Endophytic *Botryosphaeriaceae*, including five new species, associated with mangrove trees in South Africa

J. Alexander Osorio[a], Casparus J. Crous[b], Z. Wilhelm de Beer[a], Michael J. Wingfield[a], Jolanda Roux[b,*]

[a]Department of Microbiology, DST/NRF Centre of Excellence in Tree Health Biotechnology (CTHB), Forestry and Agricultural Biotechnology Institute (FABI), Faculty of Natural and Agricultural Sciences, University of Pretoria, Private Bag X20, Pretoria, 0028, South Africa.
[b]Department of Plant and Soil Sciences, DST/NRF Centre of Excellence in Tree Health Biotechnology (CTHB), Forestry and Agricultural Biotechnology Institute (FABI), Faculty of Natural and Agricultural Sciences, University of Pretoria, Private Bag X20, Pretoria, 0028, South Africa.

*Corresponding author; e-mail: jolanda.roux@fabi.up.ac.za

Abstract

Little is known regarding the fungi, especially fungal pathogens, associated with mangroves in Africa. This includes fungi in the *Botryosphaeriaceae* that comprise numerous opportunistic, stress-associated pathogens often associated with trees affected by environmental and anthropogenically generated stresses, such as those affecting mangroves. We investigated the occurrence of endophytic *Botryosphaeriaceae* along the entire distribution of mangroves in South Africa. Asymptomatic branches were collected from 10 localities and six mangrove species. Isolates resembling species of *Botryosphaeriaceae* were identified based on multi-gene sequence data of the internal transcribed spacer regions (ITS), including the 5.8S nrRNA, the beta-tubulin (*tub*2), partial translation elongation factor 1-alpha (*tef*1-α) and DNA-directed RNA polymerase II second largest subunit (*rpb*2) gene regions. Inoculation trials were conducted on healthy branches of *Avicennia marina* and *Bruguiera gymnorrhiza* to evaluate the potential pathogenicity of the collected species. Fourteen species in the *Botryosphaeriaceae* belonging to four genera, *Botryosphaeria*, *Diplodia*, *Lasiodiplodia* and *Neofusicoccum* were collected, including five new species. *Neofusicoccum* was the most prevalent genus followed by

Lasiodiplodia, with species of *Diplodia* and *Botryosphaeria* being the least frequent. The inoculation studies revealed that one of the new species, *Lasiodiplodia avicenniae* is highly pathogenic to *A. marina* and could pose a threat to the health of these trees.

Key words: Barringtonia racemosa; Ceriops taga;, Host association patterns; Lumnitzera racemosa; Rhizophora mucronata.

Introduction

Mangroves are trees adapted to grow in saline environments along estuaries and coastal regions, providing important environmental services in the areas where they occur (Tomlinson 1986; De Lacerda 2002; Spalding *et al*. 2010). Over the past few decades, the conservation status of mangroves has received increased attention due to their rapid deterioration globally. This is mostly attributed to industrial development, local human activities and associated pollution of the environment where they grow (Ellison & Farnsworth 1996; World Rainforest Movement 2002; Ellison 2015; Richards & Friess 2016), as well as drought, erosion, sedimentation and changes in water salinity (Ellison 1999; Kathiresan 2002; Upadhyay *et al.* 2002). These factors lead to stress and weakness of trees, predisposing them to colonization by opportunistic pathogens and subsequent disease syndromes (Schoeneweiss 1981; Desprez-Loustau *et al.* 2007; Marçais and Bréda 2007; Sieber 2007; Pautasso *et al.* 2015).

The *Botryosphaeriaceae* (*Fungi*, *Ascomycota*, *Botryosphaeriales*) include important stress-associated pathogens infecting a wide variety of woody-plant species globally. However, it is known that many of these fungi can also exist naturally in healthy plant tissues as symptomless endophytes (Slippers & Wingfield 2007). The biology of fungi in the *Botryosphaeriaceae* can vary depending on the degree of host stress; behaving as primary or opportunistic pathogens or saprobes (Denman *et al.* 2000; Swart *et al.* 2000; Schoch *et al.* 2006; Mehl *et al.* 2013). These fungi are, therefore, expected to play an increasingly important role in tree diseases with the predicted increases in temperature and drought stress associated with global climate change (Desprez-Loustau *et al.* 2006, Slippers & Wingfield 2007). It is

consequently realistic to expect that they could play a role in stress induced diseases of mangrove species globally.

Few reports have revealed the presence of *Botryosphaeriaceae* species in mangrove trees, mostly in the Americas and Asia. These studies include *Botryosphaeria ribis* from *Hibiscus tiliaceus*, a mangrove associate, in the Hawaiian Islands (Stevens & Shear 1929), *Fusicoccum* sp. from symptomatic branches and stems of *Rhizophora mangle* in South Florida (Rayachhetry *et al.* 1996), *Botryosphaeria* sp., *Lasiodiplodia* sp. and *Neofusicoccum* sp. from mangroves in Brazil (De Souza *et al.* 2013) and *Neofusicoccum* sp. from *Sonneratia ovata* in China (Xing *et al.* 2011).

In South Africa, six species of mangroves, namely *Avicennia marina*, *Bruguiera gymnorrhiza*, *Ceriops tagal*, *Lumnitzera racemosa*, *Rhizophora mucronata* and *Xylocarpus granatum* occur in estuaries along the east coast of the country (Steinke 1995, Taylor *et al.* 2003) (Table 1). These trees are distributed from Kosi Bay in the north of the KwaZulu-Natal Province (KZN), where all six species occur, to the Nahoon estuary, near East London in the Eastern Cape Province in the south where mostly *A. marina* and some individuals of *B. gymnorrhiza* are present (Steinke 1999). The mangrove associates, *Barringtonia racemosa* and *Hibiscus tiliaceus*, occur along riverine and coastal areas of the KwaZulu-Natal and Eastern Cape Provinces (Coates & Coates 2002, Lim 2012, South African National Biodiversity Institute–SANBI 2016).

Numerous *Botryosphaeriaceae* species have been reported from South Africa, either from symptomatic or asymptomatic native trees (e.g. Pavlic *et al.* 2007; Mehl *et al.* 2011; Jami *et al.* 2012; Slippers *et al.* 2014), and from non-native trees (e.g. Slippers *et al.* 2007; Bihon *et al.* 2011; Pillay *et al.* 2013). However, nothing is known regarding *Botryosphaeriaceae* species on mangroves in the country (Osorio *et al*. 2014). Consequently, the objective of this study was to investigate the species composition, host range and distribution of these endophytic fungi across multiple mangrove species in South Africa. In addition, inoculation studies were undertaken in order to assess the possible role that these fungi might play in disease development.

Table 1 – Mangrove species, their distribution across 10 sites of the eastern coast of South Africa and their associated Botryosphaeriaceae species.

Sampling sites and mangrove species sampled at each site	No. of branches sampled per host at each site	No. of isolates obtained per host species	No. of branches yielding isolates	(%) of isolates obtained from 20 branches	(%) of branches yielding isolates	Endophytic *Botryosphaeriaceae* isolated from each mangrove species including number of isolates from each species in parenthesis
SITE 1 Kosi Bay						
Avicennia marina	20	2	1	10.0%	5.0%	(1) *Botryosphaeria* sp. (1) *Neofusicoccum umdonicola*,
Bruguiera gymnorrhiza	20	7	5	35.0%	25.0%	(2) *N. parvum*, (5) *N. umdonicola*,
Ceriops tagal	20	3	3	15.0%	15.0%	(1) *Lasiodiplodia gonubiensis*, (1) *N. cryptoaustrale*, (1) *N. umdonicola*,
Lumnitzera racemosa	20	10	9	50.0%	45.0%	(2) *N. cryptoaustrale*, (3) *N. lumnitzerae*, (1) *N. mangroviorum*, (4) *N. umdonicola*,
Rhizophora mucronata	20	0	0	0.0%	0.0%	None
Total	100	22	18	22.0%	18.0%	
SITE 2 St. Lucia						
Avicennia marina	20	3	3	15.0%	15.0%	(2) *Diplodia estuarina*, (1) *N. parvum*,
Bruguiera gymnorrhiza	20	11	9	55.0%	45.0%	(1) *L. gonubiensis*, (2) *N. cryptoaustrale*, (1) *N. kwambonambiense*, (1) *N. mangroviorum*, (4) *N. parvum*, (2) *N. umdonicola*,
Total	40	14	12	35.0%	30.0%	
SITE 3 Mapelane						
Barringtonia racemosa	20	8	8	40.0%	40.0%	(3) *L. theobromae*, (5) *N. parvum*,
Total	20	8	8	40.0%	40.0%	
SITE 4 Richards Bay						
Avicennia marina	20	3	3	15.0%	15.0%	(1) *D. sapinea*, (2) *N. mangroviorum*,
Bruguiera gymnorrhiza	20	3	3	15.0%	15.0%	(2) *L. bruguierae*, (1) *N. umdonicola*,
Barringtonia racemosa	20	11	11	55.0%	55.0%	(3) *L. theobromae*, (8) *N. parvum*,
Rhizophora mucronata	20	9	9	45.0%	45.0%	(1) *D. estuarina*, (8) *N. cryptoaustrale*,
Total	80	26	26	32.5%	32.5%	

SITE 5 Mtunzini						
Avicennia marina	20	2	2	10.0%	10.0%	(2) *N. cryptoaustrale*,
Bruguiera gymnorrhiza	20	9	9	45.0%	45.0%	(7) *L. bruguierae*, (1) *N. luteum*, (1) *N. mangroviorum*,
Total	40	11	11	27.5%	27.5%	
SITE 6 Beachwood						
Avicennia marina	20	9	9	45.0%	45.0%	(3) *L. avicenniae*, (1) *N. cryptoaustrale*, (1) *N. luteum*, (4) *N. mangroviorum*,
Bruguiera gymnorrhiza	20	16	16	80.0%	80.0%	(2) *N. cryptoaustrale*, (1) *N. luteum*, (12) *N. mangroviorum*, (1) *N. parvum*,
Rhizophora mucronata	20	0	0	0.0%	0.0%	None
Total	60	25	25	41.7%	41.7%	
SITE 7 Isipingo						
Avicennia marina	20	2	2	10.0%	10.0%	(1) *L. avicenniae*, (1) *N. luteum*,
Bruguiera gymnorrhiza	20	0	0	0.0%	0.0%	None
Rhizophora mucronata	20	8	6	40.0%	30.0%	(2) *N. cryptoaustrale*, (1) *N. kwambonambiense*, (1) *N. luteum*, (2) *N. mangroviorum*, (1) *N. parvum*, (1) *N. umdonicola*,
Total	60	10	8	16.7%	13.3%	
SITE 8 Mgazana						
Avicennia marina	20	7	7	35.0%	35.0%	(7) *N. cryptoaustrale*,
Bruguiera gymnorrhiza	20	3	3	15.0%	15.0%	(3) *L. bruguierae*,
Rhizophora mucronata	20	5	5	25.0%	25.0%	(2) *N. mangroviorum*, (3) *N. parvum*,
Total	60	15	15	25.0%	25.0%	
SITE 9 Wavecrest						
Avicennia marina	20	0	0	0.0%	0.0%	*None*
Bruguiera gymnorrhiza	20	9	8	45.0%	40.0%	(5) *N. cryptoaustrale*, (1) *N. mangroviorum*, (3) *N. parvum*,
Total	40	9	8	22.5%	20.0%	
SITE 10 Nahoon						
Avicennia marina	20	10	10	50.0%	50.0%	(2) *D. sapinea*, (7) *N. cryptoaustrale*, (1) *N. parvum*,
Bruguiera gymnorrhiza	10	10	9	100.0%	90.0%	(4) *N. cryptoaustrale*, (1) *N. mangroviorum*, (3) *N. parvum*, (2) *N. umdonicola*,
Total	30	20	19	66.7%	63.3%	

Materials and methods

Sample collection

Ten sites where mangrove forests occur were sampled to evaluate the presence, species diversity, host range and spatial distribution of *Botryosphaeriaceae* fungi on these trees in South Africa. Samples were collected from five mangrove species at Kosi Bay (*A. marina, B. gymnorrhiza*, *C. tagal, L. racemosa* and *R. mucronata*), four at Richards Bay (*A. marina, B. gymnorrhiza, R. mucronata* and the mangrove associate *B. racemosa*), three at Beachwood and Isipingo (*A. marina, B. gymnorrhiza* and *R. mucronata*), two from Mtunzini and St. Lucia (*A. marina* and *B. gymnorrhiza*) and one mangrove associate from Mapelane (*B. racemosa*), in the KwaZulu-Natal Province. Three mangrove species were sampled at Mgazana (*A. marina, B. gymnorrhiza* and *R. mucronata*), and two from Nahoon and Wavecrest (*A. marina* and *B. gymnorrhiza*) in the Eastern Cape Province (Table 1 and Fig. 1). The sampling sites were selected to cover the entire geographic range of mangroves in South Africa and to include all mangrove species that occur in the region. At each site, 20 trees of each mangrove species were randomly selected for sample collection. One healthy branch, ~ 10–12 mm in diam and ~ 15 cm in length was collected per tree selected. Samples were placed in paper bags and taken to the laboratories of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria for isolation of possible endophytic *Botryosphaeriaceae*.

The plant material collected was firstly washed to remove traces of mud. Then, sections (1 cm long) were cut from each branch and split into four roughly equal pieces, with the bark maintained intact where possible. The surface disinfection was completed with some modifications in the procedure described by Slippers & Wingfield (2007). Small portions of branches were submerged in 70 % ethanol for 1 min, followed by 1 min in 4 % NaOCl (Bleach) and 1 min in 95 % ethanol and then rinsed in autoclaved distilled water for 1 min. Branch sections were then transferred to 2 % water agar in Petri plates and incubated at 25 ºC. Plates were examined daily for the presence of mycelial growth using a Nikon SMZ 745 dissection microscope. Single hyphal-tips from expanding colonies were transferred to 2 % MEA medium (20 g malt extract, 20 g Biolab agar in 1L distilled water) amended with 0.4 g/l streptomycin sulfate (Sigma-Aldrich, USA) and incubated at 25

˚C to obtain pure cultures. Colonies resembling species of *Botryosphaeriaceae* (floccose or cottony mycelium, grey to green, turning dark green, brownish or almost black), were separated into different morphological groups and subsequently identified using DNA sequencing and phylogenetic analyses.

