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Diversity of Endophytes in the *Botryosphaeriaceae* Differs on *Anacardiaceae* in Disturbed and Undisturbed Ecosystems in South Africa

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Abstract: *Botryosphaeriaceae* represents a diverse family of fungi with a cosmopolitan distribution and a wide host range. We studied species diversity and overlap of *Botryosphaeriaceae* on selected tree species of *Anacardiaceae* in disturbed (farming or forestry) and undisturbed (isolated and/or protected) ecosystems in the Limpopo and Mpumalanga provinces, South Africa. The disturbed sites resided at Tshikundamalema and Tshipise in Limpopo and the undisturbed sites at Nwanedi and the Mapungubwe National Park in Limpopo and the Kruger National Park in Mpumalanga. Asymptomatic branches were collected from *Mangifera indica*, *Sclerocarya birrea* and *Lannea schweinfurthii* trees in 2017 and 2018. Eleven species were identified using a multi-gene sequencing approach, including *Diplodia allocellula*, *Dothiorella brevicollis*, *Do. dulcispinae*, *Do. viticola*, *Lasiodiplodia crassispora*, *L. exigua*, *L. gonubiensis*, *L. mahajangana*, *Neofusicoccum parvum*, *Oblongocollomyces* sp. 1 and *Oblongocollomyces* sp. 2. Ten of the 11 species were identified in undisturbed ecosystems (eight species being unique), while only three species were identified in disturbed ecosystems (one species being unique). Two species were generalists on trees in disturbed and undisturbed ecosystems. *Lasiodiplodia mahajangana* was the most dominant species as it occurred on the three tree species of *Anacardiaceae*. Isolates of *N. parvum* occurred on both *S. birrea* (a native species) and *M. indica* (a non-native species) that occurred adjacent to each other in disturbed ecosystems, confirming the ability of this invasive pathogen to cross-infect native and non-native hosts and its abundance in human-disturbed environments. The findings from this study confirm the lack of host specificity for most species of *Botryosphaeriaceae*. The results also indicate that disturbance through human activity, such as clear-cutting, selective cutting and land-use changes, negatively influences the diversity of the *Botryosphaeriaceae*.

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

Citation: Ramabulana, E.; Kunjeku, E.; Slippers, B.; Coetzee, M.P.A. Diversity of Endophytes in the *Botryosphaeriaceae* Differs on *Anacardiaceae* in Disturbed and Undisturbed Ecosystems in South Africa. *Forests* **2022**, *13*, 341. https://doi.org/10.3390/f13020341

Academic Editor: Paulo A. Zaini

Received: 21 December 2021
Accepted: 15 February 2022
Published: 18 February 2022

1. Introduction

Species of *Botryosphaeriaceae* are capable of infecting a broad range of monocotyledonous, dicotyledonous and gymnospermous hosts [1]. Fungi in the *Botryosphaeriaceae* are common and known from a variety of hosts in South Africa, including commercial fruit trees [2–4], plantation trees [5,6] and native trees [7–9]. The ability of species of *Botryosphaeriaceae* to infect multiple hosts facilitates their spread and establishment in new areas, increasing their threat as potential pathogens of woody trees globally [1,10,11]. Once introduced into a new environment, these fungi can spread and infect both related and unrelated hosts [1,9,12].

In South Africa, species of *Botryosphaeriaceae* have been found overlapping on related tree species of *Anacardiaceae* [13] and *Myrtaceae* [7,12] and unrelated hosts in the

Cannabaceae, Celastraceae and *Fabaceae* [9]. Several species of *Botryosphaeriaceae*, including *Botryosphaeria dothidea*, *Lasiodiplodia mahajangana*, *L. pseudotheobromae*, *L. theobromae*, *Neofusicoccum kwambonambiense, N. vitifusiforme, N. luteum* and *N. parvum* have been found on both native and non-native hosts such as *Eucalyptus* spp. [5,6,14], *Mangifera indica* [13], *Malus* spp. [4], *Pinus* spp. [5], *Prunus* spp. [3,4], *Vachellia karroo* [15], *Sclerocarya birrea* [13], *Syzygium cordatum* [7] and *Vitis vinifera* [2].

It is likely that some prominent species that are commonly recorded on trees in agricultural and forestry ecosystems are also invaders of trees in natural stands. This is expected for species such as *B. dothidea, D. sapinea, L. theobromae* and *N. parvum* that have been reported on various hosts globally [11,16–20]. The broad distribution of these species also indicates their ability to grow under very different climatic conditions [11,20,21].

Some species, however, appear to have a narrow host range and are known from a single host or region [21]. Species that are not commonly reported in South Africa include *Botryosphaeria atrovirens, Diplodia africana, D. scrobiculata, Dothiorella brevicollis, Do. capri-amissi*, *Do. pretoriensis, Eutiarosporella urbis-rosarum, Eu. africana, Eu. graminis, Eu. tritici, Lasiodiplodia avicenniae, L. bruguierae, L. euphorbicola* and *Neofusicoccum viticlavatum* [21]. Their host records might be influenced by their rare occurrence and not necessarily provide a true reflection of their ability to infect other plants. Therefore, it is not clear whether the narrow host range that has been observed for some species of *Botryosphaeriaceae* can be attributed to host specificity or rather the sampling strategies that were followed during sample collection.

To understand the diversity of *Botryosphaeriaceae* in a region, these fungi must be isolated from a variety of woody plants growing across diverse ecosystems. While the *Botryosphaeriaceae* have been extensively studied in South Africa, they have not been widely investigated in undisturbed ecosystems. This is because most of the work on *Botryosphaeriaceae* is focused on commercially important trees in human-disturbed ecosystems such as plantations, orchards and cities [2,4–6]. Where studies on native tree species have been done in South Africa, they have revealed a great diversity of *Botryosphaeriaceae* on various native hosts such as *Vachellia* spp., *Euphorbia ingens, Pterocarpus angolensis*, *S. birrea*, mangrove species and *S. cordatum* [21].

Fungi in the *Botryosphaeriaceae* were previously identified on trees of *Anacardiaceae* in South Africa. Currently, 11 *Botryosphaeriaceae* species are known to occur on *S. birrea* [13], 10 on *M. indica* [13,22] and one on *Searsia lancea* [9]. The family *Anacardiaceae* includes more than 800 species worldwide [23]. Several genera in the family are economically important, including *Anacardium*, *Pistacia*, *Mangifera* and *Sclerocarya*. *Anacardium occidentale* and *S. birrea* are highly prized for their use in traditional medicine in rural parts of Africa [23–25].

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

2. Materials and Methods

2.1. Sample Sites, Tree Health Assessment and Sampling

Asymptomatic branches (20–30 cm long and 2–5 mm wide) showing no prior damage or disease symptoms were collected from three tree hosts, namely *S. birrea, M. indica,* and, for the first time sampled in South Africa, *L. schweinfurthii*, in five locations that included disturbed (developed and intensively farmed) and undisturbed or low disturbance (isolated and undeveloped, and/or protected parks) sites.

The disturbed sites were Tshikundamalema (Latitude 22°40′17.87 S, Longitude 30°41′27.29 E) and Tshipise (Latitude 22°36′15.59 S, Longitude 30°09′59.08 E), which are

approximately 90 km apart. The land is used for agricultural purposes for the cultivation of maize, groundnuts, vegetables, as well as for livestock production. Sample collections in Tshikundamalema were made from 49 *S. birrea* and 54 *M. indica* trees that were approximately 25 m from each other, in Tshipise, samples were collected from 30 *M. indica* and 20 *S. birrea* trees that were approximately 25 m radius from each other.

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

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

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

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

2.2. Fungal Isolations

Samples were surface disinfected by immersing ~10 cm of the branch in 10% hydrogen peroxide for 2 min, followed by rinsing twice in sterile distilled water for 1 min each. Subsequently, 12 discs (0.5 cm) were cut from each branch and plated onto 2% MEA (2% malt extract, 1.5% agar; Biolab, Midrand, South Africa) with streptomycin to inhibit the growth of bacteria. Primary isolates were incubated at 25 °C and checked daily for fungal growth. Pure cultures were obtained by transferring single hyphal tips from developing colonies resembling species of *Botryosphaeriaceae* onto clean 2% MEA.

Isolates were grouped based on culture morphology and colour characteristics using the colour chart by Rayner [26]. Isolates were grouped into 11 cultural morphogroups, from which 2–10 isolates representing the different tree species and sites were selected. In total, 78 isolates were selected for preliminary ITS identification. Of these, 52 isolates were obtained from *S. birrea* trees sampled at Tshikundamalema, Tshipise, Mapungubwe and Kruger National Park, 16 from *L. schweinfurthii* trees at Nwanedi and Kruger National Park and 10 from *M. indica* trees at Tshipise and Tshikundamalema. Isolates used in this study (Table 1) are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Table 1. Representative fungal isolates obtained from tree species of *Anacardiaceae* in this study and used in the phylogenetic analyses.

