelii	*Neofusicoccum* sp.nov1	Die-back	Mpumalanga, SA	Ndove L 2013
Po. henkelii	*Neofusicoccum ursorum*	Symptomless	Gauteng, SA	Ndove L 2013
Po. henkelii	*Spencermartinsia viticola*	Symptomless	Gauteng, SA	Ndove L 2013
Po. latifolius	*Lasiodiplodia gilanensis*	Symptomless	Western Cape, SA	Ndove L 2013
Po. latifolius	*Neofusicoccum crypto- australe*	Symptomless	Western Cape, SA	Ndove L 2013
Po. latifolius	*Neofusicoccum eucalypti*	Die-back	Gauteng, SA	Ndove L 2013
Populus sp.	*Diplodia seriata*	Diseased tissues	Western Cape, SA	Slippers et al., 2007
Protea cynaroides	*Neofusicoccum protearum*	Die-back	Western Cape, SA	Denman et al. 2003
Pr. cynaroides	*Saccharata proteae*	Symptomless	Western Cape, SA	Denman et al. 2003
Pr. eximia	*Neofusicoccum protearum*	Canker	Western Cape, SA	Denman et al. 2003
Pr. magnifica	*Diplodia seriata*	Symptomless	Western Cape, SA	Denman et al. 2003

Pr. magnifica	*Neofusicoccum protearum*	Canker & Leaf necrosis	Western Cape, SA	Denman et al. 2003
Pr. repens	*Diplodia pinea*	Symptomless	KwaZulu-Natal, SA	Smith et al. 1996b
Pr. repens	*Neofusicoccum protearum*	Canker	Western Cape, SA	Denman et al. 2003
Pr. repens	*Saccharata proteae*	Leaf necrosis	Western Cape, SA	Denman et al. 2003
Prunus armeniaca	*Diplodia seriata*	Diseased tissues	Limpopo, SA	Denman et al. 2003
P. armeniaca	*Neofusicoccum australe*	Diseased tissues	Limpopo, SA	Denman et al. 2003
P. dulcis	*Neofusicoccum australe*	Diseased tissues	Western Cape, SA	Slippers et al. 2007
P. persica	*Aplosporella prunicola*	Not available	Limpopo, SA	Damm et al. 2007b
P. persica	*Diplodia africana*	Canker & die-back	Western cape, SA	Damm et al. 2007
P. persica	*Diplodia pinea*	Canker & die-back	Western cape, SA	Damm et al. 2007
P. persica	*Diplodia seriata*	Diseased tissues	Western Cape, SA	Slippers et al. 2007
P. persica	*Neofusicoccum australe*	Canker& die-back	Western cape, SA	Damm et al. 2007
P. persica	*Neofusicoccum vitifusiforme*	Canker & die-back	Western cape, SA	Damm et al. 2007
P. persica	*Spencermartinsia viticola*	Canker & die-back	Limpopo, SA	Damm et al. 2007
P. salicina	*Diplodia mutila*	Canker & die-back	Western Cape, SA	Damm et al. 2007
P. salicina	*Diplodia seriata*	Diseased tissues	Western Cape, SA	Slippers et al. 2007
P. salicina	*Lasiodiplodia plurivora*	Canker & die-back	Western cape, SA	Damm et al. 2007
P. salicina	*Neofusicoccum australe*	Diseased tissues	Western Cape, SA	Slippers et al. 2007
P. salicina	*Neofusicoccum vitifusiforme*	Canker & die-back	Western cape, SA	Damm et al. 2007
Pterocarpus angolensis	*Lasiodiplodia theobromae*	Symptomless	Mpumalanga, SA	Mehl et al. 2011
Pt. angolensis	*Pseudofusicoccum olivaceum*	Symptomless	Mpumalanga, SA	Mehl et al. 2011
Pt. angolensis	*Pseudofusicoccum violaceum*	Symptomless	Mpumalanga, SA	Mehl et al. 2011