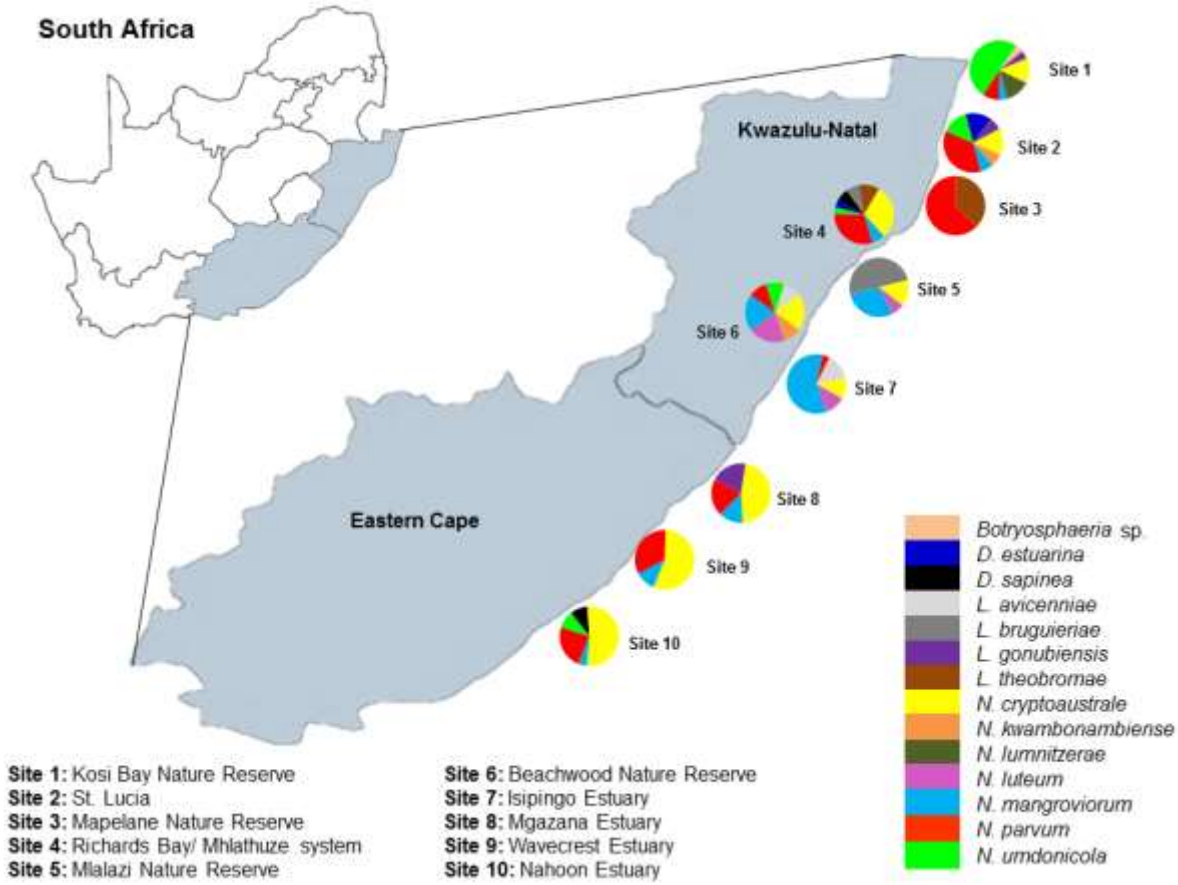

Fig 1 – Mangrove sampling sites in South Africa, also indicating the *Botryosphaeriaceae* species isolated at each sampling site.

Pure cultures resembling *Botryosphaeriaceae* species were deposited in the culture collection (CMW) of the Tree Protection Co-operative Programme (TPCP) at FABI, University of Pretoria. Duplicate cultures of novel species were deposited in the culture collection of the CBS-KNAW (Centraalbureau voor Schimmelcultures) Fungal Biodiversity Centre, Utrecht, The Netherlands. Type specimens were deposited in the herbarium of the National Collection of Fungi (PREM), Pretoria, South Africa.

Identification

DNA extraction, PCR amplification and sequencing

All fungal isolates resembling species of *Botryosphaeriaceae* were grown on MEA for 2 wks to produce sufficient mycelium for DNA extraction. To extract genomic DNA, mycelium was scraped from the surface of cultures and placed into 2 ml sterile Eppendorf tubes and freeze dried. The mycelial samples were then pulverized using 2 mm sterilized metal beads in a Mixer Mill type MM 301 Retsch^{R} tissue lyser (Retsch, Germany) for 3 min at a frequency of 30 cycles per second. Total genomic DNA was extracted following the method described by Raeder and Broda (1985). Extracted DNA was suspended in 50 µl Sabax water (Adcock Ingram, Johannesburg, South Africa). Five µl RNAse (5 mg/ml) was added to degrade the RNA in the samples and to obtain a better quality of DNA. The tubes were then incubated at 37 ºC for 60 min. The quality and quantity of the extracted DNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). All DNA samples were diluted to attain a concentration of 20 ng/µl and stored at -20 ºC.

A total of four partial gene regions, the Internal Transcribed spacer regions 1 and 2, including the 5.8S nrRNA gene (ITS), the beta-tubulin *tub*2), the translation elongation factor 1-alpha (*tef*1-α) and the DNA-directed RNA polymerase II second largest subunit (*rpb*2) regions were amplified using the polymerase chain reaction (PCR). Standard published primers were utilized for each region, including BT2a, BT2b (Glass and Donaldson 1995), ITS1, ITS4 (White *et al.* 1990), EF1F, EF2R (Jacobs *et al.* 2004) and/or EF1-688F, EF1-1251R (Alves *et al.* 2008), *rpb*2bot6F, *rpb*2bot7R (Sakalidis 2004) and/or *rpb*2lasF, *rpb*2lasR (Cruywagen *et al.* 2016).

A 25 µl reaction mixture was prepared for each PCR, containing 100 ng of DNA for the ITS (0.2 mM), 40 ng of DNA for the *tub*2 (0.2 mM), 30 ng for the *rpb*2 (0.2 mM), and 80 ng for the *tef*1-α (0.2 mM), reactions, 2.5 µl of PCR reaction buffer (10 mM Tris-HCL, 1.5 mM MgCl2, 50 mM KCL), 1 µl of each primer, 2 µl dNTP (0.2 mM) and 0.5 µl of Faststart Taq DNA Polymerase (Roche Applied Science, Germany). Sterile Sabax water was added to adjust the final reaction volumes to 25 µl. All reactions were run using the following thermal cycling conditions: initial denaturation at 94 ˚C for 4 min followed by a step of ten cycles consisting of 94 ºC for 20 s, annealing at 54 ˚C (*tub*2 and the *tef*1-α for the primers TEF1-688f and

TEF1-1251r) and 55 ºC (ITS, *tef*1-α and *rpb*2) for 48 s, and elongation at 72 ºC for 45 s, followed by a further 25 cycles of 94 ºC for 20 s, with an annealing step using the temperatures as previously indicated for each gene region for 40 s with a time increase of 5 s every cycle, and elongation for 45 s at 72 ºC. This was concluded with a final elongation step at 72 ºC for 10 min. An aliquot of 5 µl of each of the PCR products was stained with GelRed™ nucleic acid gel stain (Biotium, USA), separated on 1 % agarose gels for 20 min at 90 Volts and viewed with a Gel Doc EZ Imager (Bio-Rad Laboratories Inc.) to access the success of the PCR.

PCR products were cleaned using Sephadex G-50 columns following the instructions provided by the manufacturers (Sigma Aldrich, Sweden) and the cleaned filtrate was used in the sequencing reactions. The concentrations of the cleaned PCR products were determined using a NanoDrop ND-1000 spectrometer (NanoDrop Technologies, USA) and a concentration of DNA between 60 to 100 ng/µl was added to each sequencing reaction.

Products were sequenced with a BigDye® Terminator v. 3.1 Cycle Sequencing Kit (PE Applied Biosystems, California, USA) using the same primers and annealing temperatures that were used in the initial PCR. The sequencing products were cleaned in Sephadex G-50 columns. Where a 96 well PCR plate was used, the cleaning of the products was performed with Exonuclease I - Shrimp Alkaline Phosphatase (Exo-SAP). In this case 2 µl of the PCR product was used for sequencing, and the product was then cleaned using ethanol precipitation. PCR products were sequenced in both directions using a BigDye® Terminator v. 3.1 Cycle Sequencing Kit on an ABI PRISM 3100 DNA sequencer (Applied Biosystems, USA). Sequencing results were viewed manually and consensus sequences were assembled with CLC Main Workbench v. 7.6.1 (http://www.clcbio.com/genomics/).

Phylogenetic analyses

Sequences of the isolates obtained from the six species of mangroves were compared to data of previously published species obtained from GenBank (http://blast.ncbi.nih.gov/blast.cgi). The obtained ITS consensus sequences were used to perform BLAST searches in GenBank using BLASTn (Altschul *et al.* 1990) and to identify isolates to genus and closest species level. This information was used to generate datasets for further phylogenetic analyses. The data matrices were

aligned online using MAFFT v. 7 (Katoh & Standley 2013) and edited manually for alignment errors with MEGA v. 5 (Tamura *et al.* 2011).

The ITS region was sequenced for all isolates obtained from mangroves in this study and compared against known *Botryosphaeriaceae* species. Subsequently, an extra ITS data set comprising 17 previously described genera within the *Botryosphaeriaceae* and 13 representative sequences from mangroves were used in the final phylogenetic analyses of the ITS and 5.8S regions. *Melanops tulasnei*, was used as the outgroup for the analyses. To confirm species identities, additional data sets of relevant gene regions were compiled separately for each genus: *tub*2 and *tef*1-α for *Botryosphaeria* and *Diplodia*; *tub*2, *rpb*2 and *tef*1-α for *Lasiodiplodia* and *Neofusicoccum*. These data sets included additional isolates for each unidentified taxon, as well as extra isolates (where available) of closely related known species, including the ex-types. The *Botryosphaeria* data sets were rooted to *Neofusicoccum parvum* and *N. luteum*, the *Diplodia* data sets to *Lasiodiplodia gonubiensis* and *L. theobromae*, the *Lasiodiplodia* data sets to *Neofusicoccum cordaticola* and *N. parvum*, the *Neofusicoccum* data sets were rooted to *Lasiodiplodia gonubiensis* and *L. theobromae*.

Phylogenetic analyses of sequence data for Maximum Parsimony (MP) were performed using PAUP v. 4.0b10 (Swofford 2003). Maximum Parsimony genealogies for single genes were constructed using the heuristic search option with 100 random taxon additions and tree bisection and reconnection (TBR). Gaps were treated as missing data and all characters were unordered and of equal weight. Statistical support for nodes was obtained by performing 1000 bootstrap replicates. In addition, the consistency index (CI), homoplasy index (HI), rescaled consistency index (RC), retention index (RI) and tree length (TL) were determined for the resulting trees. Maximum Likelihood (ML) and Bayesian inference (BI) analyses were performed for each sequence data set. For both analyses, jModelTest v. 2.1.4 (Darriba *et al.* 2012) was used to infer the appropriate substitution model using Akaike information criteria (AIC) (Akaike 1974). ML analyses were performed with the program PhyML v. 3.0 (Guindon and Gascuel 2003), taking into account the proportion of invariable sites. The confidence support values for nodes were estimated using 1000 replication bootstrap analyses. Posterior probabilities were determined using BI based on a Markov Chain Monte Carlo (MCMC) algorithm performed in MrBayes v. 3.2 (Ronquist *et al.* 2012). Two independent runs were done simultaneously for 5 million

generations at every 100th generations. Burn-in values were determined with Tracer v. 1.6.0 (Rambaut *et al*. 2013) and the first 25 000 sampled trees that represented the burn-in phase were discarded and the remaining trees used to construct a majority rule consensus tree. Phylogenetic trees obtained from BI, ML and MP analyses were viewed in MEGA 5 (Tamura *et al.* 2011) or Treeview v. 1.6.6 (Page 1996).

For the description of novel species, colony morphology and microscopic features were examined from cultures growing on 2 % MEA. To induce sporulation, small portions of autoclaved *A. marina* branches of ~ 3 cm long or pine needles were oven-dried at 65 ºC and placed on the surface of water agar, after which *Botryosphaeriaceae* isolates were inoculated onto each plate. Plates were then incubated near UV-light for 1 wk, followed by two weeks in the dark at 25 ºC, one week in a cold room at 7 ºC and thereafter left to grow on a laboratory bench at approximately 25 ºC until fruiting structures were evident. Microscope slides were prepared for structures, including the pycnidia and conidia, in 85 % lactic acid, and observations were made using a Zeiss Axioskop compound microscope (Carl Zeiss, Germany). Photographic images were captured with an Axiocam digital camera and the fungal structures were measured using the Axiovision 3.1 software. Taxonomically informative characters such as the size of pycnidia, size and pigmentation of conidia and the size of paraphyses were used to compile the descriptions of novel species. Fifty measurements of length/width (l/w) were made for each relevant morphological character and the mean, standard deviation (SD) and 95 % confidence intervals were calculated, with the minimum and maximum sizes presented in parentheses as (min–) mean ± SD (–max). Morphological descriptions and nomenclatural details were deposited in MycoBank (www.MycoBank.org).