Isolate No.	Identity	Host	Location	GenBank			
				ITS	*tef1-α*	*β-tub*	*rpb2*
CMW54349	*Diplodia allocellula*	*Lannea schweinfurthii*	Nwanedi	OL441853	OL441909	OL441965	OM585579
CMW54351	*D. allocellula*	*Sclerocarya birrea*	Kruger	OL441855	OL441911	OL441967	OM585581
CMW54353	*D. allocellula*	*Lannea schweinfurthii*	Kruger	OL441857	OL441913	OL441969	OM585583
CMW57629	*Dothiorella brevicollis*	*Lannea schweinfurthii*	Nwanedi	OL441858	OL441914	OL441970	-
CMW57630	*Do. brevicollis*	*Sclerocarya birrea*	Mapungubwe	OL441859	OL441915	OL441971	-
CMW57462	*Do. dulcispinae*	*Sclerocarya birrea*	Kruger	OL441861	OL441917	OL441973	-
CMW57466	*Do. dulcispinae*	*Sclerocarya birrea*	Mapungubwe	OL441862	OL441918	OL441974	-
CMW57463	*Do. viticola*	*Sclerocarya birrea*	Mapungubwe	OL441863	OL441919	OL441975	-
CMW57464	*Do. viticola*	*Sclerocarya birrea*	Mapungubwe	OL441864	OL441920	OL441976	-
CMW54318	*Lasiodiplodia crassispora*	*Lannea schweinfurthii*	Kruger	OL441866	OL441922	OL441978	OL442021
CMW54319	*L. crassispora*	*Sclerocarya birrea*	Tshikundamalema	OL441867	OL441923	OL441979	OL442022
CMW54320	*L. crassispora*	*Sclerocarya birrea*	Mapungubwe	OL441868	OL441924	OL441980	OL442023
CMW54321	*L. crassispora*	*Mangifera indica*	Tshipise	OL441869	OL441925	OL441981	OL442024
CMW57579	*L. crassispora*	*Sclerocarya birrea*	Kruger	OL441870	OL441926	OL441982	OL442025
CMW54314	*L. gonubiensis*	*Sclerocarya birrea*	Kruger	OL441873	OL441929	OL441985	OL442028
CMW54315	*L. gonubiensis*	*Sclerocarya birrea*	Kruger	OL441874	OL441930	OL441986	OL442029

Table 1. *Cont.*

Isolate No.	Identity	Host	Location	GenBank			
				ITS	***tef1-α***	***β-tub***	***rpb2***
CMW54312	*L. exigua*	*Sclerocarya birrea*	Mapungubwe	OL441875	OL441931	OL441987	OL442030
CMW54313	*L. exigua*	*Sclerocarya birrea*	Mapungubwe	OL441876	OL441932	OL441988	OL442031
CMW54326	*L. mahajangana*	*Mangifera indica*	Tshikundamalema	OL441877	OL441933	OL441989	OL442032
CMW54329	*L. mahajangana*	*Lannea schweinfurthii*	Nwanedi	OL441878	OL441934	OL441990	OL442033
CMW54331	*L. mahajangana*	*Sclerocarya birrea*	Mapungubwe	OL441880	OL441936	OL441992	OL442035
CMW54334	*L. mahajangana*	*Sclerocarya birrea*	Kruger	OL441881	OL441937	OL441993	OL442036
CMW54357	*Neofusicoccum parvum*	*Sclerocarya birrea*	Tshipise	OL441892	OL441948	OL442004	OL442047
CMW54359	*N. parvum*	*Mangifera indica*	Tshipise	OL441893	OL441949	OL442005	OL442048
CMW54360	*N. parvum*	*Mangifera indica*	Tshikundamalema	OL441894	OL441950	OL442006	OL442049
CMW57467	*Oblongocollomyces* sp. 1	*Sclerocarya birrea*	Mapungubwe	OL441905	OL441961	OL442017	OL442060
CMW57572	*Oblongocollomyces* sp. 1	*Sclerocarya birrea*	Mapungubwe	OL441906	OL441962	OL442018	OL442061
CMW57465	*Oblongocollomyces* sp. 2	*Sclerocarya birrea*	Mapungubwe	OL441907	OL441963	OL442019	OL442062
CMW57573	*Oblongocollomyces* sp. 2	*Sclerocarya birrea*	Mapungubwe	OL441908	OL441964	OL442020	OL442063

2.3. DNA Extraction, PCR Amplification and Sequencing

Isolates selected for preliminary identification were grown on 2% MEA for 7 days and incubated at 25 °C, from which mycelia were scraped and freeze-dried. Freeze-dried mycelia were ground into powder and DNA was extracted following the protocol published by Möller et al. [27]. The resulting DNA pellets were re-suspended in 50 µL sterile SABAX water (SABAX; Adcock Ingram, Bryanston, S.A). DNA was stained with GelRed® (Biotium, Haward, CA, USA) and visualized under UV light after electrophoresis on 1% agarose gel. DNA concentrations were determined using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, NC, USA) and adjusted to a working concentration of 50 ng μL^{-1} using SABAX water.

Four gene regions that are commonly used in the systematics of *Botryosphaeriaceae* were amplified, including the internal transcribed spacer (ITS), translation elongation factor (*tef1-α*), β-tubulin (*β-tub*) and the RNA polymerase II subunit (*rpb2*). The ITS region that includes the ITS-1 spacer, 5.8S gene and ITS-2 spacer was amplified and sequenced for all the 78 isolates selected as representatives for the different morphological groups using primer pairs ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) [28]. A subset of 29 isolates was selected based on the ITS phylogeny and analysed using other gene regions for phylogenetic confirmation of species identity. For these isolates, the translation elongation factor (*tef1-α*), using primer pairs *tef1*-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and *tef1*-986R (5′-TAC TTG AAG GAA CCC TTA CC-3′) [29], βeta-tubulin (*β-tub*) gene using primers *Bt*2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and *Bt*2b (5′-ACCCTCAGTGTAGTGACCCTTG GC-3′) [30], as well as the RNA polymerase II subunit (*rpb2*) gene using primer pairs *rpb2*-LasF (5′-GGTAGCGACGTCACTCCT-3′) and *rpb2*-LasR (5′-GCGCAAATACCCAGAATCAT-3′) [31] and *rpb2*bot6F (5′-GGTAGCGACGTCACTCCC-3′) and *rpb2*bot7R (5′-GGATGGATC TCGCAATGCG-3′) [32] were amplified and sequenced.

PCR reactions consisted of ~40–50 ng genomic DNA, 0.2 µM of each primer, 0.5 U of MyTaq™ DNA polymerase (Bioline, London, UK), 5 µL MyTaq PCR reaction buffer (10 mM Tris-HCl [pH 8.3], 3.0 mM $MgCl_2$, 50 mM KCl, Roche Diagnostics, Mannheim, Germany) and PCR grade water to a final volume of 25 µL. PCR cycling conditions were 2 min at 94 °C, followed by 30 cycles at 94 °C for 30 sec, annealing at 54 °C (ITS and *rpb2*) and 56 °C (*tef1-α* and *β-tub*) for 30 sec, extension for 1 min at 72 °C and final extension for 7 min at 72 °C. PCR products were separated on 1% agarose gel stained with GelRed® and visualised under UV light. Amplicons were purified with Exosap (Mixture of Exonuclease I and FastAP Alkaline Phosphatase) (Thermo Fisher Scientific Inc. Waltham, MA, USA) following the manufacturer's specifications. Purified PCR fragments were sequenced in both directions using the same primer pairs utilised in PCR reactions. Sequencing reactions were conducted with the ABI Prism® Big Dye™ sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing of the amplicons was done at the sequencing facility of the University of Pretoria. Consensus sequences were built from the forward and reverse sequence reads using CLC Main Workbench v.7.9 (QIAGEN, Aarhus, Denmark).

2.4. DNA Sequence and Phylogenetic Analyses

Sequences generated in this study were subjected to BLASTn searches against sequences on GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (accessed on 25 February 2019) for preliminary identification. To confirm species identity, phylogenetic analyses were performed individually for each locus dataset. Reference sequences showing similarity to query sequences in BLASTn searches were retrieved from GenBank. Sequences were aligned using an online interface of MAFFT v.7 server [33]. Alignments were verified and manual adjustments were made where necessary. The best nucleotide substitution model for each dataset was determined with jModelTest v.0.1.1 [34] using the Akaike Information Criterion (AIC) to select the model that optimally fits the data. The models HKY + I + G, HKY + G, GTR + G and GTR + G were applied to the ITS, *tef1-α*, *β-tub*, *rpb2* datasets, respectively. Maximum likelihood (ML) phylogenetic analyses were performed for the four

datasets using RAxML v.8 [35]. To determine the robustness of the trees, bootstrap analyses were done using 1000 replicates.

Bayesian inference (BI) of phylogenetic trees was done using MrBayes v.3.2.6 [36]. Four simultaneous Markov chains were run for 3000000 generations and trees were sampled every 100th generation. The first 7500 trees representing the burn-in phase of the analyses were discarded and the remaining 22500 trees were used for calculating posterior probabilities (PP) based on a majority rule consensus tree. Effective sampling size (ESS) values were assessed using Tracer v.1.7.1 (http://tree.bio.ed.ac.uk/software/tracer/) (accessed on 23 February 2021). Both the ML and BI phylogenetic trees were rooted to sequences of *Melanops tulasnei* (*Botryosphaeriales*; *Melanopsaceae*) as the outgroup taxa.

3. Results

3.1. Tree Health Assessment

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

Sclerocarya birrea trees were the most damaged, followed by *L. schweinfurthii* and *M. indica* (Table 2). Results obtained from the χ^2 test indicated a statistically significant correlation between health status and tree species ($p = 0.00$). Physical damage caused by elephants was evident on *S. birrea* and *L. schweinfurthii* trees at the two National Park sites (Figure 1).

Table 2. Health status of the three tree species of *Anacardiaceae* assessed in this study.

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

Kruger National Park had the highest number of infected trees, followed by Tshikundamalema, Mapungubwe National Park and Tshipise. Nwanedi had the least number of infected trees (Table 3). Statistical analyses indicated that there is an association between health status and site ($p = 0.00$).

Table 3. Health status of the *Anacardiaceae* at the five sites sampled in this study.