Pt. angolensis	*Diplodia pseudoseriata*	Healthy/ Diseased tissues	Mpumalanga, SA	Mehl et al. 2011
Pt. angolensis	*Fusicoccum atrovirens*	Symptomless	Mpumalanga, SA	Mehl et al. 2011
Pt. angolensis	*Lasiodiplodia crassispora*	Diseased tissues	Mpumalanga, SA	Mehl et al. 2011
Pt. angolensis	*Lasiodiplodia pseudotheobromae*	Healthy/ Diseased tissues	Mpumalanga, SA	Mehl et al. 2011
Pyrus communis	*Diplodia seriata*	Diseased tissues	Western Cape, SA	Slippers et al. 2007
Py. communis	*Neofusicoccum australe*	Diseased tissues	Western Cape, SA	Slippers et al. 2007
Searsia lancea	*Aplosporella javeedii*	Healthy branches	Gauteng, SA	Jami et al. 2013b
Sequoia gigantea	*Neofusicoccum parvum*	Not available	Eastern Cape, SA	Slippers et al 2004
Syzygium cordatum	*Botryosphaeria dothidea*	Canker& die-back	Eastern Cape, KwaZulu-Natal, SA	Pavlic et al. 2004, 2007
S. cordatum	*Lasiodiplodia gonubiensis*	Canker & die-back	Eastern Cape, SA	Pavlic et al. 2004, 2007
S. cordatum	*Lasiodiplodia pseudotheobromae*	Symptomless	KwaZulu-Natal, SA	Pillay et al. 2013
S. cordatum	*Lasiodiplodia theobromae*	Canker & die-back	Eastern Cape, SA	Pavlic et al. 2004, 2007
S. cordatum	*Neofusicoccum australe*	Canker & die-back	Eastern Cape, SA	Pavlic et al. 2004, 2007
S. cordatum	*Neofusicoccum cordaticola*	Symptomless and dying branches	KwaZulu-Natal, SA	Pavlic et al. 2009
S. cordatum	*Neofusicoccum crypto-australe*	Symptomless	Gauteng, SA	Maleme 2009
S. cordatum	*Neofusicoccum kwambonambiense*	Symptomless	KwaZulu-Natal, SA	Pillay et al. 2013
S. cordatum	*Neofusicoccum luteum*	Canker & die-back	Eastern Cape, SA	Pavlic et al. 2004, 2007
S. cordatum	*Neofusicoccum mangiferum*	Canker & die-back	Eastern Cape, SA	Pavlic et al. 2004, 2007
S. cordatum	*Neofusicoccum parvum*	Canker & die-back	Eastern Cape, SA	Pavlic et al. 2004, 2007
S.cordatum	*Neofusicoccum umdonicola*	Canker & die-back	Eastern Cape, SA	Pavlic et al. 2004, 2007

S. guineense	*Neofusicoccum parvum*	Canker & die-back	Eastern Cape, SA	Pavlic et al. 2004, 2007
Terminalia catappa	*Lasiodiplodia pseudotheobromae*	Die-back	KwaZulu-Natal, SA	Begoude et al. 2010
T. catappa	*Lasiodiplodia theobromae*	Die-back	KwaZulu-Natal, SA	Begoude et al. 2010
T. catappa	*Neofusicoccum parvum*	Die-back	KwaZulu-Natal, SA	Begoude et al. 2010
T. sericea	*Lasiodiplodia pseudotheobromae*	Die-back	Namibia	Begoude et al. 2012
T. sericea	*Neofusicoccum kwambonambiense*	Not available	SA	Begoude et al. 2010
T. sericea	*Neofusicoccum parvum*	Not available	SA	Begoude et al. 2010
Tibouchina urvilleana	*Neofusicoccum parvum*	Healthy/ Dead tissues	KwaZulu-Natal, SA	Heath et al. 2011
Ti. urvilleana	*Neofusicoccum mangiferum*	Healthy/ Dead tissues	KwaZulu-Natal, SA	Heath et al. 2011
Triticum sp.	*Tiarosporella tritici*	Disease tissue	Free State, SA	Crous et al 2006
Vitis vinifera	*Diplodia seriata*	Die-back	Western Cape, SA	Van Niekerk et al. 2004
V. vinifera	*Lasiodiplodia plurivora*	Canker & die-back	Western cape, SA	Damm et al. 2007
V. vinifera	*Lasiodiplodia theobromae*	Die-back	Western cape, SA	Van Niekerk et al. 2004
V. vinifera	*Neofusicoccum australe*	Die-back	Western cape, SA	Van Niekerk et al. 2004
V. vinifera	*Neofusicoccum parvum*	Die-back	Western cape, SA	Van Niekerk et al. 2004
V. vinifera	*Neofusicoccum viticlavatum*	Die-back	Western Cape, SA	Van Niekerk et al. 2004
V. vinifera	*Neofusicoccum vitifusiforme*	Die-back	Western cape, SA	Van Niekerk et al. 2004
V. vinifera	*Phaeobotryosphaeria porosa*	Die-back	Western Cape, SA	Van Niekerk et al. 2004
V. vinifera	*Spencermartinsia viticola*	Disease tissue	Western Cape, SA	Van Niekerk et al 2004
Widdringtonia nodiflora	*Neofusicoccum australe*	Diseased tissues	Western cape, SA	Slippers et al. 2005

(*More specific areas are indicated in the Figure 3)