Growth and colony characterization

To determine the colony growth average of newly discovered fungal species, 4 mm diam mycelial plugs were cut from the actively growing margins of 5-d-old colonies, placing the mycelium side down at the centers of 90-mm-diam Petri plates containing 2 % MEA. Five replicates of each isolate were incubated in the dark at temperatures ranging from 5 ºC to 35 ºC at 5 ºC intervals. The experiment was terminated once the first of the test cultures covered an entire Petri plate. Diameters of the colonies

(mm) were then measured along two perpendicular axes centered on the plugs. The experiment was repeated once. Average growth was calculated for each isolate at the different test temperatures and presented in parentheses. Colony colors (upper surface and reverse) were assessed after 7 d of growth on 2 % (MEA) at 25 ºC, under dark conditions, using the color charts of Rayner (1970), with notation characters presented in parentheses.

Pathogenicity tests

Two mangrove species, *A. marina* and *B. gymnorrhiza,* were inoculated in-field with 48 *Botryosphaeriaceae* isolates including all the species collected from the different sampling locations and mangrove hosts. Seven-d-old isolates, grown on 2 % MEA at 24 ºC, were used for inoculations. A sterile cork borer of 7 mm diam was used to make plugs in the cultures and wounds of equal size on the branches. One branch per tree (~10 mm diam), on 15 trees of each of the two tree species, was inoculated for each fungal isolate. Fifteen branches were inoculated with sterile MEA plugs for each tree species to serve as controls. Agar plugs overgrown with the mycelium of each test isolate were placed into the wounds with the mycelium facing the cambium. The inoculated wounds were sealed with masking tape to avoid desiccation and contamination. The entire experiment was repeated once.

Lesion lengths on the inoculated branches were measured after 6 wks. Portions of inoculated branches were also harvested and surface-sterilized with 70 % ethanol and rinsed with distilled water. Small (~ 2 x 2 mm) pieces of infected tissue were cut from the lesions and placed onto 2 % MEA and incubated at 24 ºC for 7 d. Hyphal tips from the emerging colonies were sub-cultured. The re-isolated fungi were identified using DNA sequences of the ITS and/or *tef*1-α gene regions to comply with the conditions of Koch's postulates.

Lesion lengths were subjected to non-parametric Wilcoxon signed-rank tests in SPSS 17. These non-parametric tests were performed because most means were not normally distributed based on the Shapiro-Wilk test performed in SPSS. Paired rank tests were also used since the tested individuals were from branches of one population. Means with error plots were then constructed for each mangrove species, with annotation as to which species were responsible for inducing the longest lesions. Since the control lesions were 10 mm in size, we also overlaid a 20

mm pathogenicity trend line to compare how many fungal isolates caused a 100 % increase in lesion length between the two mangrove species.

Botryosphaeriaceae diversity and distribution

To test for possible spatial auto-correlation between fungal community assemblages and distance between sites (whether sites closer to each other are more alike in *Botryosphaeriaceae* communities), the RELATE function in PRIMER 6 (Clarke & Gorley 2006) was used. The RELATE test is a mantel-type test that correlates two similarity matrices with each other. For this test, the Spearman's Rho with 9999 permutations was used. *Botryosphaeriaceae* response data showed high levels of heteroscedasticity, in that there were a few abundant species and many rarely observed species across the sampling region. To elevate the influence of the rare species in determining community similarity, data were square root transformed, and site similarity calculated using the Bray-Curtis measure.

To observe similarity/dissimilarity in *Botryosphaeriaceae* species richness and community assemblage composition between mangrove species and between sites, two diversity correspondence analyses (CA for sites, DCA for species) were run in CANOCO 5 (Microcomputer Power, Ithaca, New York). For these measures, data were square root transformed.

The sampling intensity from a regional and mangrove-species perspective was evaluated using a species accumulation curves (Gotelli & Colwell 2001). For the regional tally, adequate taxon representation was validated using Sobs and the abundance-based Chao 1 estimators in PRIMER 6, using 9999 permutations. Mangrove species populations were unevenly spread across the sampling region, and were present in varying abundances. Sampling adequacy within each species was thus also considered and measured using Jackknife 1 estimators with 9999 permutations (PRIMER 6).

Quantitative data for occurrence of *Botryosphaeriaceae* from mangrove species was calculated based on a modified formula used by Sarma & Hyde (2001). The probability of successful isolation was calculated as the total number of *Botryosphaeriaceae* isolates/Total number of samples examined. A sample was defined as a random branch selected from each tree species in the field. The

frequencies were converted into percentage (percentage of occurrence indicates the number of branches on which a *Botryosphaeriaceae* species was isolated), and ranked according to a modified scoring criteria of Samón-Legrá *et al.* (2014), as very frequent (>10 %), frequent (>5 % and <10 %) and infrequent (<5 %)

Results

Sample collection

A total of 530 branches, from six mangrove species (*A. marina* = 180 branches*, B. gymnorrhiza* = 170 branches*, B. racemosa* = 40 branches*, C. tagal* = 20 branches*, L. racemosa* = 20 branches*, R. mucronata* = 100 branches), were collected for this study. This resulted in 160 isolates of endophytic *Botryosphaeriaceae*. Of these, based on the proportion of isolates recovered from an individual host species, 10 isolates were obtained from *L. racemosa* (50 %, isolates obtained from 20 branches of this host species), 19 isolates from *B. racemosa* (47.5 %), 68 isolates from *B. gymnorrhiza* (40 %), 22 isolates from *R. mucronata* (22 %), 38 isolates from *A. marina* (21 %), and 3 isolates from *C. tagal* (15 %), which yielded the lowest number of isolates.

Identification

DNA extraction, PCR amplification and sequencing

DNA was extracted from the 160 isolates resembling species of *Botryosphaeriaceae* and PCR and sequence products were generated for the ITS region. Of these, 80 isolates were selected and used in smaller data sets for the ITS, *tub*2, *tef*1-α and *rpb*2 to confirm their species level identities. Sequence fragments were approximately 500–610 bp for the ITS, 390–460 bp in size for the *tub*2, 330–370 bp for the *tef*1-α, and 560–600 for the *rpb*2 gene regions. Representative sequences of all species recovered were deposited in GenBank (Table 2). Based on blast searches in GenBank, isolates from mangroves in South Africa represented at least four genera in the *Botryosphaeriaceae*.

Table 2 – Culture numbers and Genbank accession information of representative isolates of *Botryosphaeriaceae* spp. obtained from mangrove trees in South Africa.

Species	Isolate CMW No.	Host	Location	GenBank Accession Numbers			
				ITS	*tef*1-α	*tub*2	*rpb*2
Botryosphaeria sp.	CMW41226	*Avicennia marina*	Kosi Bay	KP860875	KP860718	KP860795	*
Diplodia estuarina	**CMW41231-T**	*A. marina*	Saint Lucia	KP860831	KP860676	KP860754	*
D. estuarina	**CMW41230-P**	*A. marina*	Richards Bay	KP860830	KP860675	KP860753	*
D. estuarina	**CMW41363-P**	*Rhizophora mucronata*	Richards Bay	KP860829	KP860674	KP860752	*
D. sapinea	CMW41234	*A. marina*	Nahoon	KP860827	KP860672	KP860750	*
D. sapinea	CMW41235	*A. marina*	Nahoon	KP860828	KP860673	KP860751	*
D. sapinea	CMW41362	*A. marina*	Richards Bay	KP860826	KP860671	KP860749	*
Lasiodiplodia avicenniae	**CMW41467-T**	*A. marina*	Isipingo	KP860835	KP860680	KP860758	KU587878
L. avicenniae	**DNA**	*A. marina*	Beachwood	KU587957	KU587946	KU587867	KU587879
L. avicenniae	**DNA**	*A. marina*	Beachwood	KU587956	KU587947	KU587868	KU587880
L. bruguierae	**CMW41470-T**	*Bruguiera gymnorrhiza*	Mtunzini	KP860832	KP860677	KP860755	KU587876
L. bruguierae	**CMW41614-P**	*B. gymnorrhiza*	Mtunzini	KP860833	KP860678	KP860756	KU587877
L. bruguierae	**CMW42480-P**	*B. gymnorrhiza*	Richards Bay	KP860834	KP860679	KP860757	KU587875
L. gonubiensis	CMW41227	*Ceriops tagal*	Kosi Bay	KP860838	KP860683	KP860761	KU587883
L. gonubiensis	CMW41229	*B. gymnorrhiza*	Saint Lucia	KP860839	KP860684	KP860762	KU587884
L. gonubiensis	CMW41236	*B. gymnorrhiza*	Mgazana	KP860840	KP860685	KP860763	KU587885
L. gonubiensis	CMW43762	*B. gymnorrhiza*	Mgazana	KU587954	KU587943	KU587864	KU587886
L. gonubiensis	CMW43763	*B. gymnorrhiza*	Mgazana	KU587955	KU587944	KU587865	KU587887
L. theobromae	CMW41214	*Barringtonia racemosa*	Mapelane	KP860842	KP860687	KP860765	KU587889
L. theobromae	CMW41222	*B. racemosa*	Richards Bay	KP860836	KP860681	KP860759	KU587881
L. theobromae	CMW41223	*B. racemosa*	Richards Bay	KP860837	KP860682	KP860760	KU587882
L. theobromae	CMW42341	*B. racemosa*	Mapelane	KP860843	KU587945	KU587866	*
L. theobromae	CMW41360	*B. racemosa*	Mapelane	KP860841	KP860686	KP860764	KU587888
Neofusicoccum cryptoaustrale	CMW41211	*A. marina*	Richards Bay	KP860844	KP860688	KP860766	KU587891
N. cryptoaustrale	CMW41219	*B. gymnorrhiza*	Beachwood	KP860865	KP860708	KP860785	*
N. cryptoaustrale	CMW41370	*A. marina*	Mgazana	KP860902	KP860745	KP860822	KU587942
N. cryptoaustrale	CMW42344	*R. mucronata*	Richards Bay	KP860857	KP860701	KP860778	KU587902
N. cryptoaustrale	CMW42347	*R. mucronata*	Isipingo	KP860868	KP860711	KP860788	KU587913
N. cryptoaustrale	CMW42350	*R. mucronata*	Isipingo	KP860872	KP860715	KP860792	*
N. cryptoaustrale	CMW42354	*C. tagal*	Kosi Bay	KP860878	KP860721	KP860798	KU587923

Table 2 – Culture numbers and Genbank accession information of representative isolates of *Botryosphaeriaceae* spp. obtained from mangrove trees in South Africa.

Species	Isolate CMW No.	Host	Location	GenBank Accession Numbers			
				ITS	*tef*1-α	*tub*2	*rpb*2
N. cryptoaustrale	CMW42355	*L. racemosa*	Kosi Bay	KP860879	KP860722	KP860799	*
N. cryptoaustrale	CMW42356	*L. racemosa*	Kosi Bay	KP860880	KP860723	KP860800	*
N. cryptoaustrale	CMW42357	*B. gymnorrhiza*	Saint Lucia	KP860884	KP860727	KP860804	KU587928
N. cryptoaustrale	CMW42362	*B. gymnorrhiza*	Nahoon	KP860892	KP860735	KP860812	KU587934
N. cryptoaustrale	CMW42365	*B. gymnorrhiza*	Wavecrest	KP860899	KP860742	KP860819	KU587939
N. cryptoaustrale	CMW42483	*B. gymnorrhiza*	Beachwood	KP860866	KP860709	KP860786	KU587911
N. cryptoaustrale	CMW42485	*A. marina*	Nahoon	KP860896	KP860739	KP860816	KU587936
N. kwambonambiense	CMW42349	*R. mucronata*	Isipingo	KP860870	KP860713	KP860790	KU587915
N. kwambonambiense	CMW41369	*B. gymnorrhiza*	Saint Lucia	KP860886	KP860729	KP860806	KU587930
N. lumnitzerae	**CMW41469-T**	*Lumnitzera racemosa*	Kosi Bay	KP860881	KP860724	KP860801	KU587925
N. lumnitzerae	**CMW41228-P**	*L. racemosa*	Kosi Bay	KP860882	KP860725	KP860802	KU587926
N. lumnitzerae	**CMW41613-P**	*L. racemosa*	Kosi Bay	KU587958	KU587948	KU587869	KU587924
N. luteum	CMW41218	*B. gymnorrhiza*	Beachwood	KP860863	KP860706	KP860783	KU587909
N. luteum	CMW41220	*A. marina*	Isipingo	KP860867	KP860710	KP860787	KU587912
N. luteum	CMW42348	*R. mucronata*	Isipingo	KP860869	KP860712	KP860789	KU587914
N. luteum	CMW41359	*B. gymnorrhiza*	Mtunzini	KP860846	KP860690	KP860768	KU587893
N. luteum	CMW42482	*A. marina*	Beachwood	KP860862	KP860705	KP860782	KU587908
N. mangroviorum	**CMW41365-T**	*A. marina*	Beachwood	KP860859	KP860702	KP860779	KU587905
N. mangroviorum	**CMW42481-P**	*B. gymnorrhiza*	Mtunzini	KP860848	KP860692	KP860770	KU587895
N. mangroviorum	**CMW42487-P**	*R. mucronata*	Mgazana	KP860900	KP860743	KP860820	KU587940
N. mangroviorum	CMW41216	*A. marina*	Richards Bay	KP860854	KP860698	KP860775	KU587901
N. mangroviorum	CMW42340	*A. marina*	Richards Bay	KP860845	KP860689	KP860767	KU587892
N. mangroviorum	CMW41615	*B. gymnorrhiza*	Mtunzini	KP860847	KP860691	KP860769	KU587894
N. mangroviorum	CMW41466	*A. marina*	Beachwood	KP860860	KP860703	KP860780	KU587906
N. mangroviorum	CMW41217	*A. marina*	Beachwood	KP860861	KP860704	KP860781	KU587907
N. mangroviorum	CMW42346	*B. gymnorrhiza*	Beachwood	KP860864	KP860707	KP860784	KU587910
N. mangroviorum	CMW41221	*R. mucronata*	Isipingo	KP860871	KP860714	KP860791	KU587916
N. mangroviorum	CMW42351	*R. mucronata*	Isipingo	KP860873	KP860716	KP860793	KU587917
N. mangroviorum	CMW42359	*B. gymnorrhiza*	Nahoon	KP860887	KP860730	KP860807	*
N. mangroviorum	CMW42486	*B. gymnorrhiza*	Wavecrest	KP860898	KP860741	KP860818	KU587938

Table 2 – Culture numbers and Genbank accession information of representative isolates of *Botryosphaeriaceae* spp. obtained from mangrove trees in South Africa.