	Healthy	<25% Dieback Branches	25–50% Dieback Branches	>50% Dieback Branches	Appearing to Be Dying or Nearly Dead	Total
Tshikundamalema	0	51	42	10	0	103
Tshipise	0	24	24	2	0	50
Nwanedi	0	28	2	0	0	30
Mapungubwe	6	11	26	7	4	54
Kruger	0	101	47	14	5	167

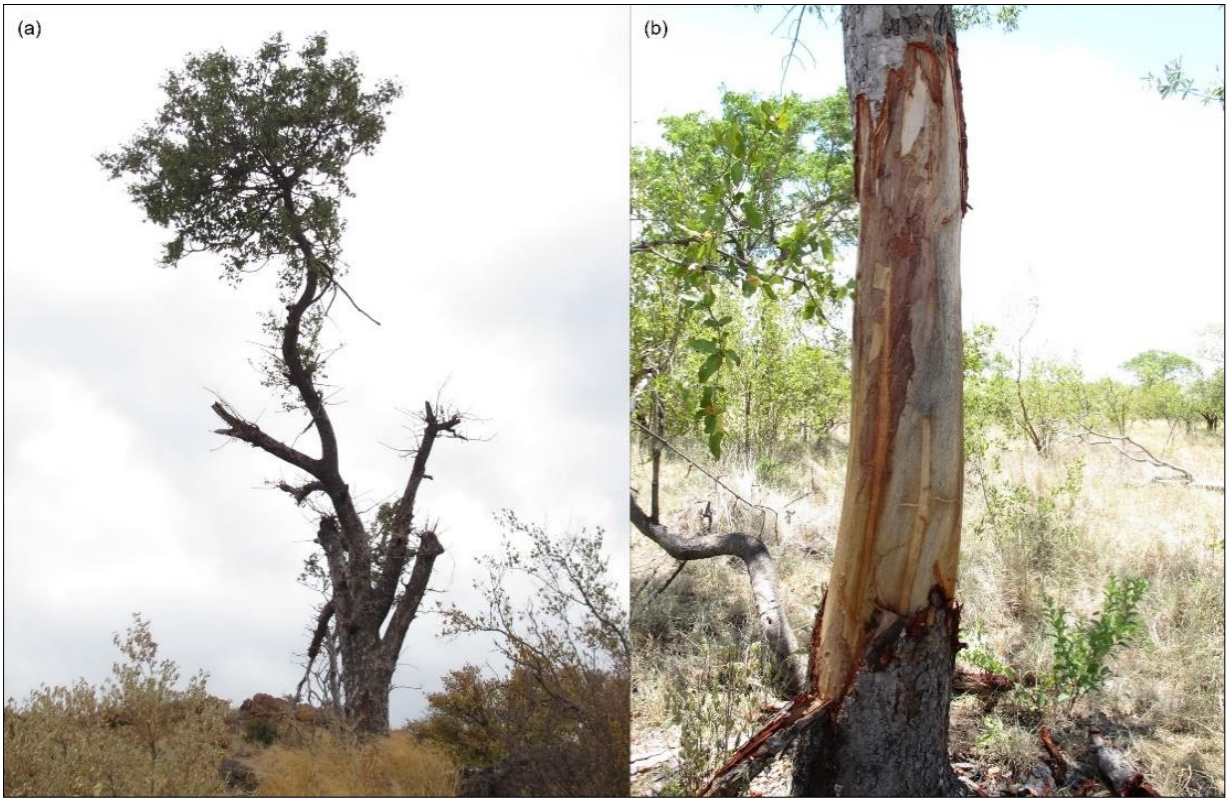

Figure 1. Trees with dieback and damage by elephants at the two National Parks; (**a**) Damaged *Sclerocarya birrea* tree at Mapungubwe National Park; (**b**) Bark stripped *Lannea schweinfurthii* tree at Kruger National Park.

3.2. Fungal Isolation and Preliminary Identification

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

3.3. Phylogenetic Analyses and Confirmation of Species Identification

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

The datasets for the ITS, *tef-1α* and *β-tub* gene regions included 29 isolates obtained in this study and 63 representatives from GenBank for ITS, 60 for *tef-1α* and 54 for *β-tub* (Table 4). The *rpb2* dataset included 23 sequences generated in this study. This was because amplicons for the *rpb2* gene region could not be obtained for *Dothiorella* isolates even after using two primer sets.

The topologies of the trees generated from ML and BI analyses of the four datasets were similar in the separation of clades representing genera of *Botryosphaeriaceae*. However, some clades of interest did not receive bootstrap (BS) and posterior probability (PP) support. Some isolates obtained in this study failed to show the same groupings found in other phylogenetic trees and were not considered congruent. For example, the *Lasiodiplodia* sub-clade (including *L. citricola*, *L. magnoliae*, *L. pseudotheobromae* and *L. vaccinii*) on the ITS phylogeny (Figure 2). Most isolates on the *tef-1α* phylogeny did not form monophyletic clades with species of *Botryosphaeriaceae* (Figure 3). Some rearrangements were also observed in the backbone of individual gene trees, such as the position of the clade accommodating isolates corresponding to *L. crassispora* on the *β-tub* phylogeny (Figure 4). The most variable locus

was *rpb2* which could distinguish between most species of *Botryosphaeriaceae* (Figure 5). Five main clades corresponding to *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum* and *Oblongocollomyces*/*Sphaeropsis* were identified.

Table 4. Reference sequences obtained from GenBank and used for phylogenetic analyses in this study (species names are as indicated in the GenBank record).

Species	Country	Strain Number	GenBank			
			ITS	*tef1-α*	*rpb2*	*β-tub*
Diplodia allocellula	South Africa	CBS 130408 CMW 36468	JQ239397	JQ239384	None	JQ239378
D. allocellula	South Africa	CMW 36469	NR_111701	JQ239385	None	JQ239379
D. eriobotryicola	Spain	CBS 140851	NR_152462	KT240193	None	MG015806
D. sapinea	Netherlands	CBS 393.84	DQ458895	DQ458880	None	DQ458863
D. sapinea	South Africa	CBS 109726	KX464094	KX464568	KX463956	KX464800
D. sapinea	Australia	CBS 189.37	KX464099	KX464573	KX463957	KX464808
Dothiorella brevicollis	South Africa	CMW 36464	JQ239404	JQ239391	None	JQ239372
Do. brevicollis	South Africa	CBS 130411 CMW 36463	NR_111703	JQ239390	None	JQ239371
Do. dulcispinae	South Africa	CMW 36461	JQ239401	JQ239388	None	JQ239374
Do. dulcispinae	Namibia	CMW 36460	NR_111702	JQ239387	None	JQ239373
Do. longicollis	Australia	CBS 122068 CMW 26166	NR_136999	EU144069	KX463972	KF766246
Do. longicollis	Australia	CMW 26165	EU144053	EU144068	None	None
Do. oblonga	South Africa	CBS 121765 CMW 25407	EU101301	EU101345	None	KX464862
Do. oblonga	South Africa	CBS 121766 CMW 25408	NR_137689	EU101346	None	KX464863
Do. plurivora	Iran	CBS 124724 IRAN 1557C	KC898225	KC898208	None	KX464874
Do. plurivora	USA, California	CBS 120999	KX464125	KX464617	None	KX464870
Do. pretoriensis	South Africa	CBS 130404 CMW 36480	JQ239405	JQ239392	None	JQ239376
Do. pretoriensis	South Africa	CMW 36481	JQ239406	JQ239393	None	JQ239377
Do. viticola	South Africa	CMW 37928	JX283730	JX283741	None	JX283717
Do. viticola	South Africa	CMW 37933	JX283735	JX283743	None	JX283719
Do. viticola	Spain	CBS 117009	MH863011	AY905559	DQ677985	EU673104
Do. viticola	South Africa	STE-U5048	AY343373	AY343336	EF204479	None
L. americana	USA, Arizona	CERC 1962 CFCC 50066	KP217060	KP217068	None	KP217076

Table 4. *Cont.*

Species	Country	Strain Number	GenBank			
			ITS	***tef1-α***	***rpb2***	***β-tub***
L. americana	USA, Arizona	CERC 1961 CFCC 50065	KP217059	KP217067	None	KP217075
Lasiodiplodia chonburiensis	Thailand	MFLUCC 16-0376	MH275066	MH412773	None	MH412742
L. citricola	Iran	IRNKB3	MN634040	MN633994	None	None
L. citricola	Iran	CBS 124707 IRAN 1522C	GU945354	GU945340	KP872455	KU887505
L. citricola	Iran	CBS 124706 IRAN 1521C	GU945353	GU945339	KP872456	KU887504
L. crassispora	Venezuela	CMW 13488	DQ103552	DQ103559	KP872458	KU887507
L. crassispora	Australia	CBS 118741 WAC 12533	NR_111194	EU673303	KP872457	KU887506
L. exigua	Tunisia	CBS 137785 BL104	KJ638317	KJ638336	KU696355	KU887509
L. exigua	Tunisia	BL184	KJ638318	KJ638337	None	None
L. gonubiensis	South Africa	CBS 115812 CMW 14077	DQ458892	DQ103566	KP872464	KU887512
L. gonubiensis	South Africa	CBS 116355 CMW 14078	AY639594	DQ103567	KP872465	KU887513
L. lignicola	Thailand	MFLUCC 11-0435	JX646797	KP872375	KP872470	JX646845
L. lignicola	India	SUF161	MT081525	None	None	None
L. magnoliae	China	MFLUCC 18-0948	MK499387	MK568537	None	MK521587
L. mahajangana	Madagascar	CMW 27801	FJ900595	FJ900641	KP872471	FJ900630
L. mahajangana	Madagascar	CMW 27818	FJ900596	FJ900642	KU696366	FJ900631
L. margaritacea	Australia	CBS 122519 CMW 26162	NR_136998	EU144065	KP872473	KX464903
L. pandanicola	Thailand	MFLUCC 16-0265	MH275068	MH412774	None	None
L. pandanicola	China	GBLZ16BO-008	MN540679	None	None	MN539183
L. pseudotheobromae	Costa Rica	CBS 116459 CMW 40939	EF622077	EF622057	KU696376	EU673111

Table 4. *Cont*.