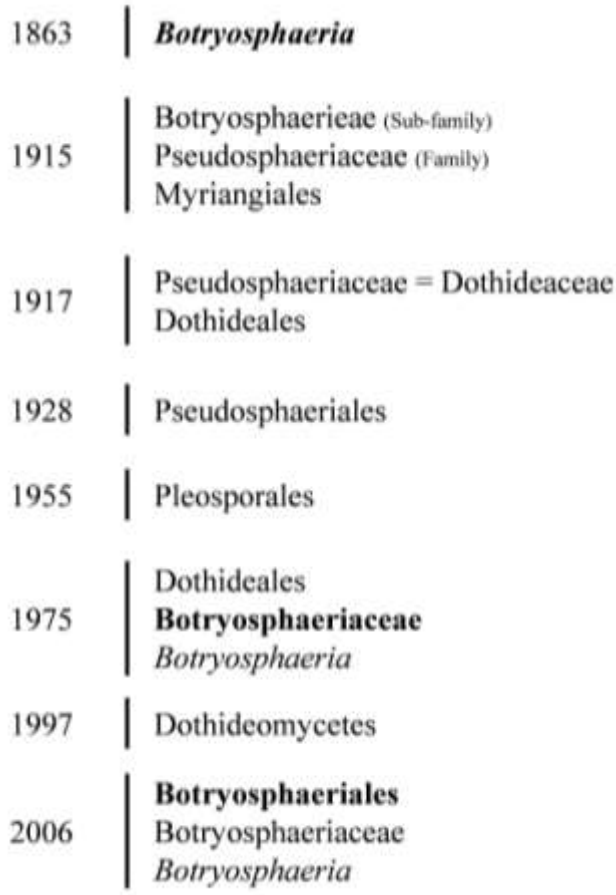
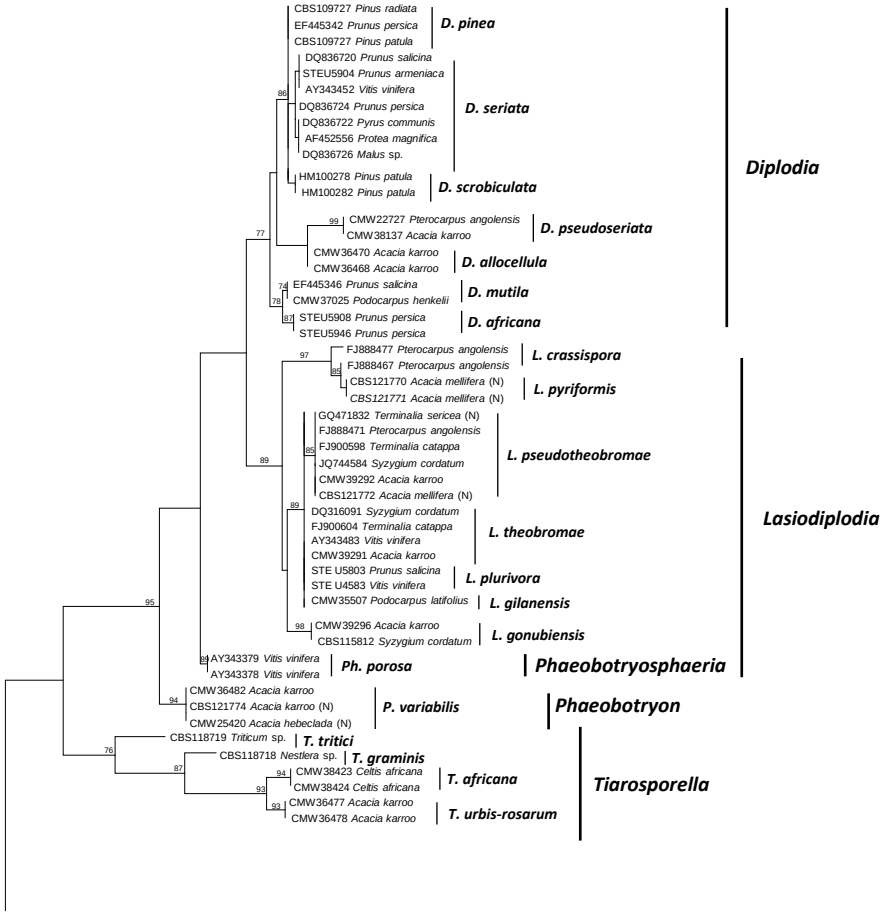