Species	Isolate CMW No.	Host	Location	GenBank Accession Numbers			
				ITS	*tef*1-α	*tub*2	*rpb*2
N. mangroviorum	CMW41364	*A. marina*	Beachwood	KU587959	KU587949	KU587870	KU587904
N. parvum	CMW41213	*B. racemosa*	Mapelane	KP860849	KP860693	KP860771	KU587896
N. parvum	CMW41215	*B. racemosa*	Mapelane	KP860851	KP860695	KP860773	KU587898
N. parvum	CMW41224	*B. racemosa*	Richards Bay	KP860874	KP860717	KP860794	KU587919
N. parvum	CMW41225	*B. racemosa*	Richards Bay	KP860852	KP860696	KP860774	KU587899
N. parvum	CMW41233	*A. marina*	Nahoon	KP860895	KP860738	KP860815	KU587935
N. parvum	CMW41361	*B. racemosa*	Mapelane	KP860850	KP860694	KP860772	KU587897
N. parvum	CMW41368	*A. marina*	Saint Lucia	KP860853	KP860697	KP860774	KU587900
N. parvum	CMW42352	*B. gymnorrhiza*	Kosi Bay	KP860876	KP860719	KP860796	KU587921
N. parvum	CMW42358	*B. gymnorrhiza*	Saint Lucia	KP860885	KP860728	KP860805	KU587929
N. parvum	CMW42361	*B. gymnorrhiza*	Nahoon	KP860891	KP860734	KP860811	KU587933
N. parvum	CMW42364	*B. gymnorrhiza*	Wavecrest	KP860897	KP860740	KP860817	KU587937
N. parvum	CMW42366	*R. mucronata*	Mgazana	KP860901	KP860744	KP860821	KU587941
N. parvum	CMW43755	*B. racemosa*	Richards Bay	KU587960	KU587950	KU587871	KU587918
N. umdonicola	CMW41265	*B. gymnorrhiza*	Nahoon	KP860889	KP860732	KP860809	KU587932
N. umdonicola	CMW41367	*L. racemosa*	Kosi Bay	KP860883	KP860726	KP860803	KU587927
N. umdonicola	CMW42345	*B. gymnorrhiza*	Richards Bay	KP860858	KU587951	KU587872	*
N. umdonicola	CMW42353	*B. gymnorrhiza*	Kosi Bay	KP860877	KP860720	KP860797	KU587922
N. umdonicola	CMW43757	*A. marina*	Kosi Bay	KU587961	KU587952	KU587873	KU587920
N. umdonicola	CMW43760	*B. gymnorrhiza*	Saint Lucia	KU587962	KU587953	KU587874	KU587931

Legend: ***** = Isolate not included in the phylogenetic analyses for the RPB2 gene region. **CMW**: Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

T = ex-holotype; P = ex-paratype

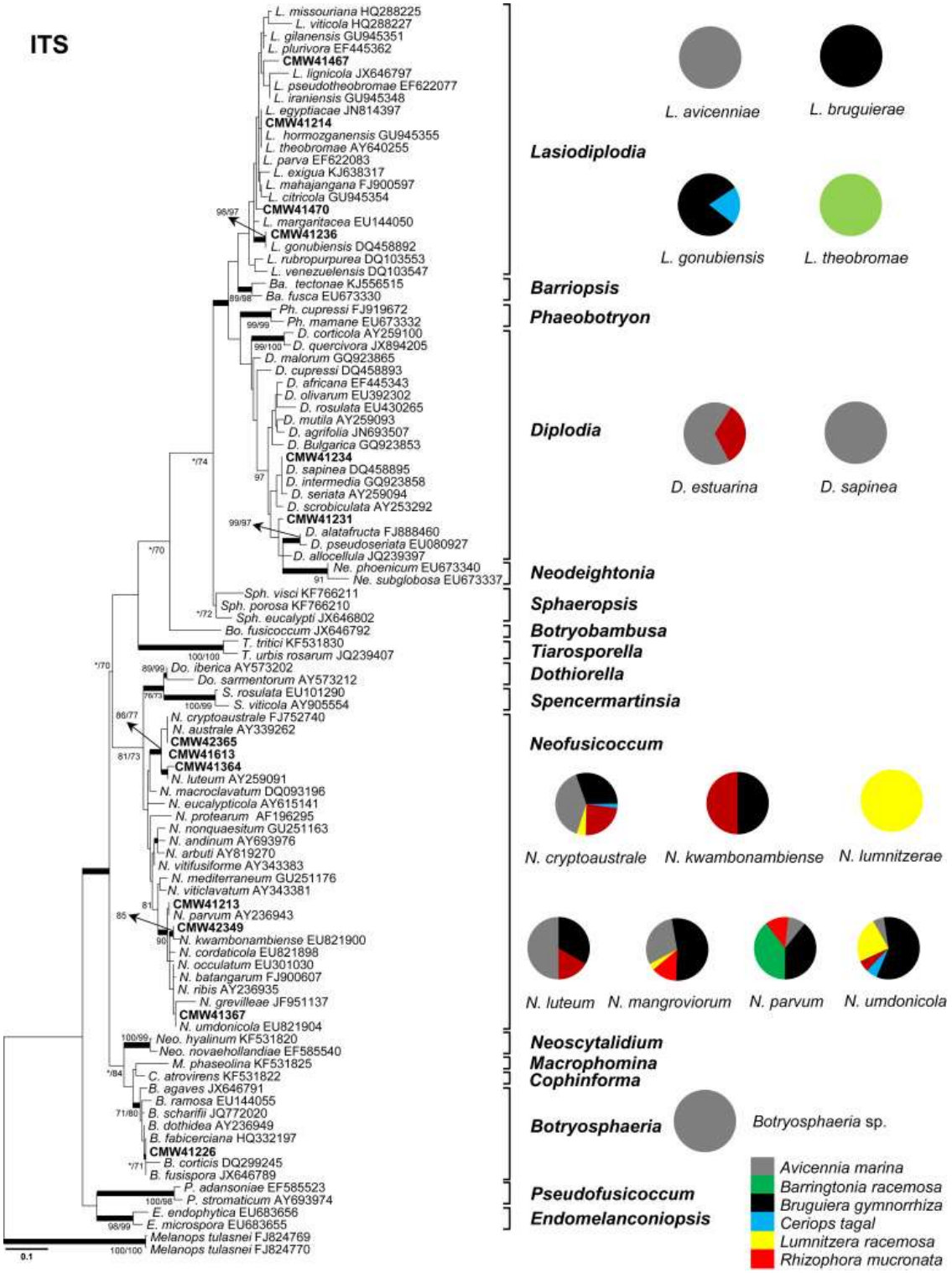

Fig 2 – Phylogram obtained from BI, ML and MP analyses of the ITS data set. Isolates from mangroves group into the genera *Botryosphaeria, Diplodia, Lasiodiplodia* and *Neofusicoccum*. (Isolates obtained in this study are printed in **bold** and the equivalent genera inside the box). BI posterior probabilities ≥95 % are represented by thick branches. Bootstrap support values >70 % are indicated near the nodes as MP/ML. * = bootstrap support values <70 %. Colors of pie charts indicate the host from where *Botryosphaeriaceae* species were isolated (five true mangroves and the mangrove associate *B. racemosa*).

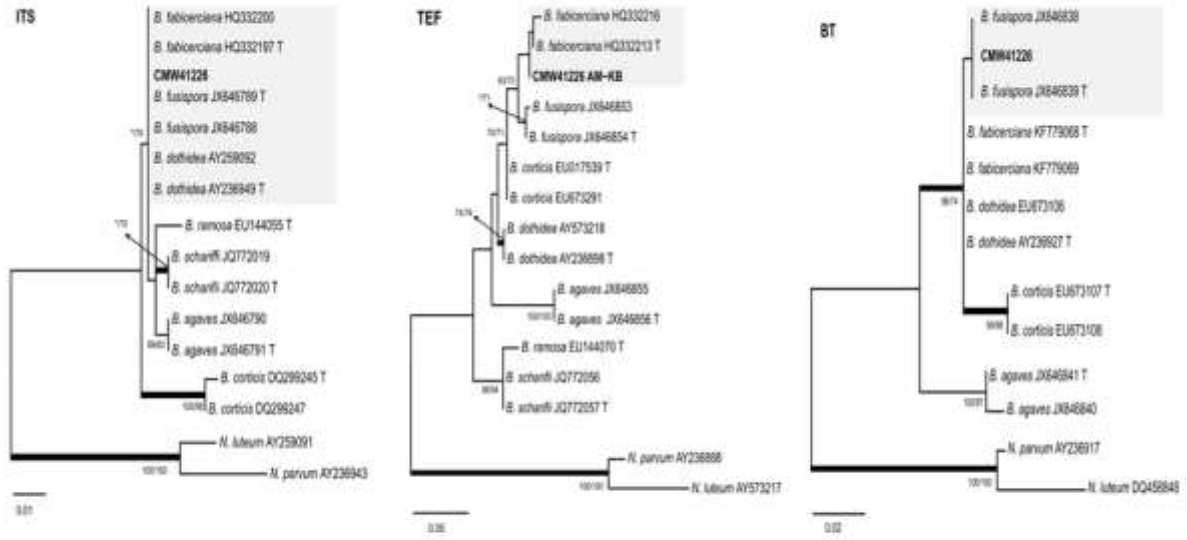

Fig 3 – Phylograms of species in the genus *Botryosphaeria* obtained from BI, ML and MP analyses of the ITS, TEF-1α and βT gene regions. The isolate obtained from mangroves (in **bold** font) groups with different species of *Botryosphaeria* and do not provide strong support to clarify its placement. BI posterior probabilities ≥95 % are represented by thick branches. Bootstrap support values >70 % are indicated near the nodes as MP/ML. * = bootstrap support values <70 %.

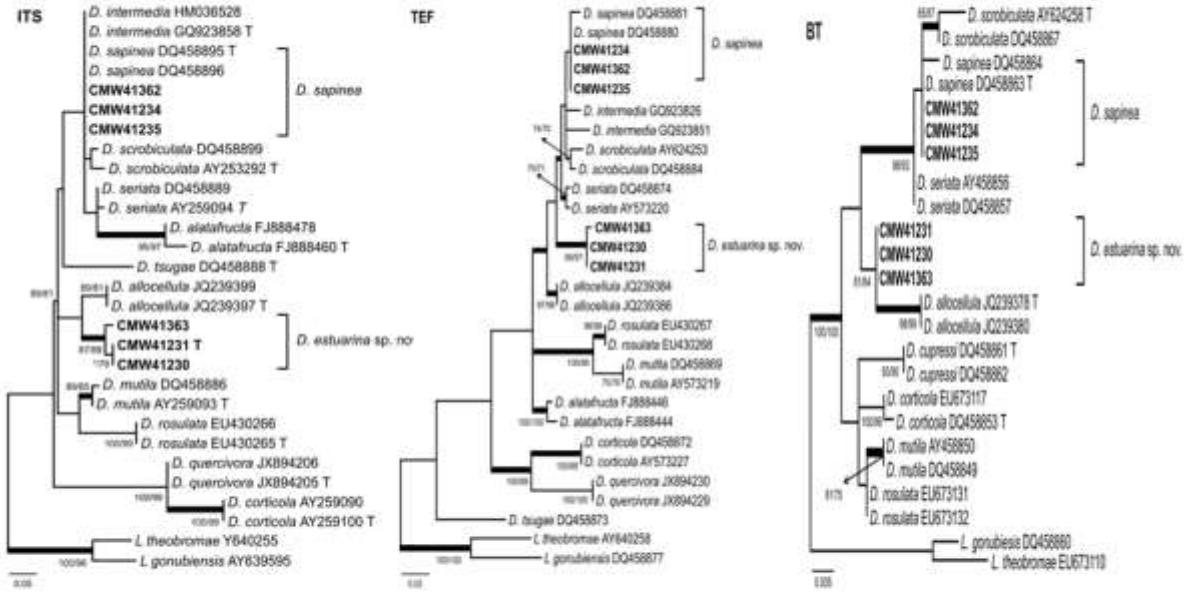

Fig 4 – Phylograms of species in the genus *Diplodia* obtained from BI, ML and MP analyses of the ITS, *tef*1-α and *tub*2 gene regions. The isolates obtained from mangroves (in **bold** font) group with *Diplodia sapinea* and a new taxon, *D. estuarina* sp. nov. BI posterior probabilities ≥95 % are represented by thick branches. Bootstrap support values >70 % are indicated near the nodes as MP/ML. * = bootstrap support values <70 %.