Species	Country	Strain Number	GenBank			
			ITS	***tef1-α***	***rpb2***	***β-tub***
L. pseudotheobromae	Zaire	CBS 374.54	KX464139	KX464633	None	KX464906
L. pseudotheobromae	China	BJFU ZYP151106-14	KX499902	KX499940	KX499977	None
L. pyriformis	Namibia	CBS 121771 CMW 25415	EU101308	EU101353	KU696379	KU887528
L. pyriformis	Namibia	CMW 25416	EU101309	EU101354	None	None
L. pyriformis	Namibia	CBS 121770 CMW 25414	NR_136993	EU101352	KP872483	KU887527
L. theobromae	Papua New Guinea	CBS 164.96 CMW 50942	AY640255	AY640258	KU696383	KU887532
L. theobromae	unknown	CBS 111530	EF622074	EF622054	KU696382	None
L. vaccinii	China, Beijing	CGMCC 3.19022	MH330318	MH330327	MH330321	MH330324
L. vaccinii	China, Beijing	CGMCC3.19256	MK157139	MK157166	MK157148	MK157157
Neofusicoccum parvum	South Africa	CMW 41213	KP860849	KP860693	KU587896	KP860771
N. parvum	New Zealand	CBS 138823 CMW 9081	NR_119487	AY236888	EU821963	AY236917
N. ribis	USA, New York	CMW 7772	AY236935	AY236877	EU863170	AY236906
N. ribis	USA, New York	CMW 7773	AY236936	AY236878	EU863169	AY236907
N. umdonicola	South Africa	CMW 14058	EU821904	EU821874	EU821934	EU821844
N. umdonicola	South Africa	CBS 123646 CMW 14060	EU821905	EU821875	EU821935	KF766145
Oblongocollomyces variabilis	Namibia/RSA	CMW 36482	JX283726	JX283738	None	JX283714
O. variabilis	Namibia	CBS 121774 CMW 25419	NR_136994	EU101357	KX464053	JX283715
Sphaeropsis porosa	South Africa	CBS 110496 CPC 5132	NR_119492	AY343340	KX464076	EU673130
Melanops tulasnei	Germany	CBS 116805	FJ824769	None	None	FJ824780
M. tulasnei	Germany	CBS 116806	FJ824770	FJ824775	KX463998	FJ824781

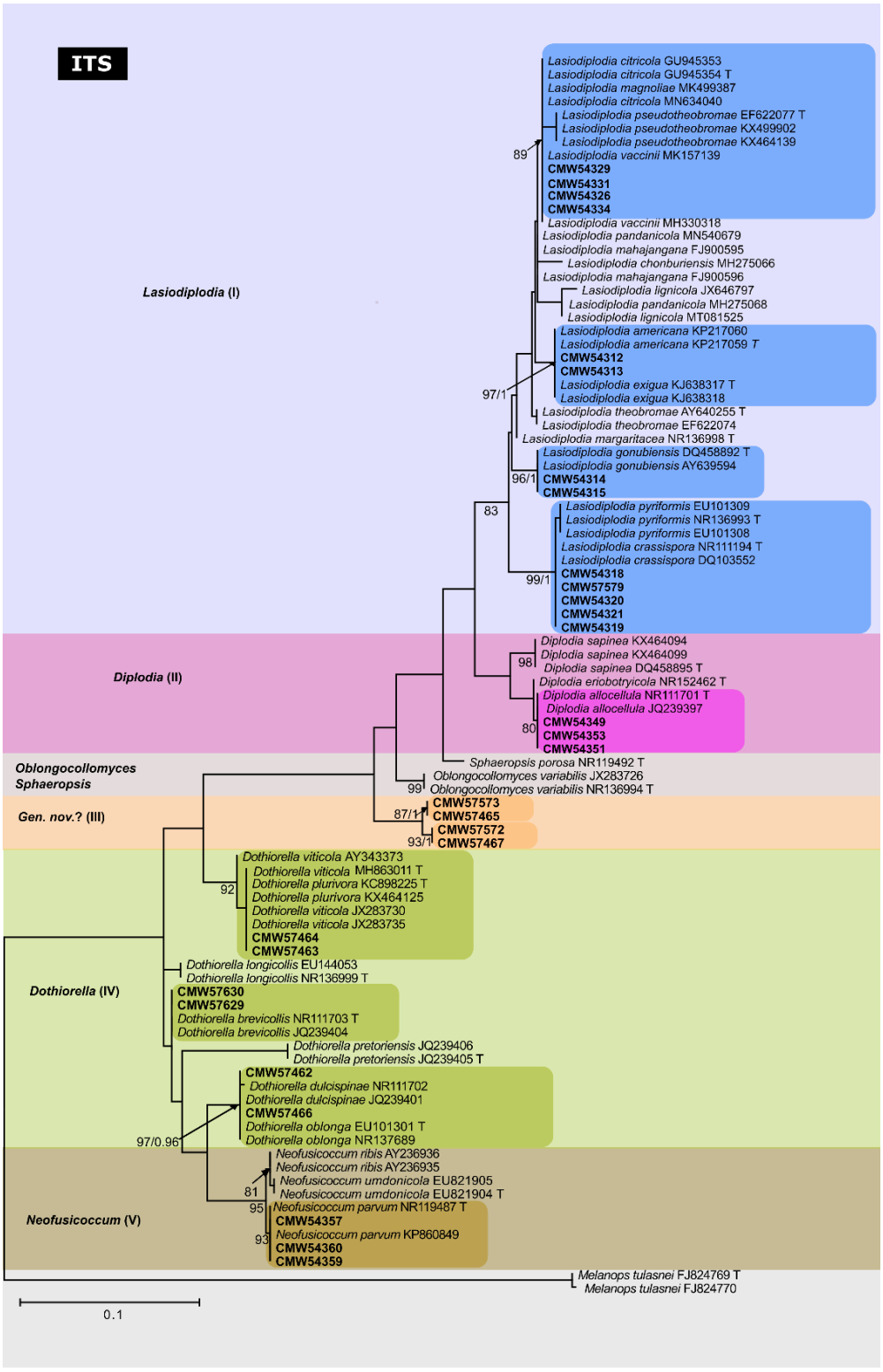

Figure 2. Maximum likelihood (ML) phylogenetic tree resulting from analyses of the ITS dataset. Bootstrap support values above 70% and Bayesian posterior probability values above 0.95 are shown at the nodes. Isolates in bold were obtained in this study. The tree was rooted to *Melanops tulasnei*.

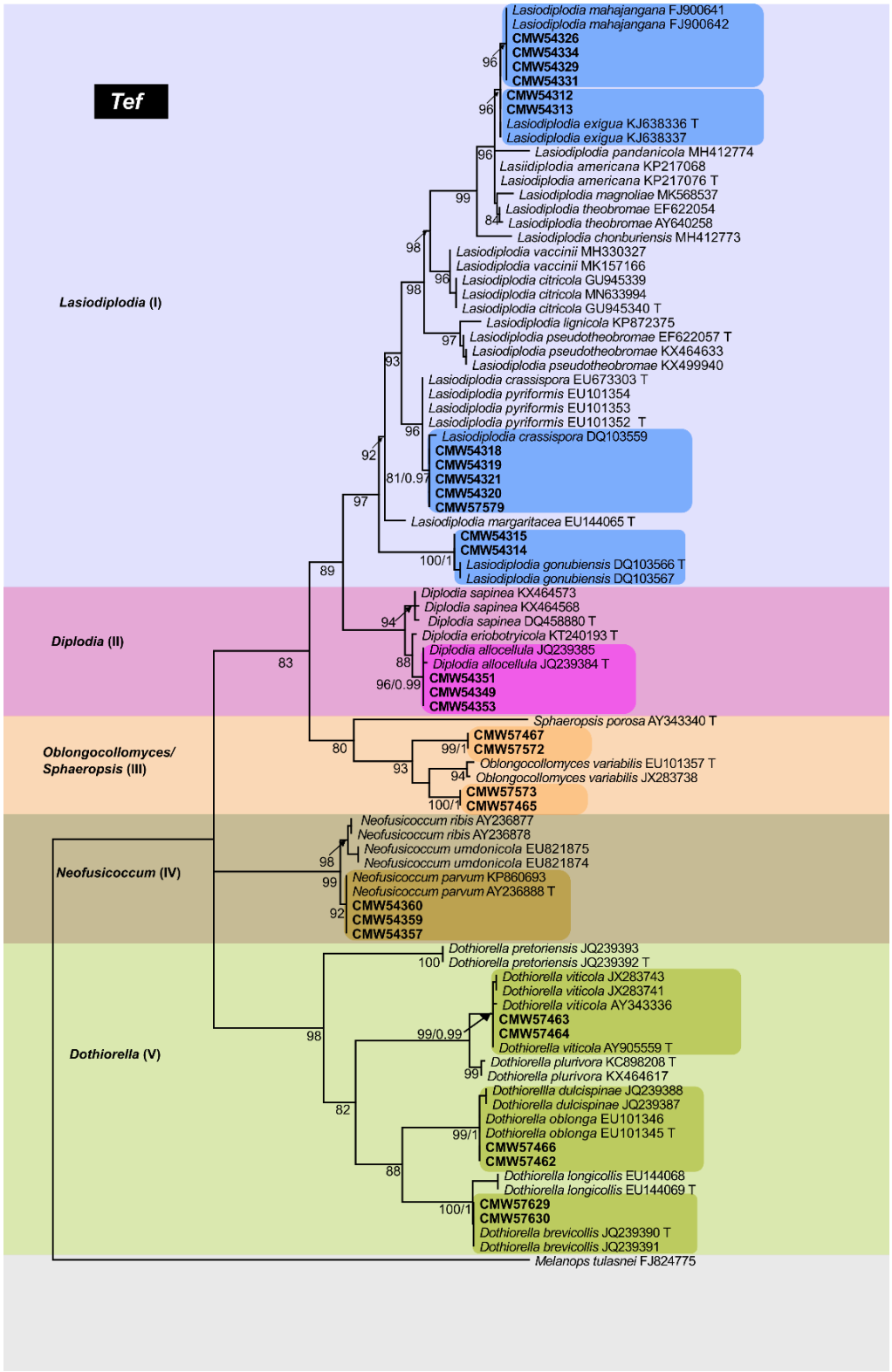

Figure 3. Maximum likelihood phylogenetic tree obtained from analyses of the *tef-1α data* including species of *Botryosphaeriaceae*. Bootstrap values >70% and posterior probabilities values >0.95 are shown at the nodes. Isolates in bold were obtained during this study. The tree was rooted to an isolate of *Melanops tulasnei*.