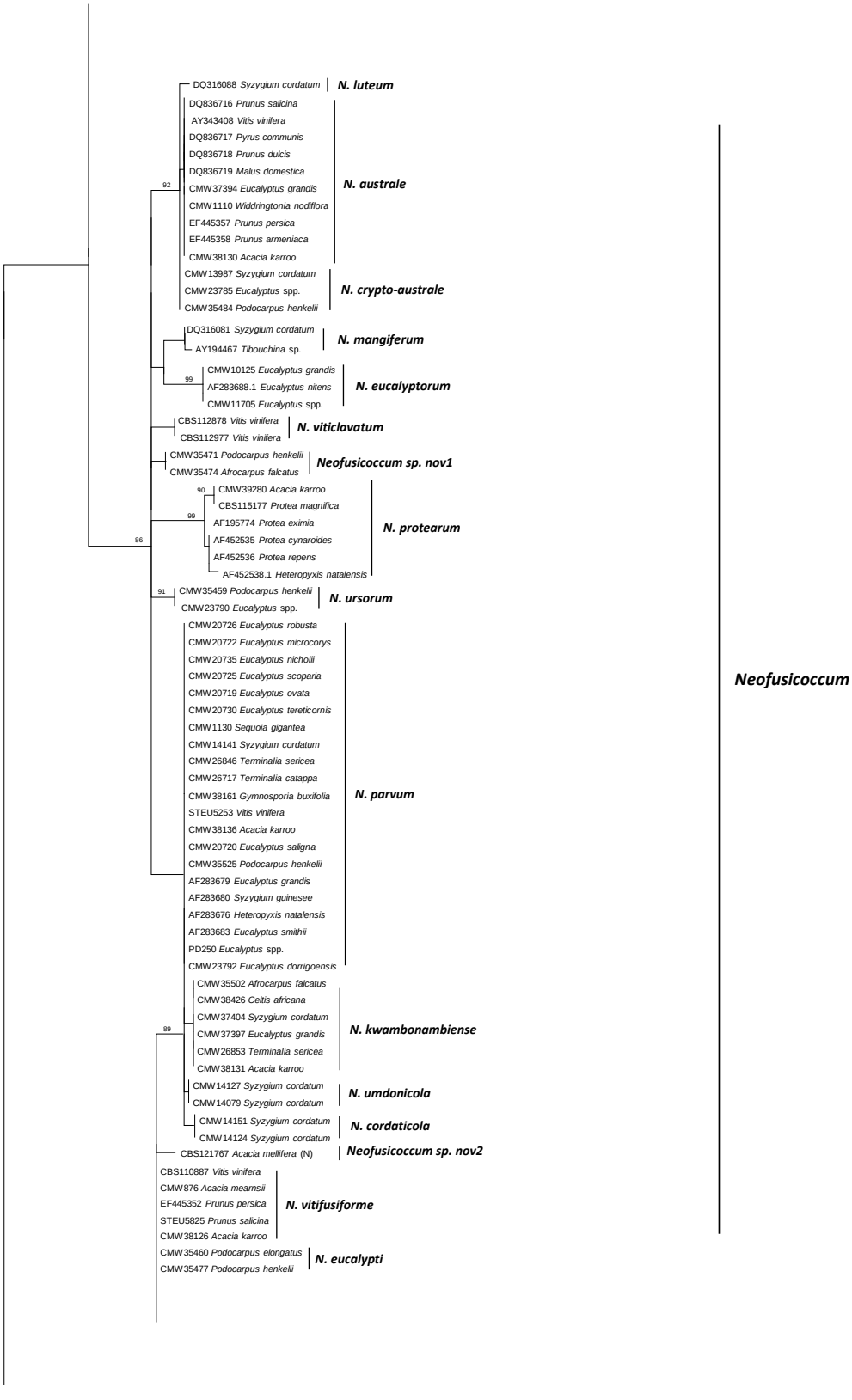
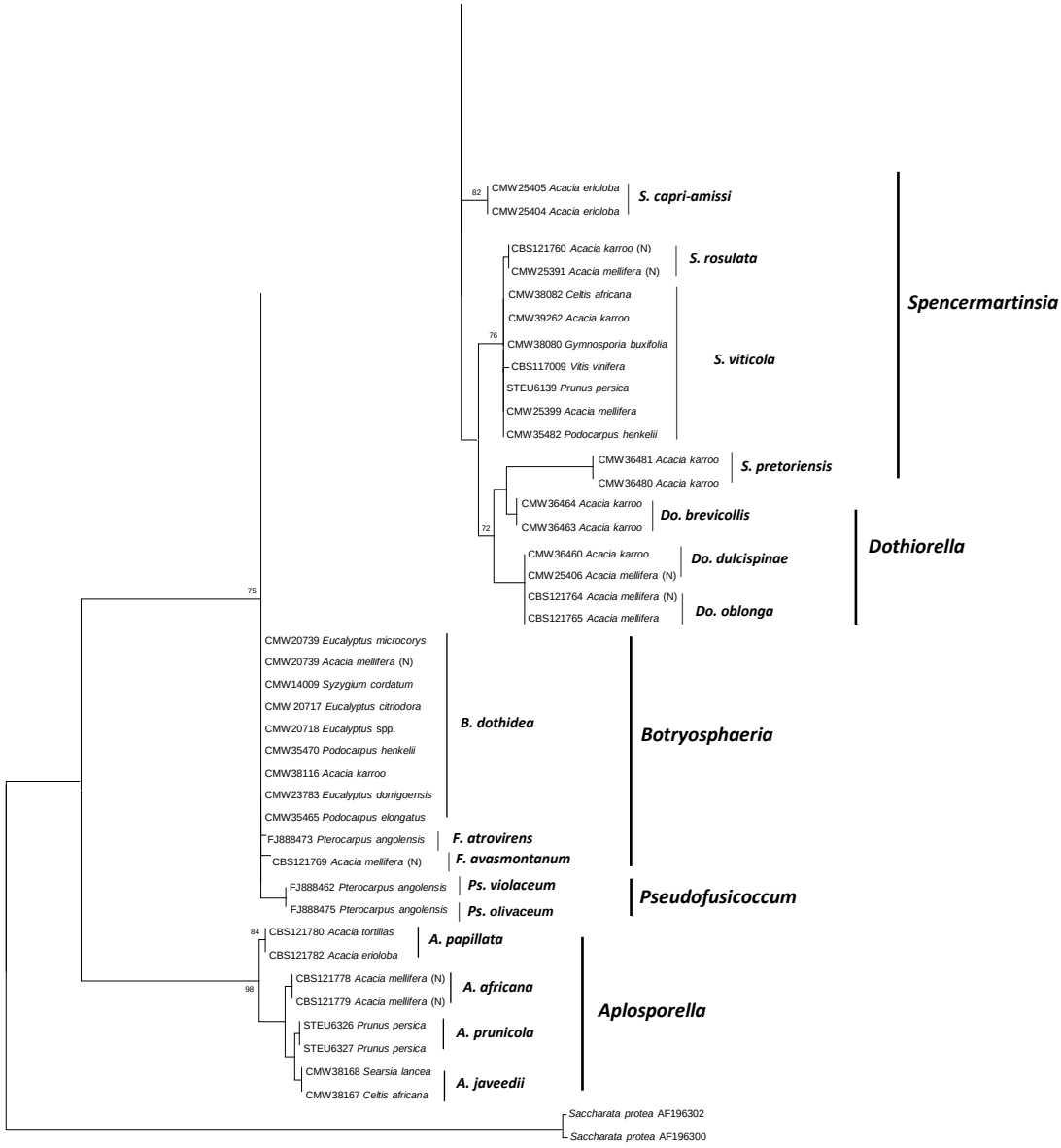