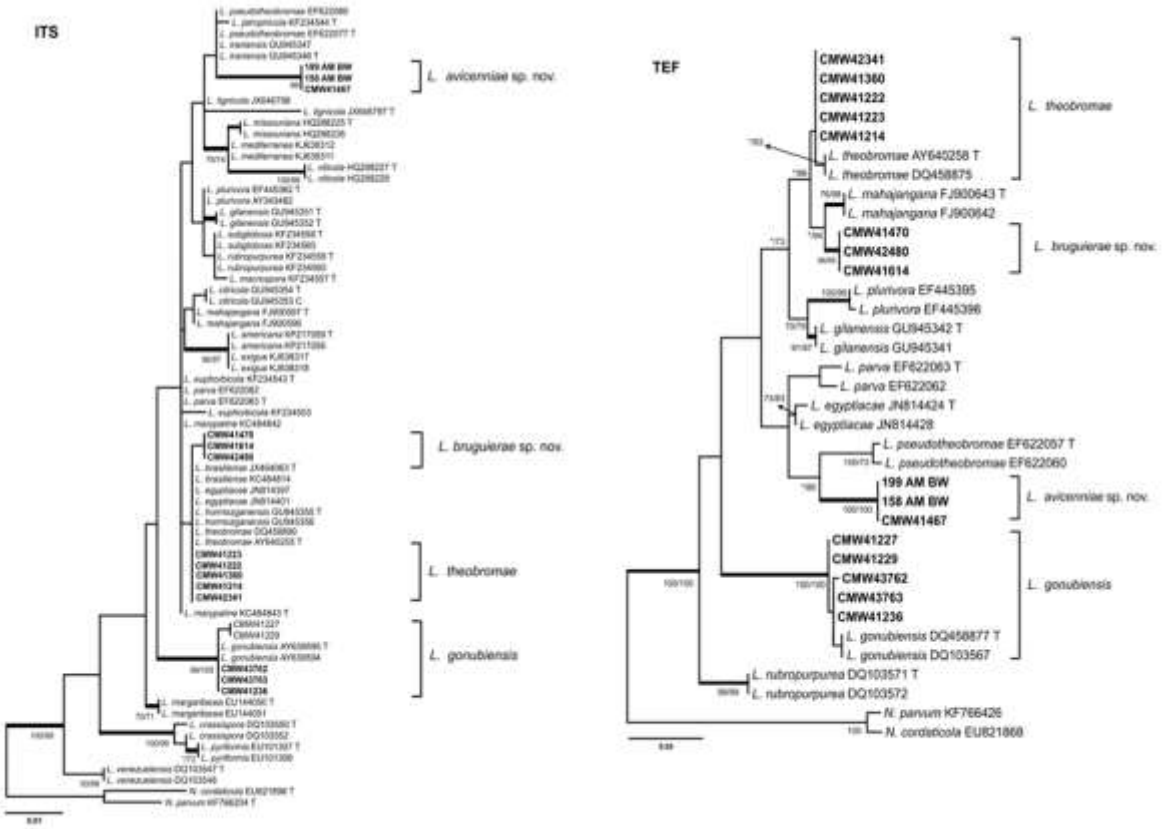

Fig 5 – Phylograms of species in the genus *Lasiodiplodia* obtained from BI, ML and MP analyses of the ITS and *tef*1-α genes. The isolates obtained from mangroves (in **bold** font) form four species in the Lasiodiplodia, including two new taxa (Species names are near to brackets). BI posterior probabilities ≥95 % are represented by thick branches. Bootstrap support values >70 % are indicated near the nodes as MP/ML. * = bootstrap support values <70 %.

Phylogenetic analyses

Alignment of ITS sequence data for the 160 isolates of *Botryosphaeriacea*e identified the presence of four clades. These represented the genera *Botryosphaeria* (one isolate from one tree), *Diplodia* (six isolates from six trees), *Lasiodiplodia* (24 isolates from 24 trees) and *Neofusicoccum* (129 isolates from 123 trees) (Fig. 2). Based on these results, all isolates were further analyzed within genera. The numbers of characters, substitution models used, as well as other statistical results generated from the phylogenetic analyses of all data sets are presented in Table 3.

Only one isolate (CMW41226) of *Botryosphaeria* was obtained and this was from *A. marina*. The placement of this isolate could not be fully resolved. The ITS sequence was identical to that of *B. dothidea, B. fabicerciana* and *B. fusispora* (Fig. 3). The *tef*1-α tree showed a close relationship with *B. fabicerciana,* while based on

Table 3 – Information on the sequence dataset, maximum parsimony (**MP**) and Maximum Likelihood (**ML**) trees for each gene region.

		Gene Region														
		Botryosphaeria			*Diplodia*			*Lasiodiplodia*				*Neofusicoccum*				Genera
		ITS	*tef*1-α	*tub*2	ITS	*tef*-1α	*tub*2	ITS	*tub*2	*tef*-1α	*rpb*2	ITS	*tef*1-α	*tub*2	*rpb*2	ITS
MP	No.AC	509	365	448	610	328	461	508	423	328	565	507	353	394	602	590
	No.EC	470	307	413	556	253	421	460	375	222	431	447	274	341	498	404
	No.IC	39	58	35	54	75	40	48	48	106	134	60	79	53	104	186
	No.MPT	15	2	2	10000	1	36	6	12	100	6	18	1000	58	84	1000
	TL	48	76	38	76	109	57	61	60	241	181	83	116	66	129	683
	(CI)	0.917	0.868	0.974	0.882	0.826	0.789	0.836	0.817	0.643	0.807	0.819	0.845	0.879	0.891	0.470
	(RI)	0.926	0.911	0.979	0.939	0.912	0.91	0.932	0.934	0.897	0.922	0.983	0.980	0.983	0.984	0.871
	(RC)	0.850	0.791	0.953	0.828	0.753	0.719	0.780	0.763	0.577	0.744	0.805	0.828	0.864	0.878	0.409
	(HI)	0.083	0.132	0.026	0.118	0.174	0.211	0.164	0.183	0.357	0.193	0.181	0.155	0.121	0.109	0.530
ML	SM	TIM1 +G	TPM3uf +G	HKY +G	HKY+I	TrN+I	TrN+G	SYM+I	TIM3 +G	TrN+G	TIM1 +G	TIM1ef +I	TPM3uf+ G	TIM3+ G	TIM3+ G	TrN+I+G
	GS	0.149	0.419	0.232	-	-	0.013	-	0.251	0.416	0.384	-	0.439	0.552	0.296	0.720
	(nst)	6	6	2	2	6	6	6	6	6	6	6	6	6	6	6
	No.T	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

MP = Maximum Parsimony, (No. AC) = Number of aligned characters, (No.EC) = Number of excluded characters, (No. IC) = Number of informative characters (No. MPT) = Number of most parsimonious trees, (TL) = Tree length, (CI) Consistency index, (RI) = Retention index (RC) = Rescaled consistency index, (HI) = Homoplasy index.

ML = Maximum Likelihood, (SM) = Substitution model, (GS) = Gamma shape, (nst) = No. of substitution sites, (No. T) = Number of trees.

the *tub*2 locus it grouped with *B. fusispora.* No *rpb*2 analysis was performed for this genus since limited DNA sequence data are available in public databases for this gene region. Based on these results, and because only one isolate was available, it is treated as *Botryosphaeria* sp.

Six isolates were identified as species of *Diplodia* based on ITS sequence data. These isolates represented two groups and the six isolates were used in subsequent analyses. Eleven species previously described for this genus were included in the phylogenetic analyses. The tree topologies for the ITS, *tef*1-α and *tub*2 reconstructed using MP, ML and BI were similar (Fig. 4) and the isolates from mangroves consistently separated into two distinct species, *Diplodia sapinea* and an undescribed taxon, closely related to, but distinct from *D. allocellula*.

Twenty-four isolates of *Lasiodiplodia* were recovered from asymptomatic branches. The phylogenetic analysis of this genus included 30 previously described species for the ITS gene region, the *tef*1-α included nine previously described taxa, while the phylogenetic analyses (MP, ML and BI) of the *tub*2 and the *rpb*2 gene regions included eight previously described species (Figs. 5, 6). These isolates were identified as *L. gonubiensis*, *L. theobromae* and two undescribed taxa. The topologies of the phylogenetic trees for the ITS, *tef*1-α, *tub*2 and *rpb*2 gene regions were similar showing a consistency in the clades in which the mangrove isolates grouped.

One hundred and twenty nine isolates were identified as belonging to the genus *Neofusicoccum*. Phylogenetic analyses included 15 previously described species and 57 isolates selected from mangroves, which were separated into six taxa including *N. luteum*, *N. kwambonambiense*, *N. parvum*, *N. umdonicola*, and two undescribed species (Figs. 6, 7).

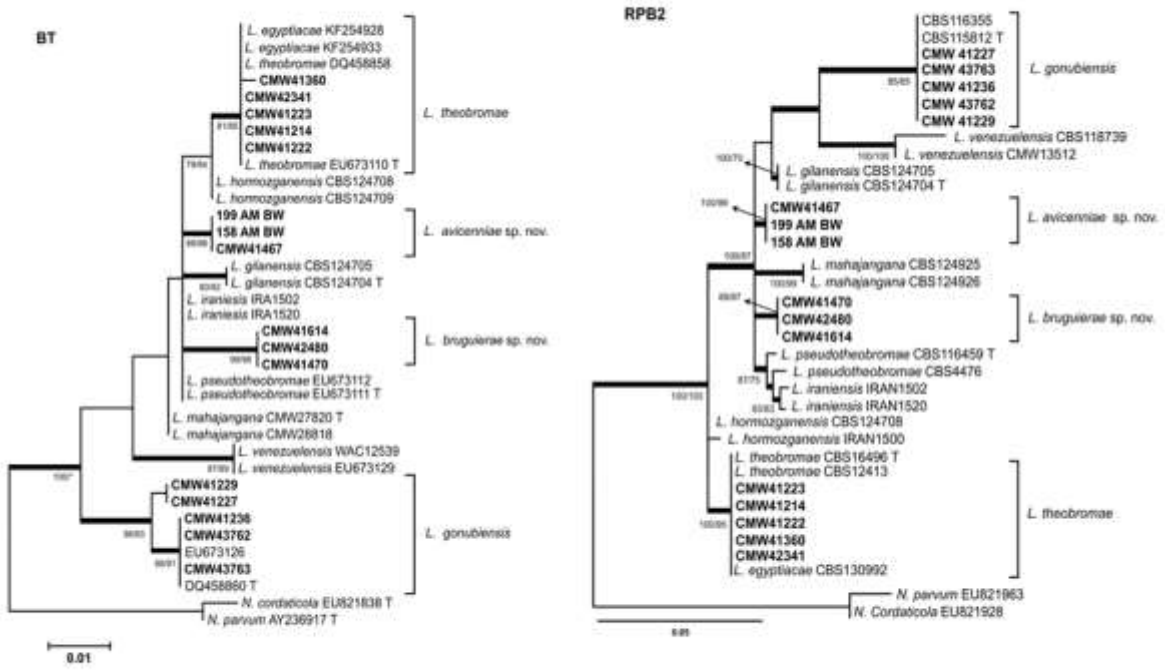

Fig 6 – Phylograms of species in the genus *Lasiodiplodia* obtained from BI, ML and MP analyses of the *tub*2 and *rpb*2 genes. The isolates obtained from mangroves (in **bold** font) group with four species in the *Lasiodiplodia*, including two new taxa, where the most pathogenic fungus found in this study is *L*. *avicenniae* (Species names are near to brackets). BI posterior probabilities ≥95 % are represented by thick branches. Bootstrap support values >70 % are indicated near the nodes as MP/ML. * = bootstrap support values <70 %.

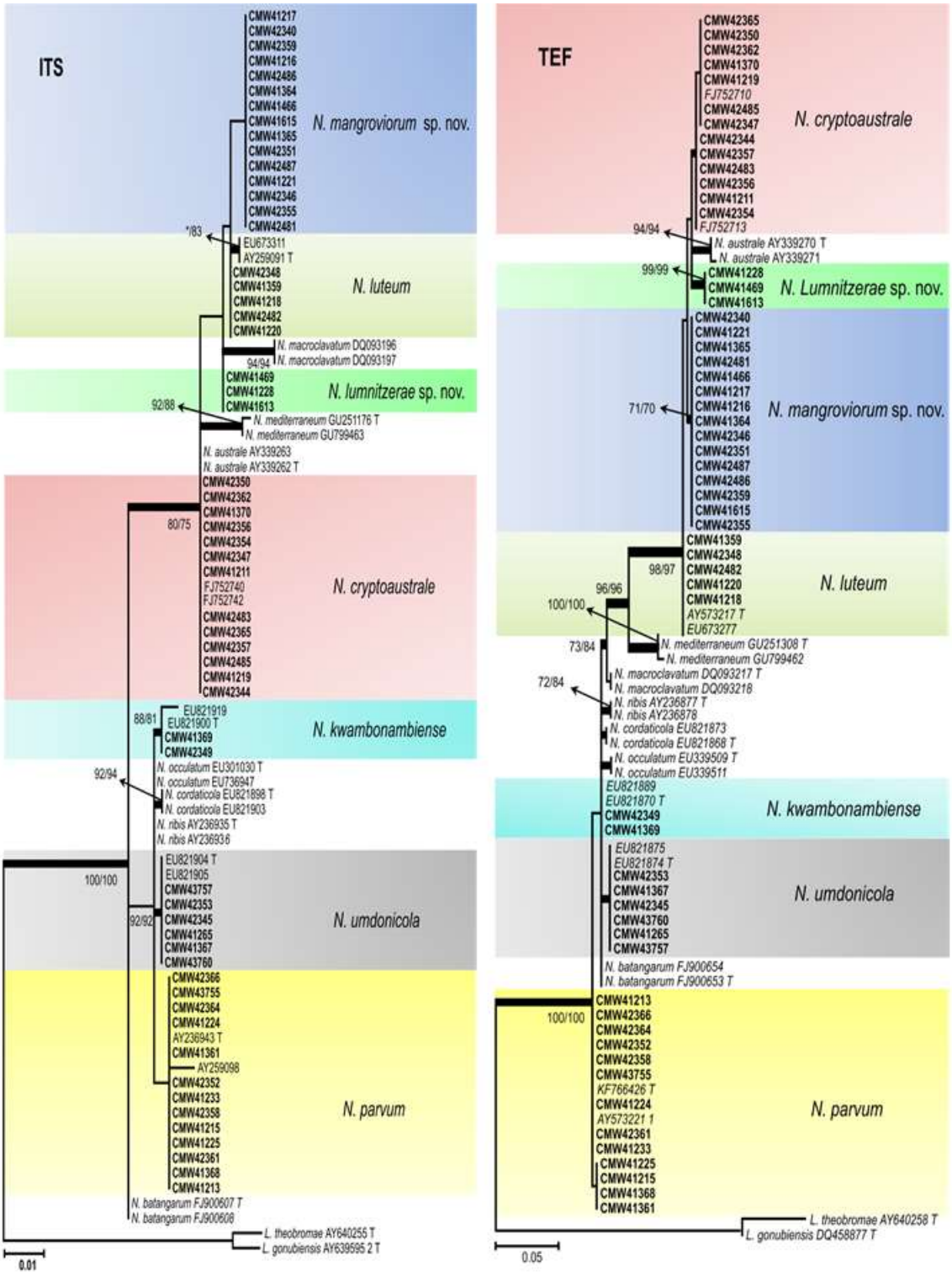

Fig 7 – Phylograms of species in the genus *Neofusicoccum* obtained from BI, ML and MP analyses of the ITS and *tef*1-α gene regions. The isolates obtained from mangroves (in **bold** font) group with seven species in the *Neofusicoccum*, including three new taxa (Species names are near to brackets). BI posterior probabilities ≥95 % are represented by thick branches. Bootstrap support values >70 % are indicated near the nodes as MP/ML. * = bootstrap support.