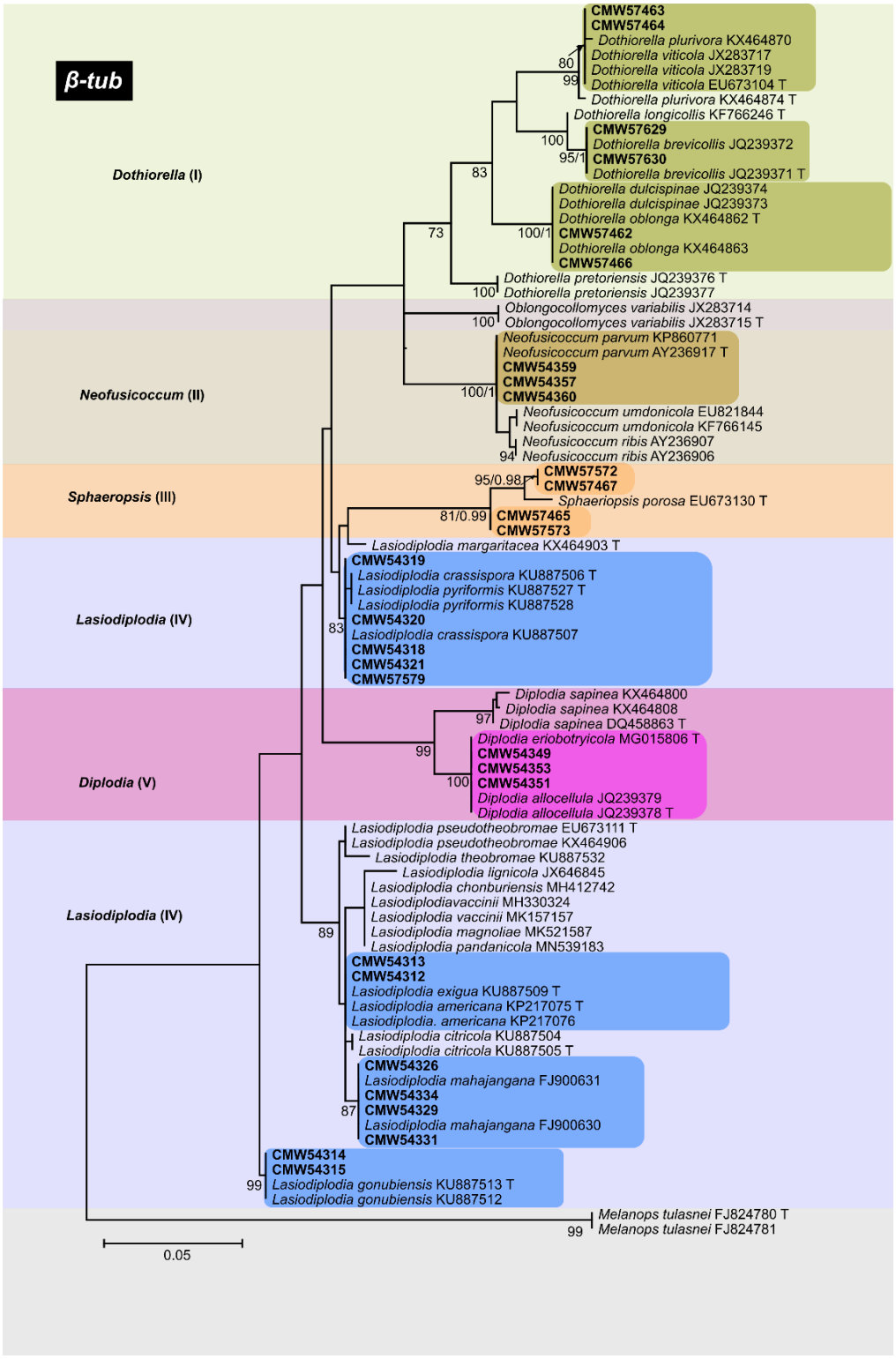

Figure 4. Phylogenetic tree obtained from ML and BI analyses of the *β-tub* dataset. Bootstrap values (>70%) and PP values (>0.95) appear at the nodes. Isolates in bold were obtained during this study. The tree was rooted to isolates of *Melanops tulasnei*.

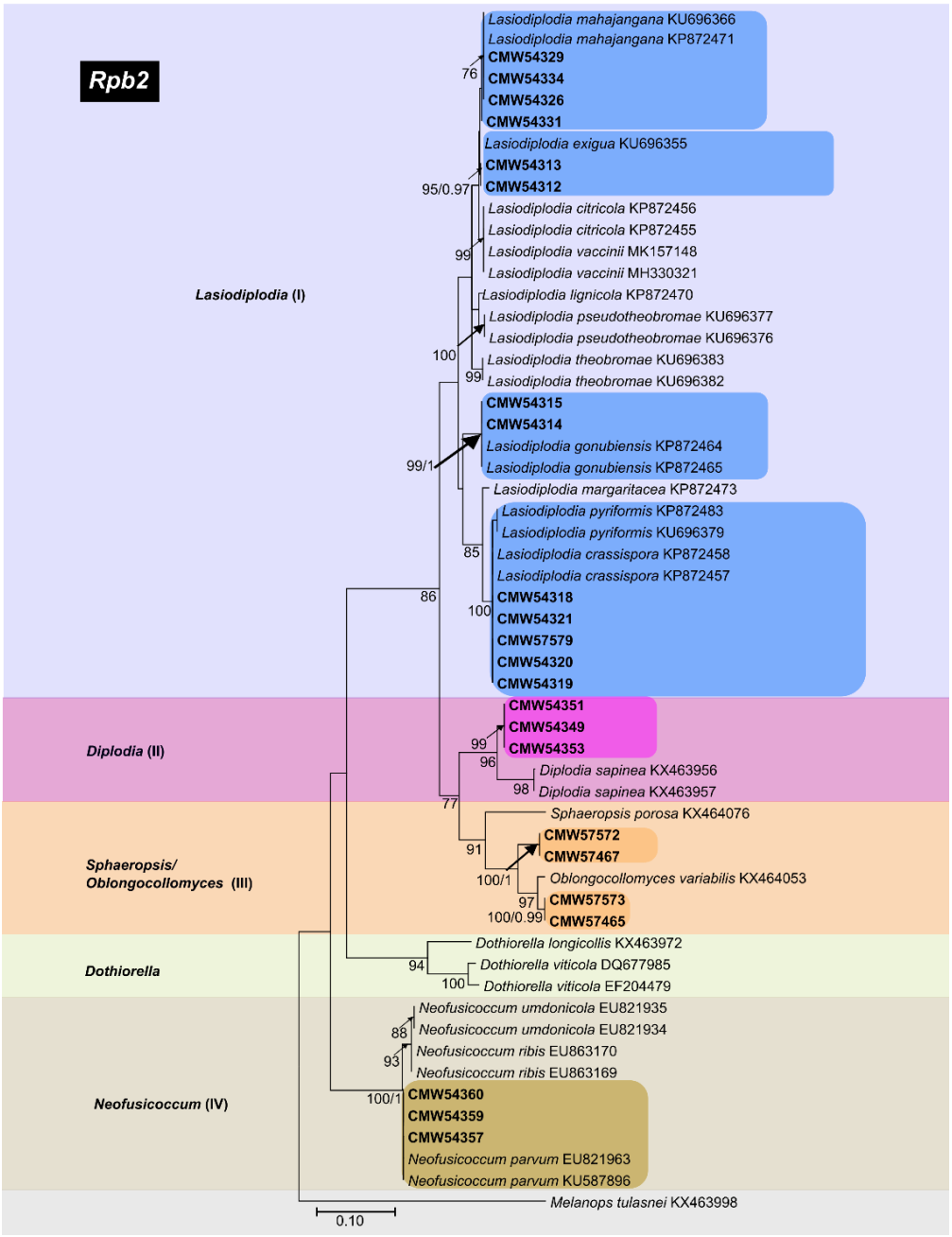

Figure 5. Maximum likelihood (ML) phylogenetic tree based on analyses of the *rpb2* dataset showing relationship between isolates obtained in this study (in bold) and species of *Botryosphaeriaceae*. Bootstrap values (>70%) and PP values (>0.95) appear at the nodes. The tree was rooted to an isolate of *Melanops tulasnei*.

3.3.1. ITS Phylogeny

The phylogeny obtained from ML and BI analyses of the ITS sequence dataset grouped isolates obtained in this study into five clades representing five genera of *Botryosphaeriaceae* (Figure 2). The five clades represented *Lasiodiplodia* (clade I), *Diplodia* (clade II), *Oblongocollomyces*/*Sphaeropsis* (clade III), *Dothiorella* (clade IV) and *Neofusicoccum* (clade V). In the *Lasiodiplodia* clade, four isolates (CMW54326, CMW54329, CMW54331 and CMW54334) could not be identified. These isolates clustered with *L. citricola*, *L. magnoliae*, *L. pseudotheobromae* and *L. vaccinii*. Isolates CMW54312 and CMW54313 grouped with *L. americana* and *L. exigua*. Two isolates, CMW54314 and CMW54315 formed a monophyletic clade with *L. gonubiensis*. Isolates CMW54318, CMW54319, CMW54320, CMW54321 and CMW57579

formed a polytomy with *L. crassispora*, separate from *L. pyriformis*. The *Diplodia* clade included *D. allocellula*, *D. eriobotryicola* and *D. sapinea* sequences. Three isolates obtained in this study (CMW54349, CMW54351 and CMW54353) formed a polytomy with *D. allocellula,* separate from *D. eriobotrycola.* Clade III included four isolates (CMW57573, CMW57465, CMW57573 and CMW57467) that formed a distinct clade from previously described species of *Botryosphaeriaceae*. The four isolates formed two sub-clades indicating that they possibly represent two closely related but distinct species. These isolates could not be assigned to a species based on ITS sequence data. These isolates were analyzed separately based on the ITS, *tef-1α*, *β-tub* and *rpb2* datasets (Figure S1). Clade IV included *Dothiorella* sequences. Two isolates, CMW57463 and CMW57464 grouped with *Do. plurivora* and *Do. viticola*. Isolates CMW57629 and CMW57630 formed a polytomy within the genus based on the ITS sequence data. Isolates CMW57462 and CMW57466 grouped with *Do. dulcispinae* and *Do. oblonga*. The *Neofusicoccum* clade included *N. parvum, N. ribis* and *N. umdonicola* sequences, with isolates obtained in this study (CMW54357, CMW54359 and CMW54360) corresponding to *N. parvum*.