Figure. 1. Summarised taxonomic history of Botryosphaeriaceae (Crous et al. 2006)

Figure 2. Maximum Likelihood (ML) tree of ITS ribosomal DNA of Botryosphaeriales. Bootstrap values above 60 % are given at the nodes. The tree was rooted to *Saccharata protea* (AF196302 and AF196300). **N** = Namibia, where the species have been isolated.


CBS109727 Pinus radiata
EF445342 Prunus persica
CBS109727 Pinus patula
D. pinea
DQ836720 Prunus salicina
STEU5904 Prunus armeniaca
AY343452 Vitis vinifera
D. seriata
DQ836724 Prunus persica
DQ836722 Pyrus communis
AF452556 Protea magnifica
DQ836726 Malus sp.
HM100278 Pinus patula
HM100282 Pinus patula
D. scrobiculata
CMW22727 Pterocarpus angolensis
CMW38137 Acacia karroo
D. pseudoseriata
CMW36470 Acacia karroo
CMW36468 Acacia karroo
D. allocellula
EF445346 Prunus salicina
CMW37025 Podocarpus henkelii
D. mutila
STEU5908 Prunus persica
STEU5946 Prunus persica
D. africana
Diplodia
FJ888477 Pterocarpus angolensis
FJ888467 Pterocarpus angolensis
L. crassispora
CBS121770 Acacia mellifera (N)
CBS121771 Acacia mellifera (N)
L. pyriformis
GQ471832 Terminalia sericea (N)
FJ888471 Pterocarpus angolensis
FJ900598 Terminalia catappa
JQ744584 Syzygium cordatum
CMW39292 Acacia karroo
CBS121772 Acacia mellifera (N)
L. pseudotheobromae
DQ316091 Syzygium cordatum
FJ900604 Terminalia catappa
AY343483 Vitis vinifera
CMW39291 Acacia karroo
L. theobromae
STE U5803 Prunus salicina
STE U4583 Vitis vinifera
L. plurivora
CMW35507 Podocarpus latifolius
L. gilanensis
CMW39296 Acacia karroo
CBS115812 Syzygium cordatum
L. gonubiensis
Lasiodiplodia
AY343379 Vitis vinifera
AY343378 Vitis vinifera
Ph. porosa
Phaeobotryosphaeria
CMW36482 Acacia karroo
CBS121774 Acacia karroo (N)
CMW25420 Acacia hebeclada (N)
P. variabilis
Phaeobotryon
CBS118719 Triticum sp.
T. tritici
CBS118718 Nestlera sp.
T. graminis
CMW38423 Celtis africana
CMW38424 Celtis africana
T. africana
CMW36477 Acacia karroo
CMW36478 Acacia karroo
T. urbis-rosarum
Tiarosporella