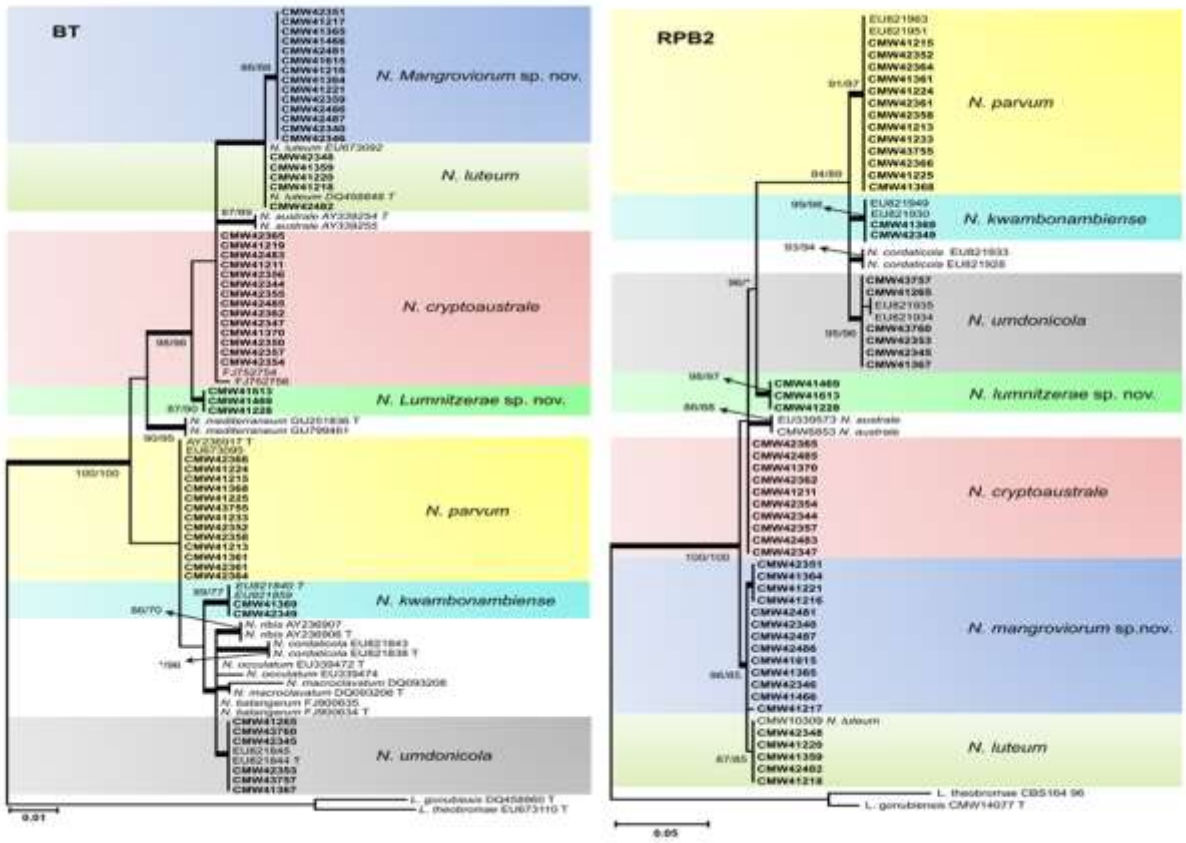

Fig 8 – Phylograms of species in the genus *Neofusicoccum* obtained from BI, ML and MP analyses of the *tub*2 and *rpb*2 gene regions. The isolates obtained from mangroves (in **bold** font) group with seven species in the *Neofusicoccum*, including three new taxa (Species names are near to brackets). BI posterior probabilities ≥95 % are represented by thick branches. Bootstrap support values >70 % are indicated near the nodes as MP/ML. * = bootstrap support.

Taxonomy

Five novel taxa in the *Botryosphaeriaceae* were obtained from asymptomatic branches of mangrove species sampled in this study and these are described here as new taxa. No sexual morphs were found in culture and descriptions are based on morphological characteristics of the asexual morphs.

***Diplodia estuarina* sp. nov.** J.A Osorio, Jol. Roux & Z.W. de Beer (**Fig. 9**)
MycoBank MB812009

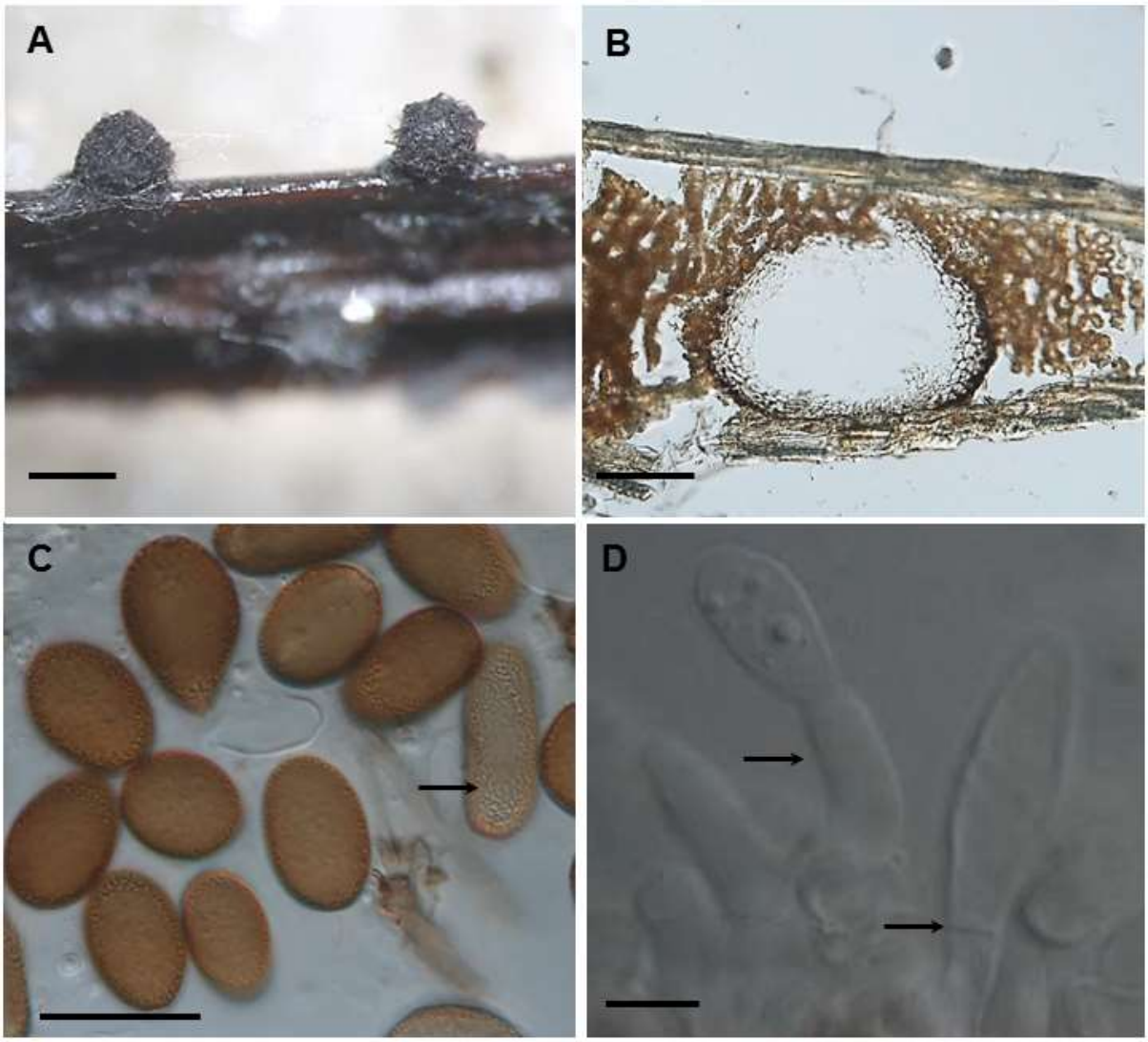

Fig 9 – *Diplodia estuarina* sp. nov. ex-holotype (CMW41231) (**A**) Erumpent conidiomata on pine needles. (**B**) Immersed conidiomata in pine needle tissue. (**C**) Conidia with variable shapes and showing rough contents (arrow). (**D**) Conidia attached to conidiogenous cell (arrow) and showing annellation (arrow). Bars: A=500 µm, B=100 µm, C=20 µm, D=10 µm, D=5 µm.

Etymology: Epithet refers to the ecosystem (estuary) where mangroves occur. *Sexual morph* unknown. *Conidiomata* pycnidial, produced most often in cultures older than 4 wks, solitary or aggregated, unilocular, globose, non-papillate, wall composed of dark brown textura angularis (167–)331–387(–585) µm wide, immersed in media to erumpent at maturity on pine needles, sometimes fruiting structures agglomerate, forming clumps on water agar (WA). *Conidiogenous cells* hyaline, holoblastic, lageniform to cylindrical, (11–)12–16(–20) × (1.2–)1.5–2.5(–3) µm. *Paraphyses* hyaline, septate, unbranched, rounded tips, (2–)2.7 × 3.6(–4) µm. *Conidia* hyaline becoming pale brown to dark brown with age, rarely forming 1

septum, guttulate, both ends broadly rounded, variable in shape, oblong, ellipsoid to ovoid, internally roughened, thick walled, (20–)25–26(–29) × (9–)11–11.5(–15) µm.

Culture characteristics: Colonies on MEA at 25 ºC in darkness for 7 d, mycelium white at first, becoming grayish olive (21""), darkest around the plug and white on the outer areas of colonies, olivaceous (21''m) on the reverse side, floccose mycelium. Cardinal temperatures for growth: minimum ≤ 10 ºC, maximum ≥ 30 ºC, optimum 25 ºC. Colonies reaching an average of 11 mm at 10 ºC, 26 mm at 15 ºC, 41 mm at 20 ºC, 54 mm at 25 ºC, 22 mm at 30 ºC after 4 days. No growth at 5 ºC and 35 ºC.

Specimens examined: SOUTH AFRICA, KWAZULU-NATAL PROVINCE: St. Lucia and Richards Bay, from asymptomatic branches of *Avicennia marina* and *Rhizophora mucronata*. Collectors J.A. Osorio & Jol. Roux. **Holotype** (PREM 61247, ex-holotype CMW41231 = CBS 139666); **Paratype** (ex-paratype cultures CMW41230 = CBS 139667, CMW41363 = CBS 139668).

Habitat: Asymptomatic branches of *A. marina* and *R. mucronata*.
Known distribution: St. Lucia estuary and Richards Bay (KwaZulu-Natal Province, South Africa).

Notes: *Diplodia estuarina* is phylogenetically closely related to *D. allocellula,* differing in 11 bases in the *tef*1-α sequence and five bases in *tub*2 sequence. Pycnidia in *D. estuarina* are arranged solitary or aggregated, and are considerably wider (387 µm) compared to those of *D. allocellula* that are solitary and 100 µm wide. Conidiogenous cells in *D. estuarina* are smaller, (12–16 × 1.5–2.5 µm), than those of *D. allocellula,* (13.4–23.6 × 4.2–5).