3.3.2. *Tef-1α* Phylogeny

The *tef-1α* phylogeny separated sequences from the isolates obtained in this study into five clades. These clades represented *Lasiodiplodia* (clade I), *Diplodia* (clade II), *Oblongocollomyces/Sphaeropsis* (clade III), *Neofusicoccum* (clade IV) and *Dothiorella* (clade V) (Figure 3). The *Lasiodiplodia* clade included isolates (CMW54326, CMW54329, CMW54331 and CMW54334) that grouped with *L. mahajangana*. Two isolates, CMW54312 and CMW54313 formed a polytomy with *L. exigua*. Isolates CMW54318, CMW54319, CMW54320, CMW54321 and CMW57579 grouped with *L. crassispora.* Isolates CMW54314 and CMW54315 grouped with *L. gonubiensis*. The *Diplodia* clade included *D. allocellula*, *D. eriobotryicola* and *D. sapinea* sequences, with isolates obtained in this study (CMW54349, CMW54351 and CMW54353) corresponding to *D. allocellula*. Clade III included four isolates that could not be identified to species level based on the *tef-1α* sequence data. Two isolates (CMW57573 and CMW57465) formed a sister clade with *O. variabilis.* The other two isolates (CMW57467 and CMW57572) formed a sister clade with both *O. variabilis* and the other two isolates (CMW57573 and CMW57465). These isolates could not be assigned to a species because *O. variabilis* is the only species in the genus, and the four isolates did not form a monophyletic group with this species. Therefore, these isolates are referred to as *Oblongocollomyces* sp. 1 (CMW57467 and CMW57572) and *Oblongocollomyces* sp. 2 (CMW57573 and CMW57465). The *Neofusicoccum* clade included isolates (CMW54357, CMW54359 and CMW54360) that formed a monophyletic clade with *N. parvum.* Clade V included *Dothiorella* sequences. Isolates CMW57463 and CMW57464 grouped with *Do. viticola.* Isolates CMW57462 and CMW57466 formed a polytomy with *Do. oblonga*, separate from *Do. dulcispinae*. Isolates CMW57629 and CMW57630 formed a polytomy with *Do. brevicollis,* separate from *Do. longicollis*.

3.3.3. *β-Tub* Phylogeny

The phylogeny emerging from ML and BI analyses of the *β-tub* dataset separated isolates obtained in this study into clades corresponding to five genera of *Botryosphaeriaceae* including *Dothiorella* (clade I), *Neofusicoccum* (clade II), *Sphaeropsis* (clade III), *Lasiodiplodia* (IV) and *Diplodia* (V) (Figure 4). The *β-tub* phylogeny varied from the other gene trees on the placement of some isolates obtained in this study. For example, the *Lasiodiplodia* clade including isolates corresponding to *L. crassispora* formed a separate clade from other *Lasiodiplodia* species. In the *Dothiorella* clade, two isolates (CMW57463 and CMW57464) grouped with *Do. plurivora* and *Do. viticola*. Isolates CMW57629 and CMW57630 grouped with *Do. brevicollis.* Isolates CMW57462 and CMW57466 grouped with *Do. dulcispinae* and *Do. oblonga*. The *Neofusicoccum* clade included *N. parvum, N. ribis* and *N. umdonicola* sequences. Isolates obtained in this study (CMW54357, CMW54359 and CMW54360) grouped with *N. parvum*. The four isolates (CMW57467, CMW57572, CMW57465 and CMW57573) that

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

3.3.4. *rpb2* Phylogeny

The *rpb2* phylogeny separated isolates obtained in this study into four clades. These clades represented *Lasiodiplodia* (clade I), *Diplodia* (clade II), *Oblongocollomyces/Sphaeropsis* (clade III) and *Neofusicoccum* (clade IV) (Figure 5). Four isolates in the *Lasiodiplodia* clade (CMW54326, CMW54329, CMW54331 and CMW54334) grouped with *L. mahajangana*. Two isolates, CMW54312 and CMW54313 grouped with *L. exigua.* Isolates CMW54314 and CMW54315 formed a monophyletic clade with *L. gonubiensis*. Isolates CMW54318, CMW54319, CMW54320, CMW54321 and CMW57579 formed a polytomy with *L. crassispora*, separate from *L. pyriformis*. The *Diplodia* clade included *D. sapinea* sequences only because reference sequences for other *Diplodia* species for the *rpb2* gene region are not available on public databases. Three isolates (CMW54349, CMW54351 and CMW54353) identified as *D. allocellula* on the ITS, *tef1-α* and *β-tub* phylogenetic trees formed a sister clade with *D. sapinea* indicating that they reside in the same genus. Clade III included *O. variabilis* and *S. porosa* sequences. Two isolates obtained in this study (CMW57465 and CMW57573) formed a sister clade with *O. variabilis*. The other two isolates (CMW57467 and CMW57572) formed a sister clade with both *O. variabilis* and the other two isolates (CMW57573 and CMW57465). These isolates could not be assigned to a species based on the *rpb2* sequence data and are, thus, referred to as *Oblongocollomyces* sp. 1 (CMW57467 and CMW57572) and *Oblongocollomyces* sp. 2 (CMW57465 and CMW57573). The *Dothiorella* clade did not include any isolates obtained in this study because amplicons for *Dothiorella* isolates could not be obtained despite efforts to sequence two different primer sets. The *Neofusicoccum* clade included *N. parvum, N. ribis* and *N. umdonicola* sequences. Three isolates obtained in this study (CMW54357, CMW54359 and CMW54360) grouped with *N. parvum*.

3.4. Species Diversity Distribution

The phylogenies obtained from ML and BI analyses of the four loci distinguished isolates obtained in this study as *D. allocellula*, *Do. brevicollis*, *Do. dulcispinae/Do. oblonga*, *Do. plurivora/Do. viticola*, *L. americana/L. exigua*, *L. crassispora/L. pyriformis*, *L. gonubiensis*, *L. citricola/L. magnoliae/L. pseudotheobromae/L. vaccinii*, *L. mahajangana*, *N. parvum*, *Oblongocollomyces* sp. 1 and *Oblongocollomyces* sp. 2.

Isolates CMW54312 and CMW54313 that grouped with *L. americana* and *L. exigua* are treated as *L. exigua* based on a previous study that reduced *L. americana* to synonymy with *L. exigua* [37]. Four isolates (CMW54326, CMW54329, CMW54331 and CMW54334) that grouped with *L. citricola*, *L. magnoliae*, *L. pseudotheobromae* and *L. vaccinii* on the ITS phylogeny grouped with *L. mahajangana* on the *tef1-α*, *β-tub* and *rpb2* phylogenies. Therefore, these isolates are treated as *L. mahajangana*. Isolates CMW54318, CMW54319, CMW54320, CMW54321 and CMW57579 that grouped with *L. crassispora* and *L. pyriformis* are treated as *L. crassispora* as they formed unresolved groups and based on an earlier study that reduced *L. pyriformis* to synonymy with *L. crassispora* [38]. Similarly, isolates CMW57462 and CMW57466 that grouped with *Do. dulcispinae* and *Do. oblonga* are treated as *Do. dulcispinae* as they formed unresolved groups and based on a study that reduced *Do. oblonga* to synonymy with *Do. dulcispinae* [38]. Isolates CMW57463 and CMW57464 that

grouped with *Do. plurivora* and *Do. viticola* are treated as *Do. viticola* based on analyses of the *tef1-α* sequence data.

Eleven species of *Botryosphaeriaceae* including *D. allocellula*, *Do. brevicollis*, *Do. dulcispinae*, *Do. viticola*, *L. crassispora*, *L. exigua*, *L. gonubiensis*, *L. mahajangana*, *N. parvum*, *Oblongocollomyces* sp. 1 and *Oblongocollomyces* sp. 2 were identified as endophytes on tree species of *Anacardiaceae* in this study. Identities of the 11 species were assigned to all 404 *Botryosphaeriaceae* isolates based on the combined results of the morphogroup identification and phylogenetic analyses of the ITS, *tef1-α*, *β-tub* and *rpb2* sequence datasets (Table 5).

Table 5. Number of *Botryosphaeriaceae* isolates identified based on morphological and phylogenetic grouping.

Location	Host	Identity	Number of Isolates
Tshikundamalema	*Sclerocarya birrea*	*Lasiodiplodia crassispora*	10
		Lasiodiplodia mahajangana	7
		Neofusicoccum parvum	3
Tshikundamalema	*Mangifera indica*	*Lasiodiplodia crassispora*	28
		Lasiodiplodia mahajangana	26
		Neofusicoccum parvum	4
Tshipise	*Sclerocarya birrea*	*Lasiodiplodia mahajangana*	25
		Neofusicoccum parvum	4
Tshipise	*Mangifera indica*	*Lasiodiplodia crassispora*	3
		Lasiodiplodia mahajangana	5
		Neofusicoccum parvum	34
Kruger	*Lannea schweinfurthii*	*Diplodia allocellula*	25
		Lasiodiplodia crassispora	27
		Lasiodiplodia mahajangana	6
Kruger	*Sclerocarya birrea*	*Diplodia allocellula*	29
		Dothiorella dulcispinae	6
		Lasiodiplodia crassispora	9
		Lasiodiplodia gonubiensis	2
		Lasiodiplodia mahajangana	7
Nwanedi	*Lannea schweinfurthii*	*Dothiorella brevicollis*	34
		Lasiodiplodia mahajangana	20
Mapungubwe	*Sclerocarya birrea*	*Dothiorella brevicollis*	20
		Dothiorella dulcispinae	2
		Dothiorella viticola	6
		Lasiodiplodia crassispora	15
		Lasiodiplodia exigua	3
		Lasiodiplodia mahajangana	40
		Oblongocollomyces sp. 1	2
		Oblongocollomyces sp. 2	2

The number of *Botryosphaeriaceae* species identified on the three tree species of *Anacardiaceae* varied across the different sampling sites in disturbed and undisturbed ecosystems (Figure 6). The highest species diversity was observed on *S. birrea* in Mapungubwe National Park with eight species identified.

There was variation in species diversity and distribution of *Botryosphaeriaceae* on the trees of *Anacardiaceae* in disturbed and undisturbed ecosystems (Figure 7). Eight species were unique to trees in undisturbed ecosystems, two occurred on trees in both disturbed and undisturbed ecosystems and only one species was unique to trees in disturbed ecosystems.