DQ316088 Syzygium cordatum
N. luteum
DQ836716 Prunus salicina
AY343408 Vitis vinifera
DQ836717 Pyrus communis
DQ836718 Prunus dulcis
DQ836719 Malus domestica
92
CMW37394 Eucalyptus grandis
N. australe
CMW1110 Widdringtonia nodiflora
EF445357 Prunus persica
EF445358 Prunus armeniaca
CMW38130 Acacia karroo
CMW13987 Syzygium cordatum
CMW23785 Eucalyptus spp.
N. crypto-australe
CMW35484 Podocarpus henkelii
DQ316081 Syzygium cordatum
N. mangiferum
AY194467 Tibouchina sp.
CMW10125 Eucalyptus grandis
99
AF283688.1 Eucalyptus nitens
N. eucalyptorum
CMW11705 Eucalyptus spp.
CBS112878 Vitis vinifera
N. viticlavatum
CBS112977 Vitis vinifera
CMW35471 Podocarpus henkelii
Neofusicoccum sp. nov1
CMW35474 Afrocarpus falcatus
90
CMW39280 Acacia karroo
CBS115177 Protea magnifica
99
AF195774 Protea eximia
N. protearum
86
AF452535 Protea cynaroides
AF452536 Protea repens
AF452538.1 Heteropyxis natalensis
91
CMW35459 Podocarpus henkelii
N. ursorum
CMW23790 Eucalyptus spp.
CMW20726 Eucalyptus robusta
CMW20722 Eucalyptus microcorys
CMW20735 Eucalyptus nicholii
CMW20725 Eucalyptus scoparia
CMW20719 Eucalyptus ovata
CMW20730 Eucalyptus tereticornis
CMW1130 Sequoia gigantea
CMW14141 Syzygium cordatum
CMW26846 Terminalia sericea
CMW26717 Terminalia catappa
CMW38161 Gymnosporia buxifolia
N. parvum
STEU5253 Vitis vinifera
CMW38136 Acacia karroo
CMW20720 Eucalyptus saligna
CMW35525 Podocarpus henkelii
AF283679 Eucalyptus grandis
AF283680 Syzygium guinesee
AF283676 Heteropyxis natalensis
AF283683 Eucalyptus smithii
PD250 Eucalyptus spp.
CMW23792 Eucalyptus dorrigoensis
CMW35502 Afrocarpus falcatus
CMW38426 Celtis africana
CMW37404 Syzygium cordatum
89
N. kwambonambiense
CMW37397 Eucalyptus grandis
CMW26853 Terminalia sericea
CMW38131 Acacia karroo
CMW14127 Syzygium cordatum
N. umdonicola
CMW14079 Syzygium cordatum
CMW14151 Syzygium cordatum
N. cordaticola
CMW14124 Syzygium cordatum
CBS121767 Acacia mellifera (N)
Neofusicoccum sp. nov2
CBS110887 Vitis vinifera
CMW876 Acacia mearnsii
EF445352 Prunus persica
N. vitifusiforme
STEU5825 Prunus salicina
CMW38126 Acacia karroo
CMW35460 Podocarpus elongatus
N. eucalypti
CMW35477 Podocarpus henkelii
Neofusicoccum


82
CMW25405 Acacia erioloba
CMW25404 Acacia erioloba
S. capri-amissi
CBS121760 Acacia karroo (N)
CMW25391 Acacia mellifera (N)
S. rosulata
CMW38082 Celtis africana
CMW39262 Acacia karroo
76
CMW38080 Gymnosporia buxifolia
CBS117009 Vitis vinifera
STEU6139 Prunus persica
CMW25399 Acacia mellifera
CMW35482 Podocarpus henkelii
S. viticola
Spencermartinsia
CMW36481 Acacia karroo
CMW36480 Acacia karroo
S. pretoriensis
CMW36464 Acacia karroo
CMW36463 Acacia karroo
Do. brevicollis
72
CMW36460 Acacia karroo
CMW25406 Acacia mellifera (N)
Do. dulcispinae
CBS121764 Acacia mellifera (N)
CBS121765 Acacia mellifera
Do. oblonga
Dothiorella
75
CMW20739 Eucalyptus microcorys
CMW20739 Acacia mellifera (N)
CMW14009 Syzygium cordatum
CMW 20717 Eucalyptus citriodora
CMW20718 Eucalyptus spp.
CMW35470 Podocarpus henkelii
CMW38116 Acacia karroo
CMW23783 Eucalyptus dorrigoensis
CMW35465 Podocarpus elongatus
B. dothidea
Botryosphaeria
FJ888473 Pterocarpus angolensis
F. atrovirens
CBS121769 Acacia mellifera (N)
F. avasmontanum
FJ888462 Pterocarpus angolensis
Ps. violaceum
FJ888475 Pterocarpus angolensis
Ps. olivaceum
Pseudofusicoccum
84
CBS121780 Acacia tortillas
CBS121782 Acacia erioloba
A. papillata
98
CBS121778 Acacia mellifera (N)
CBS121779 Acacia mellifera (N)
A. africana
STEU6326 Prunus persica
STEU6327 Prunus persica
A. prunicola
CMW38168 Searsia lancea
CMW38167 Celtis africana
A. javeedii
Aplosporella
Saccharata protea AF196302
Saccharata protea AF196300