***Lasiodiplodia avicenniae* sp. nov.** J.A. Osorio, Jol. Roux & Z.W. de Beer (Fig. 10) MycoBank MB812010

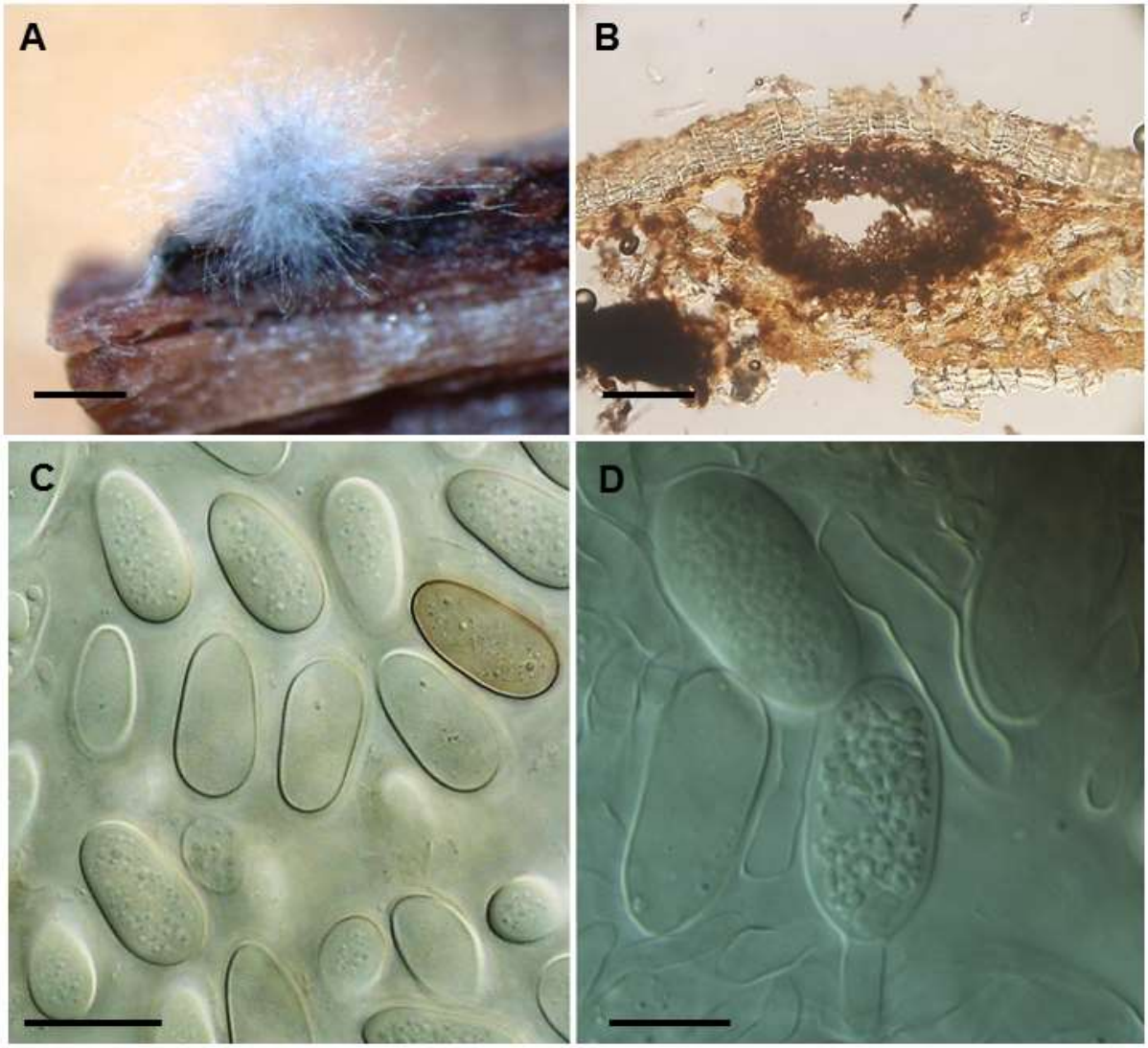

Fig 10 – *Lasiodiplodia avicenniae* sp. nov. ex-holotype (CMW41467) (**A**) Globose pycnidium covered with aerial mycelia on sticks of *A. marina* on water agar. (**B**) Immersed conidiomata. (**C**) Hyaline and light brown aseptate conidia (**D**) hyaline conidia attached to the conidiogenesis cells. (**E**) Brown conidium with septum and rough content. (**F**) Septate-brown conidium showing striated content. Bars: A–B=100 µm, C,E,F=20 µm, D=10 µm.

Etymology: Epithet refers to the host genus, *Avicennia marina,* from which the fungus was isolated.

Sexual morph unknown. *Conidiomata* pycnidial, dark brown to black, covered with dense mycelium on *A. marina* branches on water agar (WA), solitary, globose to pyriform, wall composed of layers of *textura angularis* (238–)317–485(–560) µm wide, immersed in the tissue, oozing conidia after 3 wks. *Paraphyses,* hyaline, thin-walled, septate, apex rounded, sometimes very long reaching up to 170 µm long, 2–4 µm wide. *Conidiogenous cells* holoblastic, hyaline, cylindrical, smooth, proliferating percurrently to form 1–2 annellations, (6–)9–11(–15) × (3–)3.5–4.2(– 6) µm. *Conidia,*

shape variable, obpyriform, thick walled, mostly ellipsoid sometimes constricted in the middle, apex and base rounded, granular contents when hyaline, becoming brown, 1-septate with longitudinal striations when mature, (19–) 24–26(-30) × (9–)12–12.5(–15) µm.

Culture characteristics: Colonies on MEA at 25 ºC in darkness for 7 d, initially white, becoming grayish olive (21"") on the surface; mycelium floccose except around the inoculum plug where the mycelium is flattened, outer area of colony white on the surface and reverse; Cardinal temperatures for growth: min ≤ 10 ºC, max ≥ 35 ºC, optimum between 25–30 ºC. Colonies reaching an average of 7 mm at 10 ºC, 32 mm at 15 ºC, 58 mm at 20 ºC, 86 mm at 25 ºC, 86 mm at 30 ºC, 38 mm at 35 ºC after 4 days. No growth at 5 ºC.
Specimens examined: SOUTH AFRICA, KWAZULU-NATAL PROVINCE: Beachwood and Isipingo, from asymptomatic branches of *Avicennia marina*. Collectors J.A Osorio & Jol. Roux. **Holotype** (PREM 61249, ex-holotype cultures CMW41467 = CBS 139670).
Habitat: Asymptomatic branches of *A. marina*.
Known distribution: Beachwood and Isipingo in Durban (KwaZulu-Natal Province, South Africa).

Notes: *Lasiodiplodia avicenniae* is phylogenetically closely related to *L. iraniensis, L. jatrophicola* that have recently been considered as synonyms by Rodríguez *et al*. 2016 and *L. pseudotheobromae* in the ITS region, differing in seven bases compared with *L. iraniensis* = *L. jatrophicola*, and eight bases with *L. pseudotheobromae*. Based on the *tef*1-α sequence, only *L. pseudotheobromae* is the closest related species, differing in 18 bases, and an insertion of four bases in *L. avicenniae*. Furthermore, the phylogenetic analyses of the *tub*2 and *rpb*2 gene regions showed that *L. avicenniae* forms a clade independent from the species in this genus. The paraphyses of *L. avicenniae* are septate, and significantly longer (170 µm) than those of *L. pseudotheobromae* (58 µm). Conidial shape is variable in *L. avicenniae*, obpyriform, ellipsoid and sometimes constricted in the middle and smaller (24–26 × 12–12.5 µm) compared with those of *L. pseudotheobromae,* which are ellipsoid and widest at the middle (23.5–32 × 14–18 µm).

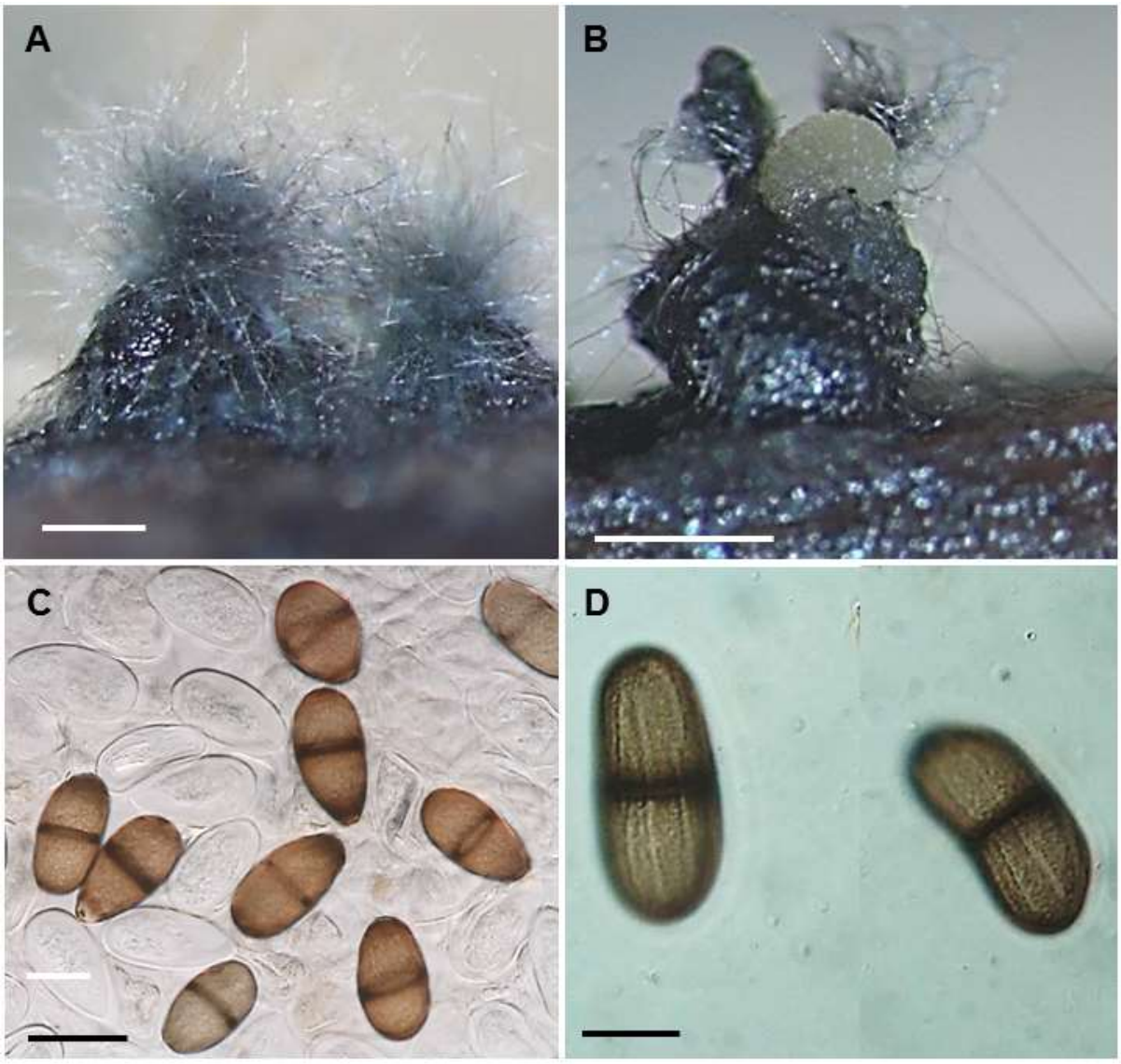

Fig 11 – *Lasiodiplodia bruguierae* sp. nov. (CMW41470) (**A**) Conidiomata covered with aerial mycelia on sticks of *A. marina* on water agar. (**B**) Subglobose pycnidium oozing conidia. (**C**) Hyaline aseptate and dark brown septate conidia. (**D**) Longitudinal striations on mature conidia. Bars: A–B=500 µm, C=20 µm, F=10 µm.

***Lasiodiplodia bruguierae* sp. nov.** J.A. Osorio, Jol. Roux & Z.W. de Beer (Fig. 11) MycoBank MB812011

Etymology: Epithet refers to the host, *Bruguiera gymnorrhiza*, from which the fungus was isolated.

Sexual morph unknown. *Conidiomata* pycnidial, produced on *A. marina* sticks on WA within 1–2 wks, solitary or aggregated, conical to subglobose, papillate, dark-brown to black, covered with dense aerial mycelium, (352–)382–622(–754) µm, oozing conidia within 3 wks. *Paraphyses* not observed. *Conidiogenous cells* hyaline, subcylindrical, holoblastic, (13)11–21(–23) × (2.7–)3–5 µm. *Conidia* observed in

culture, originally hyaline with granulose content and aseptate, becoming dark brown, 1-septate with longitudinal striations, thick walled, mostly ellipsoid to ovoid, (19–) 25–26(–32) × (11–)12–13(–15) µm.

Culture characteristics: Colonies on MEA at 25 ºC in darkness for 7 d, spreading rapidly with flattened mycelium at the center and abundant floccose mycelium in the outer areas, reaching the lid of Petri dish , gradually becoming olivaceous buff (21'"d) to smoke grey (21""d) on the surface, reverse olivaceous (21""), darker around the plug. Cardinal temperatures for growth: minimum ≤ 15 ºC, maximum ≥ 35 ºC optimum 25 ºC. Colonies reaching an average of 23 mm at 15 ºC, 54 mm at 20 ºC, 71 mm at 25 ºC, 65 mm at 30 ºC, 25 mm at 35 ºC after 4 days. No growth at 5 ºC and 10 ºC.

Specimens examined: SOUTH AFRICA, KWAZULU-NATAL PROVINCE: Mlalazi Nature Reserve, Mtunzini, from asymptomatic branches of *Bruguiera gymnorrhiza*. Collectors J.A Osorio & Jol. Roux. **Holotype** (PREM 61248, ex-holotype cultures CMW41470 = CBS 139669); **Paratype** (ex-paratype cultures CMW41614 = CBS 139638, CMW 42480 = CBS 141453).
Habitat: Asymptomatic branches of *B. gymnorrhiza*.
Known distribution: Mtunzini and Richards Bay (KwaZulu-Natal Province, South Africa).
Notes: *Lasiodiplodia bruguierae* is phylogenetically closely related to *L. brasiliense*, *L. hormozganensis* and *L. theobromae* in the ITS region, differing in one base. However, based on the *tef*1-α gene region, the closest related species is *L. mahajangana*, but differing in six bases and an insertion of one base in *L. bruguierae*. In addition, the phylogenetic analyses of the *tub*2 and *rpb*2 gene regions show that *L. bruguierae* forms an independent clade from other *Lasiodiplodia* species. The pycnidia of *L. bruguierae* are solitary or aggregated and considerably wider (622 µm) than those of *L. mahajangana,* which are solitary and 200 µm wide. Conidiogenous cells are longer (11–21 µm) than those of *L. mahajangana* (10.5–18 µm). Moreover, the conidia are considerably longer on *L. bruguierae* (25–26 µm) compared to those of *L. mahajangana* (15.5–19 µm). The colony growth in culture differs between these species with *L. mahajangana* growing faster, covering a 90

mm Petri plate in 3 d, with an optimum temperature at 25–30 ºC, while *L. bruguierae* grows and average of 65 mm in 4 d, with an optimum temperature at 25 ºC.