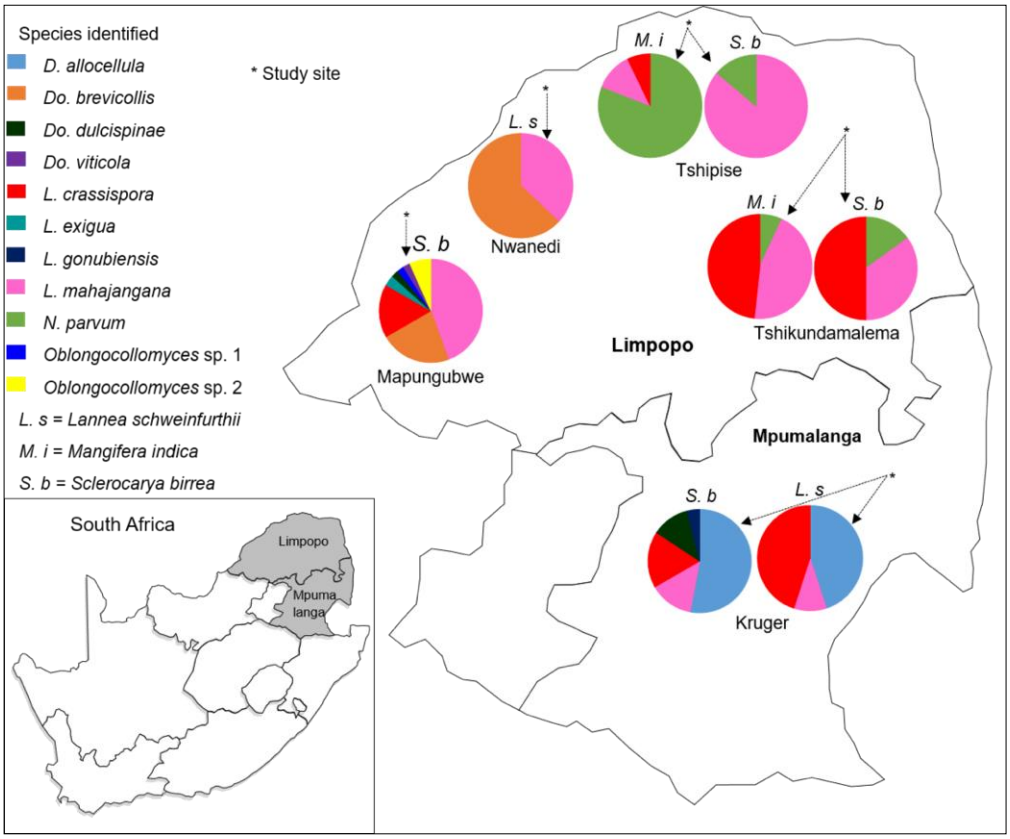

Figure 6. Map indicating species diversity and distribution of the *Botryosphaeriaceae* on *Sclerocarya birrea*, *Mangifera indica* and *Lannea schweinfurthii* trees in Mapungubwe, Nwanedi, Tshipise, Tshikundamalema and Kruger National Park. Insert: Map of South Africa with the two provinces in which the sampling sites reside shown in grey.

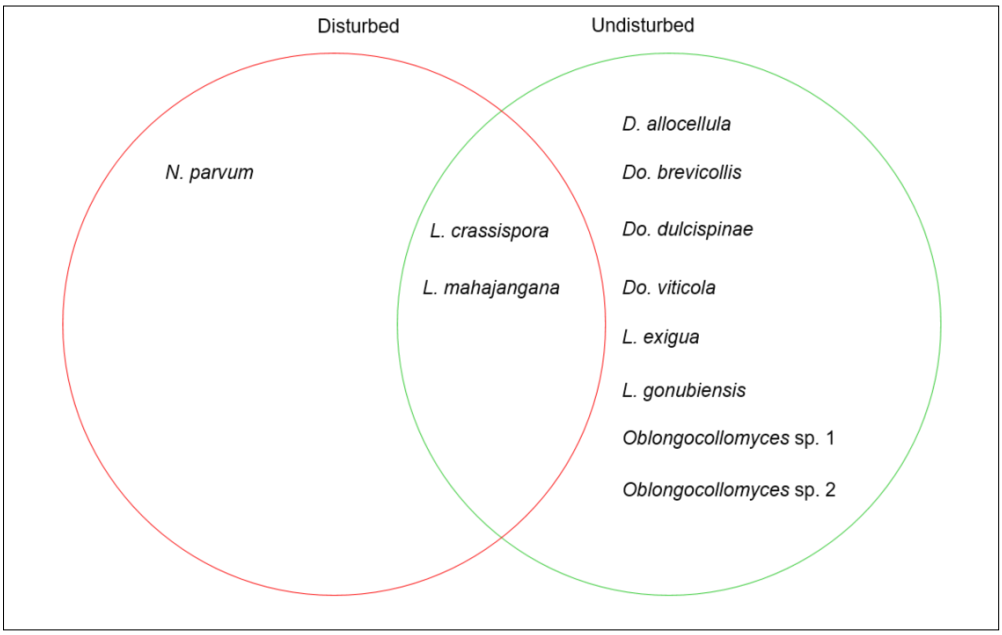

Figure 7. Venn diagram indicating species diversity and distribution of the *Botryosphaeriaceae* on tree species of the *Anacardiaceae* in disturbed (including *S. birrea* and *M. indica* at Tshikundamalema and Tshipise) and undisturbed (including *S. birrea* and *L. schweinfurthii* at Nwanedi, Mapungubwe and Kruger National Park) ecosystems.

Some species of *Botryosphaeriaceae* displayed the ability to infect both native and non-native *Anacardiaceae* (Figure 8). *Lasiodiplodia crassispora* and *L. mahajangana* overlapped on all three tree species of the *Anacardiaceae*. *Neofusicoccum parvum* overlapped between *S. birrea* (native) and *M. indica* (non-native) where they were growing next to each other at the two disturbed ecosystems. *Diplodia allocellula* and *Do. brevicollis* overlapped between native *Anacardiaceae* (*S. birrea* and *L. schweinfurthii*) in undisturbed ecosystems. Some species were only found on one tree species. These included *Do. dulcispinae, Do. viticola, L. exigua, L. gonubiensis, Oblongocollomyces* sp. 1 and *Oblongocollomyces* sp. 2 on *S. birrea*.

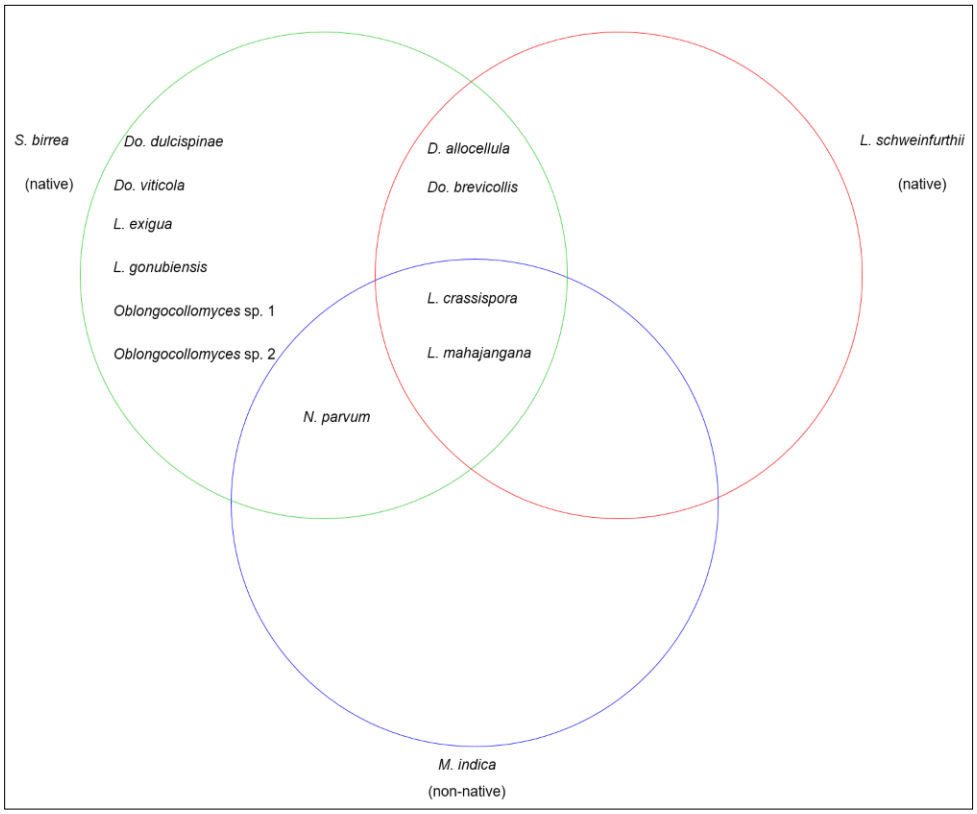

Figure 8. Venn diagram showing species overlap of *Botryosphaeriaceae* on *Sclerocarya birrea*, *Lannea schweinfurthii* and *Mangifera indica*.

4. Discussion

In this study, we report the presence of at least 11 *Botryosphaeriaceae* species, namely *D. allocellula*, *Do. brevicollis*, *Do. dulcispinae*, *Do. viticola*, *L. crassispora*, *L. exigua*, *L. gonubiensis*, *L. mahajangana*, *N. parvum*, *Oblongocollomyces* sp. 1 and *Oblongocollomyces* sp. 2 on three tree species of *Anacardiaceae* in the north-eastern parts of South Africa. The majority of these species (10) occurred on trees in undisturbed (isolated and/or protected) ecosystems, compared to only three species in disturbed (agriculture and forestry) ecosystems. Three species overlapped on both native and non-native species. Some species identified in this study are reported for the first time in South Africa or on specific hosts.