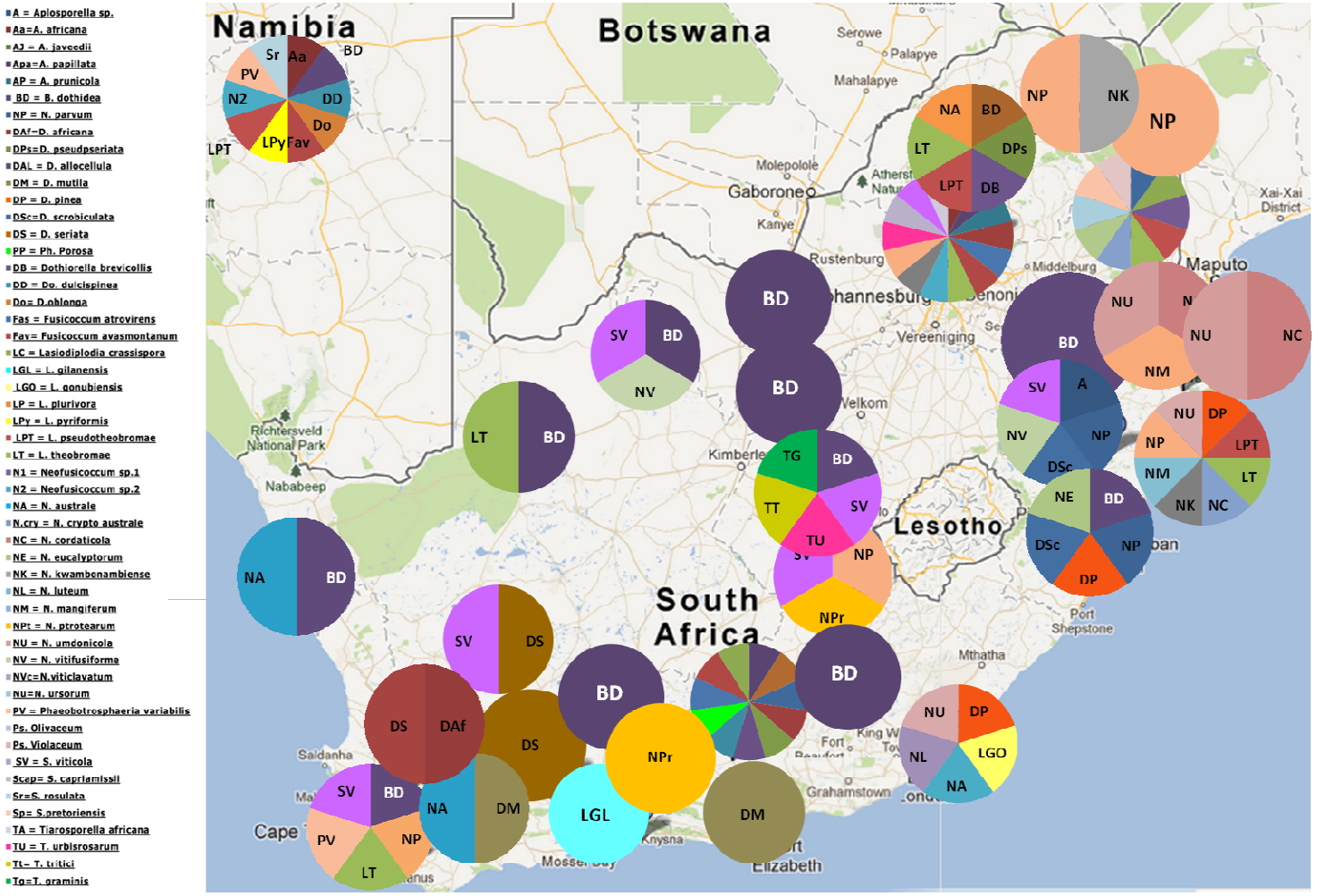


Figure. 3. Diversity of Botryosphaeriaceae species in each published site in South Africa.