The high species diversity recorded on trees in undisturbed ecosystems reflects conclusions from a previous study that biodiversity in natural ecosystems increases fungal diversity compared to ecosystems frequently disturbed by human activities such as clear-cutting, selective cutting and land-use changes [39]. Species such as *D. allocellula*, *Do. brevicollis*, *Do. dulcispinae*, *Do. viticola*, *L. exigua*, *L. gonubiensis, Oblongocollomyces* sp. 1 and *Oblongocollomyces* sp. 2 were identified on trees in undisturbed ecosystems only. The influence of human activity on *Botryosphaeriaceae* species composition was previously reported by Pavlic et al. [40]. In that study, *N. parvum* was predominant on *S. cordatum* in disturbed stands and absent in undisturbed stands where other *Neofusicoccum* species dominated.

Our results indicate that activities between the two ecosystems (including deforestation in disturbed ecosystems, opposed to rich plant diversity in undisturbed ecosystems) influence *Botryosphaeriaceae* species composition. The absence of *N. parvum* on trees in undisturbed ecosystems despite its known presence in the region suggests that undisturbed ecosystems are more resilient to invasion by some invasive *Botryosphaeriaceae*. This hypothesis is worth testing, as this would imply a potential influence of conserved areas on the spread of invasive species of *Botryosphaeriaceae*. At the same time, such areas might serve as a reservoir of new species that might infect agricultural and forestry tree species.

Lasiodiplodia crassispora and *L. mahajangana* were common on the three tree species in disturbed and undisturbed ecosystems. Organisms with a broad niche are often prominent colonizers of disturbed environments and are also successful invaders in new regions [40,41]. The presence of *L. crassispora* and *L. mahajangana* in both ecosystems was not surprising from the perspective that *Lasiodiplodia* species occurs predominantly in tropical and subtropical regions, such as those sampled in this study [8,13,31]. Furthermore, the two species have been reported in the region before and on tree species of *Anacardiaceae* [13]. *Lasiodiplodia crassispora* has been reported on *P. angolensis* in Mpumalanga [42] and *S. birrea* in Limpopo and Mpumalanga [13], while *L. mahajangana* is known to occur on *Adansonia digitata*, *E. ingens* and *S. birrea* in Limpopo and Mpumalanga provinces [8,13,31].

Neofusicoccum parvum was the only species unique to trees in disturbed, agricultural ecosystems. *Neofusicoccum parvum* was also the dominant species overlapping between *S. birrea* (native) and *M. indica* (non-native) trees in disturbed ecosystems. These results confirm the ease with which this important global pathogen spreads between native and non-native hosts in human-disturbed ecosystems [12,13,40]. The abundance and distribution of *Neofusicoccum* species, and *N. parvum* in particular, on *Syzygium cordatum,* has been linked to environmental disturbance and host composition through human activities [40]. Our results provide further support for this hypothesis.

Our study is not the first to investigate species overlap of *Botryosphaeriaceae* on native and non-native *Anacardiaceae* in South Africa. Mehl et al. [13] also reported an overlap of *N. parvum* on *S. birrea* and *M. indica* in the Hoedspruit area, Mpumalanga Province. The absence of *N. parvum* on *S. birrea* trees in undisturbed areas suggests that the fungus is not indigenous to native hosts, but it is spreading from a non-native (*M. indica*) to a native (*S. birrea*) host in disturbed ecosystems, rather than *vice versa.*

This study represents the first report of *L. exigua* in South Africa. *Lasiodiplodia exigua* was described from *Retama raetam* in Tunisia and *Pistacia vera* in the United States [43]. The fungus has also been reported to cause canker, dieback, discolouration and streaks on grapevine wood in Turkey and Mexico [44,45]. In the present study, *L. exigua* was isolated from *S. birrea* at Mapungubwe National Park. It is curious that this species was identified on a native host in an isolated low disturbance site only, even though it is known from other hosts globally. This demonstrates the ability of this fungus to spread between continents and hosts. Our results indicate that other species that are thought to not occur in the country might exist on unsampled hosts in conserved areas.

Dothiorella brevicollis, *Do. dulcispinae*, *Do. viticola*, *Oblongocollomyces* sp. 1 and *Oblongocollomyces* sp. 2 on *S. birrea*, and *L. crassispora* on *M. indica* are first reports on these hosts, albeit that these hosts were sampled before for the *Botryosphaeriaceae* in South Africa. *Dothiorella brevicollis* and *Do. dulcispinae* appear to have a narrow host range and limited distribution in South Africa, while on the other hand, *Do. viticola* appears to have a much wider host and geographic distribution. *Dothiorella brevicollis* and *Do. dulcispinae* were both described from *V. karroo* in South Africa and they have not been reported on any other host elsewhere [46]. Therefore, *S. birrea* is the second host for the two species. *Dothiorella viticola* was first described as a saprophyte from declining *V. vinifera* in Spain [47]. In South Africa, the fungus has been reported on *Celtis africana*, *Gymnosporia buxifolia*, *Prunus persica*, *Podocarpus henkelii*, *Senegalia mellifera*, *V. karroo* and *V. vinifera* [21]. *Lasiodiplodia crassispora* is known to occur on *M. indica* in Brazil [48]. On native hosts, *L. crassispora* has been reported on *P. angolensis* [42] and *S. birrea* [13] in Mpumalanga. These species are not necessarily

new introductions, but their discovery might have been influenced by the more extensive (geographic and host) sampling in this study compared to previous efforts.

The six species, *Do. brevicollis*, *Do. dulcispinae, Do. viticola, L. exigua, Oblongocollomyces* sp. 1 and *Oblongocollomyces* sp. 2 recorded on *S. birrea* for the first time in this study increases the number of *Botryosphaeriaceae* species known on this host to 17. Some species known to occur on *S. birrea* in South Africa were not identified in this study. These include *B. fabicerciana, L. iraniensis, N. mediterraneum, N. umdonicola* and *N. vitifusiforme* [13]. Except for *N. umdonicola* and *N. vitifusiforme,* the other three species that were not isolated in this study are possibly alien and they were recently identified in South Africa [13]. Timing and location of sampling likely influence these outcomes of species diversity on this host and more *Botryosphaeriaceae* can be expected in previously unsampled areas.

The three fungal species identified on *M. indica*, namely *L. crassispora*, *L. mahajangana* and *N. parvum* are well-known pathogens of this host worldwide [22,48–50]. These species have been reported to cause mango diseases in countries such as Brazil [48], Egypt [49] and Thailand [22]. Only two of the identified species, *L. mahajangana* and *N. parvum* are known to occur on *M. indica* in South Africa [13,22]. This is the first study to report the presence of *L. crassispora* on *M. indica* in South Africa, which increases the number of *Botryosphaeriaceae* species known to occur on *M. indica* in South Africa to 11.

To our knowledge, this is the first study to consider the presence of *Botryosphaeriaceae* on *L. schweinfurthii* in South Africa. Four species, including *D. allocellula*, *Do. brevicollis*, *L. crassispora* and *L. mahajangana* were identified on this host in undisturbed ecosystems. *Diplodia allocellula* has been reported on *V. karroo* in Gauteng [46] and *S. birrea* in Mpumalanga [13]. *Dothiorella brevicollis* has only been reported on *V. karroo* and was isolated on *S. birrea* in this study, making *L. schweinfurthii* a third host for the fungus. *Lasiodiplodia mahajangana* is known to occur on *Adansonia digitata* [31], *E. ingens* [8], *M. indica* and *S. birrea* [13] in Limpopo and Mpumalanga, while *L. crassispora* is known to occur on *S. birrea* and *Pterocarpus angolensis* in Mpumalanga and Kwa-Zulu Natal [13,42]. The presence of these fungi on *L. schweinfurthii* supports the assumption that species of *Botryosphaeriaceae* occur on virtually all woody tree species that have been sampled and many species remain undiscovered on native hosts in unexplored areas [9].

We isolated four isolates for which sequences could not be identified to species level. These sequences clustered with *Oblongocollomyces* and *Sphaeropsis* species confirming that the two genera are closely related. These sequences were phylogenetically close to *O. variabilis* but formed separate sub-clades indicating that they represent two species in the genus *Oblongocollomyces*. *Oblongocollomyces* was introduced as a monotypic genus to accommodate *O. variabilis* [51]. In earlier studies, this species was identified as *Sphaeropsis variabilis*. *Oblongocollomyces* differ from *Sphaeropsis* by forming long conidiomatal necks with conidia that can be up to 3-septate [51]. *Oblongocollomyces variabilis* is the only species in the genus thus far and we refer to the two undescribed species as *Oblongocollomyces* sp. 1 and *Oblongocollomyces* sp. 2. The four isolates were obtained from *S. birrea* trees in Mapungubwe National Park. Our findings mirror that of Jami et al. [21] that reported 52 species of *Botryosphaeriales* on 32 native hosts in South Africa, of which 27 species were described as new taxa on native hosts in previously unsampled areas in the region [21]. It is thus not surprising to find these potential new species from a native host (*S. birrea*) in an area (Mapungubwe) that has never been sampled.

Results obtained from analyses of data on tree health assessment show that dieback is common on tree species of *Anacardiaceae* in the north-eastern parts of the Limpopo and Mpumalanga provinces. Various species of *Botryosphaeriaceae* have been reported to cause dieback on various trees globally [8,48,52], but none of the species identified in this study could be consistently linked to these symptoms. This study will, however, lay a foundation for future studies to investigate *Botryosphaeriaceae* species associated with dieback on these trees.

5. Conclusions

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

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/f13020341/s1, Figure S1: Genealogical concordance phylogenetic species recognition (GCPSR) based on analyses of the ITS, *tef-1α*, *β-tub* and *rpb2* sequence data. Bootstrap values above 70% and PP values above 0.95 are shown at the nodes. Isolates in bold were obtained in this study. The trees are rooted to isolates of *Melanops tulasnei* (CBS116805, CBS116806).

Author Contributions: E.R. conducted laboratory work, analyzed the data, and drafted the manuscript. E.K., M.P.A.C. and B.S. conceived the study, assisted with the analyses, contributed to and assisted in writing the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Department of Science and Innovation (DSI)-National Research Foundation (NRF) through the Centre of Excellence in Plant Health Biotechnology (CPHB), grant number 40945.

Acknowledgments: We thank the Department of Science and Innovation (DSI)-National Research Foundation (NRF), Centre of Excellence in Plant Health Biotechnology (CPHB), the University of Venda and the University of Pretoria, South Africa, for financial support.

Conflicts of Interest: The authors declare no conflict of interest.

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