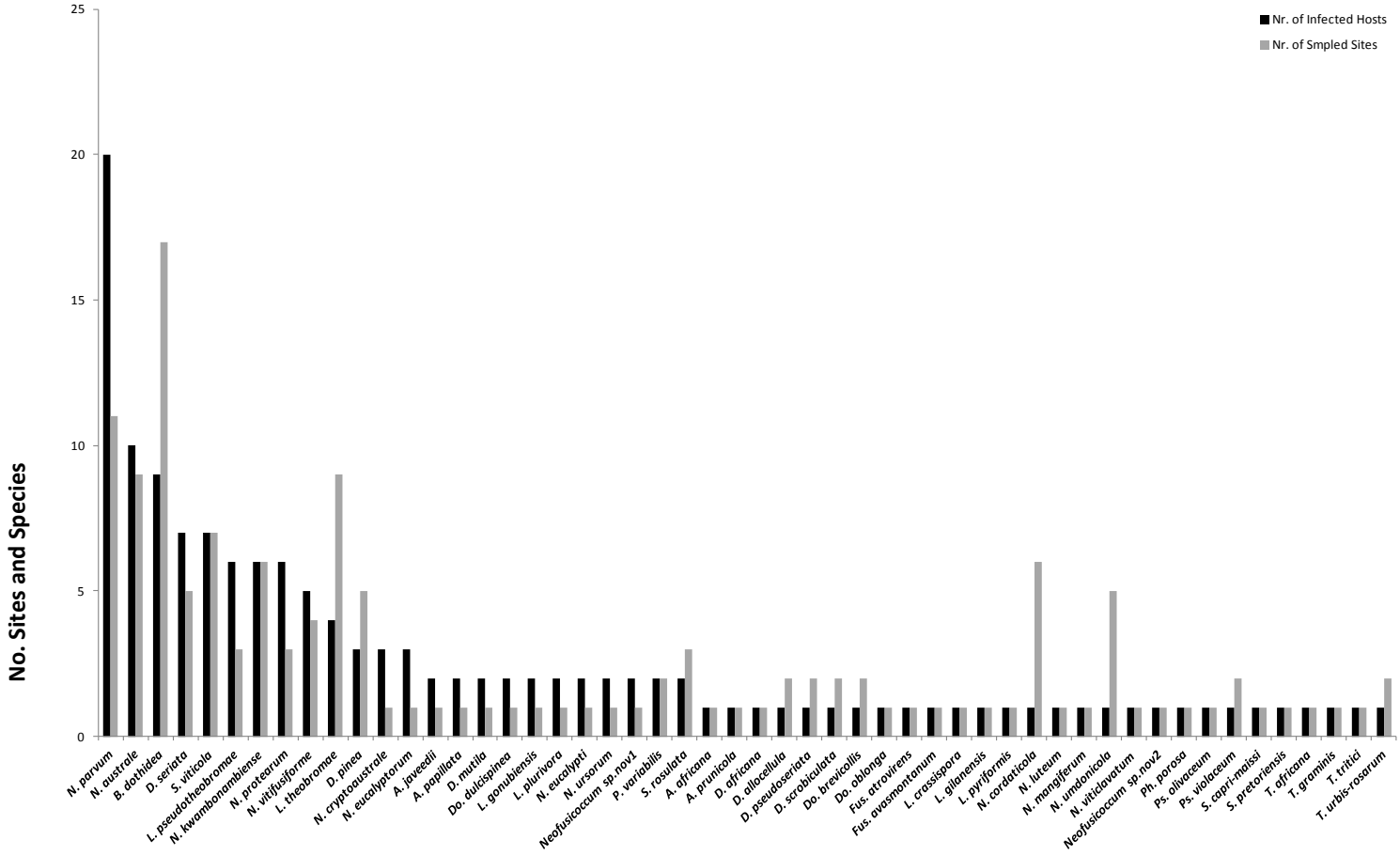


Figure. 4. Frequency of Botryosphaeriaceae species and number of sampled sites (per species) from all previous published studies across South Africa on various hosts.

Summary

Taxonomy and ecology of Botryosphaeriaceae associated with *Acacia karroo* in South Africa

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The Botryosphaeriaceae are common and diverse members of fungal communities that infect native and non-native woody plants. They can be serious pathogens and involved in diseases on native trees, but commonly occur as endophytes. These fungi are also often moved locally and globally with plant material. To interpret patterns of movement as well as host and geographic association, a clear taxonomic and species identification framework is required. The aims of studies in this thesis were to consider the species diversity, structure and variation over time of Botryosphaeriaceae associated with the native tree *Acacia karroo*, which occurs commonly across the South African landscape. The patterns of overlap of Botryosphaeriaceae between *A. karroo* and three other native trees, namely *Celtis africana*, *Searsia lancea* and *Gymnosporia buxifolia* at a single location was also considered. Finally, the diversity of Botryosphaeriaceae associated with healthy tissue types, compared with those from die-back symptoms on *A. karroo* was studied. These questions were answered by sampling *A. karroo* from 23 sites across its distribution in South Africa, with more intensive sampling done in the Tshwane area over three years. In total, 19 species of the Botryosphaeriaceae were identified, of which seven were newly described. There were clear patterns in geographic structure, with some species found only in some parts of the country. There was no significant variation in species composition over time and on different tissue types in the Tshwane area. In the comparison between healthy and diseased material, a higher diversity was found to infect the healthy material. While all the tested species could infect *A. karroo* and give rise to lesions, only a few caused significant lesions and occurred sufficiently widely to be considered important

pathogens. Results of this study showed that a rich diversity of Botryosphaeriaceae can exist on a single native plant, even in the absence of obvious disease. Furthermore, that significant structure exists in these species communities. To adequately sample this diversity and to delimit overall patterns in species occurrence and host preference, these studies suggest that rigorous sampling across more diverse geographical areas are required, even when studying a single host plant.