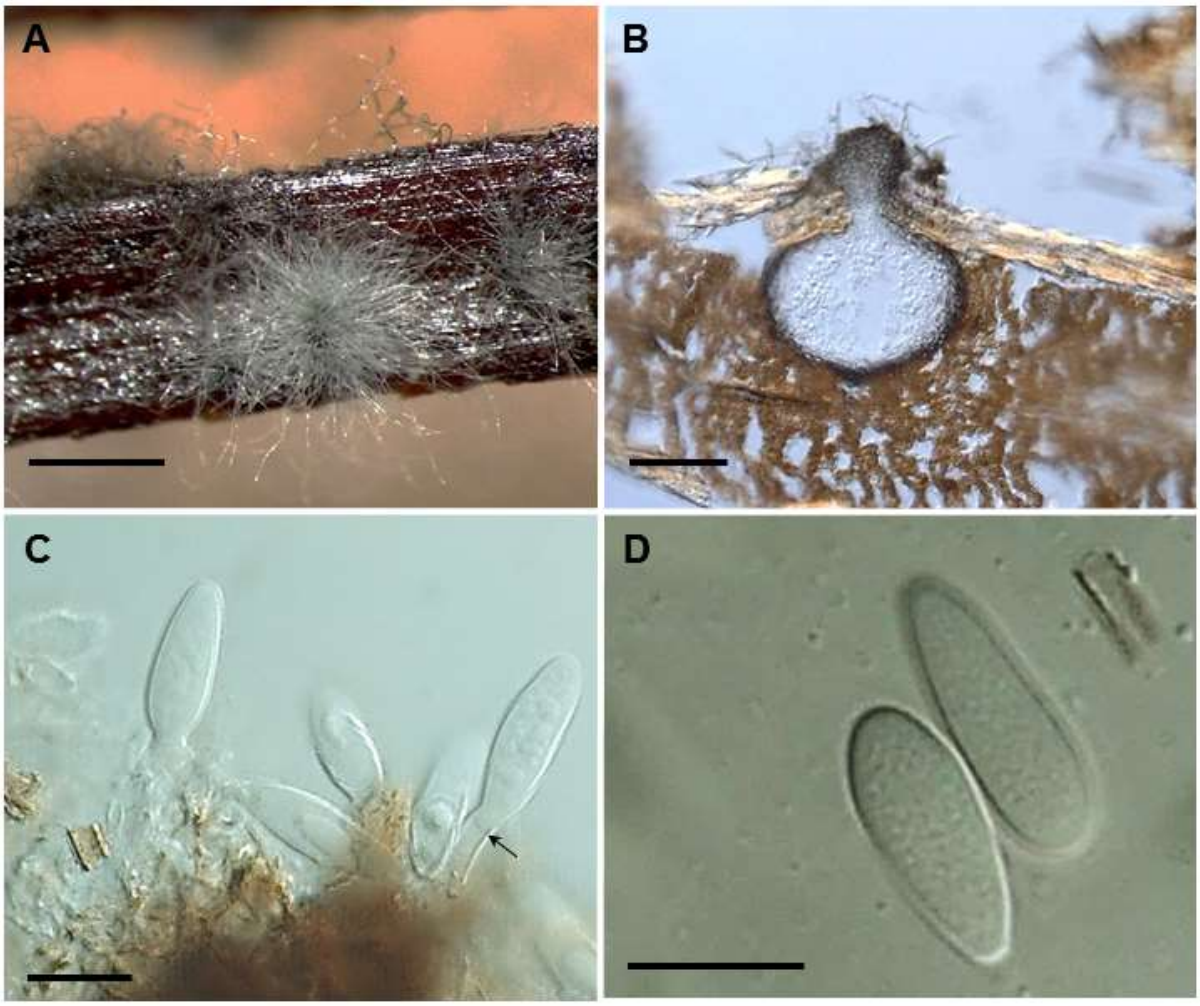

Fig 12 – *Neofusicoccum lumnitzerae* sp. nov. (CMW41469) (**A**) Pycnidium covered with aerial mycelia. (**B**) Immersed pycnidium in pine needle tissue. (**C**) Conidia attached to conidiogenous cells, showing annellation (arrow). (**D**) Conidia with granulose content. Bars: A=500 µm, B=100 µm, C,D=10 µm.

***Neofusicoccum lumnitzerae* sp. nov.** J.A. Osorio, Jol. Roux & Z.W. de Beer (Fig. 12)

MycoBank MB812012

Etymology: Epithet refers to the host, *Lumnitzera racemosa*, from which the fungus was isolated.

Sexual morph unknown. *Conidiomata* pycnidial, produced on sterilized pine needles on WA within 3–4 wks, dark brown to black, superficial or immersed in the plant tissue becoming erumpent, solitary or gregarious, globose, rarely papillate, covered with gray aerial mycelium, wall composed of outer layers of dark brown *textura*

angularis, (101–)136–170(–264) µm. *Paraphyses* not observed. *Conidiogenous cells* hyaline, smooth, cylindrical, (5–)7–9(–10) × (1.2–)2–2.3 (–3) µm. *Conidia* hyaline, thin-walled, aseptate, rarely 1-septate when mature, ellipsoidal to obclavate, with granular contents, (13–)17–18 (–21) × (5–)6.5–7(–8.5) µm.

Culture characteristics: Colonies on MEA at 25 ºC in darkness for 7 d, initially white, gradually becoming smoke-grey (21""d) on the surface, reverse side becoming olivaceous (21""), floccose at the center, growing denser and cottony towards the edges. Cardinal temperatures for growth: min ≤ 10 ºC, max ≥ 35 ºC, optimum between 25–30 ºC. Colonies reaching an average of 6 mm at 10 ºC, 21 mm at 15 ºC, 54 mm at 20 ºC, 86 mm at 25 ºC, 86 mm at 30 ºC, 3 mm at 35 ºC after 4 days. No growth at 5 ºC.

Specimens examined: SOUTH AFRICA, KWAZULU-NATAL PROVINCE: Kosi Bay, from asymptomatic branches of *Lumnitzera racemosa*. Collectors J.A Osorio & Jol. Roux. **Holotype** (PREM 61251, ex-holotype cultures CMW41469 = CBS 139674), **Paratype** (ex-paratype cultures CMW41228 = CBS 139675, CMW41613 = CBS 139676).

Habitat: Asymptomatic branches of *L. racemosa*
Known distribution in South Africa: Kosi Bay, KwaZulu-Natal Province, South Africa.

Notes: *Neofusicoccum lumnitzerae* is phylogenetically closely related to *N. australe* and *N. cryptoaustrale*, differing with eight bases in the *tef*1-α sequence, five bases in the *tub*2 sequence and six in the *rpb*2 when compared with *N. australe*. It differs with six bases in the *tef*-1α and three in the *tub*2 when compared with *N. cryptoaustrale*. Morphologically, *N*. *lumnitzerae* can be discriminated from both species by its shorter conidiogenous cells (7–9 µm) than those of *N. australe* (10−14 µm) and *N*. *cryptoaustrale* (11.5–12.5 µm). The conidia of *N. lumnitzerae* are rarely septate to maximum 1-septate with age, ellipsoidal to obclavate and significantly shorter (17–18 µm) than the fusiform conidia of *N. australe* (23−26 × 5−6 µm) and larger than the *Dichomera* synasexual conidia (10.5–14.5 × 9–11 µm) produced by the same species. Additionally, the conidia in *N. cryptoaustrale* are fusiform, 1-2-septate with age and longer (20.5–21 × 5–6 µm) than those of *N. lumnitzerae*. Colony growth

differs between species, for instance, colonies of *N. lumnitzerae* grow faster (86 mm in 4 d), with an optimum temperature at 25–30 ºC, compared with *N. australe* which reaches an average of 48 mm in 4 d, with an optimum temperature at 25 ºC. *Neofusicoccum lumnitzerae* colonies grow more slowly in culture than *N. cryptoaustrale*, which reaches 90 mm in 3 d, with an optimum temperature at 25 ºC.

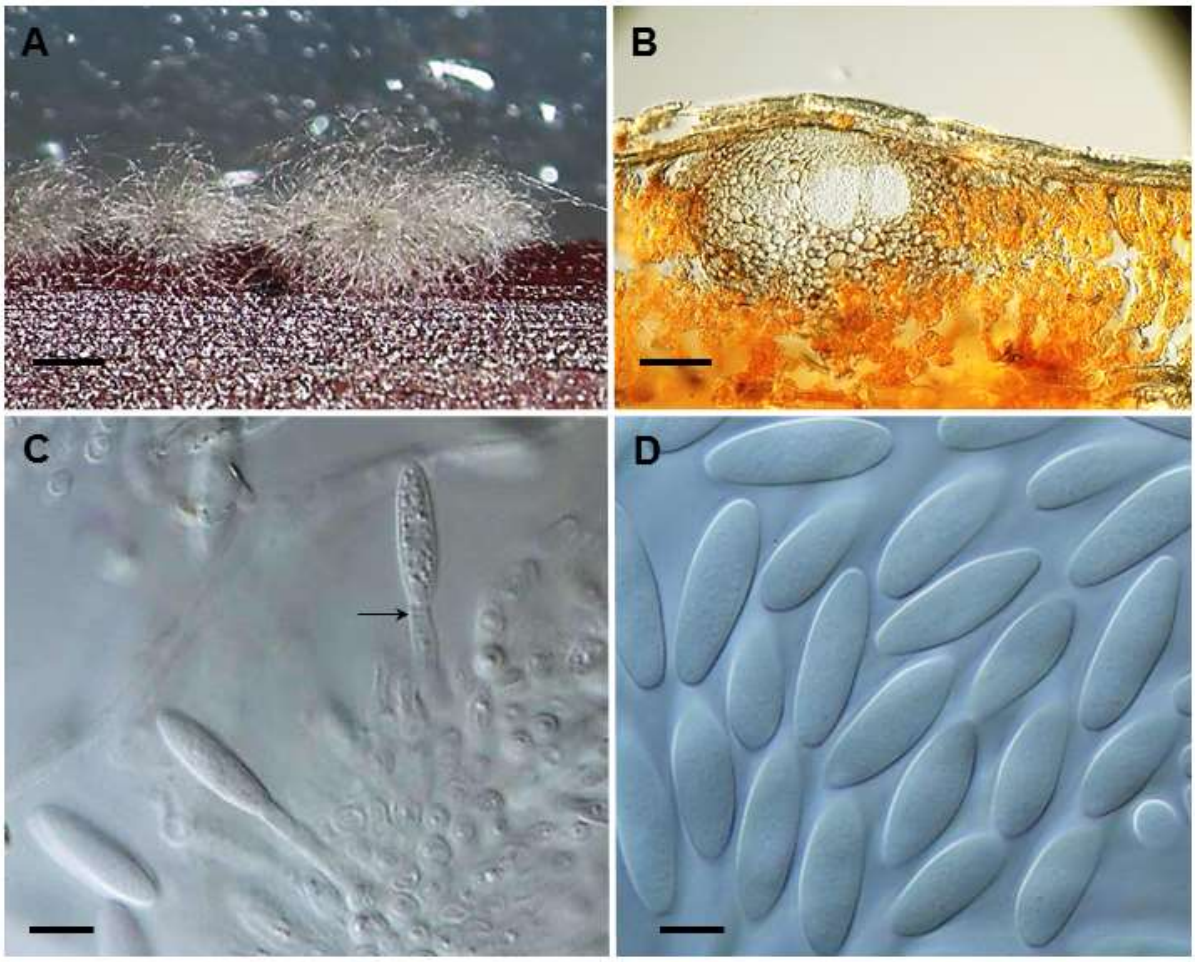

Fig 13 – *Neofusicoccum mangroviorum* sp. nov. (CMW41365) (**A**) Superficial and (**B**) immersed pycnidium. (**C**) Conidiogenous cells with annellations (arrow). (**D**) Hyaline conidia. Bars: A=500 µm, B=50 µm, C,D=10 µm.

***Neofusicoccum mangroviorum* sp. nov.** J.A. Osorio, Jol. Roux & Z.W. de Beer (Fig. 13).

MycoBank MB814641

Etymology: Epithet refers to the type of trees (mangroves) from which the fungus was isolated.

Sexual morph unknown. *Conidiomata* produced on sterilized pine needles on WA within 4 wks, covered by dense, pale olive aerial mycelia, walls light brown to dark brown, semi-immersed to immersed, becoming erumpent, solitary or becoming

gregarious, papillate or not, internal structures start forming after ~ 8 wks (136–)186–224(–300) µm wide. *Conidiogenous cells* holoblastic, hyaline, cylindrical, smooth, proliferating percurrently with 1-2 annellations, lining inner wall of pycnidium, (4.7–)5–8.5(–11) × (2–)2.1–3(–3.7) µm. *Conidia* ellipsoid, hyaline, thin walled, aseptate, smooth, both ends sub-obtuse, (15–)17–18(–20) × (6–)6.5–7(7.5) µm.

Culture characteristics: Colonies on MEA at 25 ºC in darkness for 7 d, initially white, gradually becoming smoke grey (21""d), producing yellow pigments diffusing into the agar, cottony mycelia, growing more densely at the center. Cardinal temperatures for growth: min ≤ 15 ºC, max ≥ 30 ºC, optimum 25 ºC. Colonies reaching an average of 11 mm at 15 ºC, 31 mm at 20 ºC, 53 mm at 25 ºC, 44 mm at 30 ºC after 4 days. No growth at 5 ºC and 35 ºC.
Specimens examined: SOUTH AFRICA, KWAZULU-NATAL PROVINCE: Beachwood, Kosi Bay, Mtunzini, Mgazana, from asymptomatic branches of *A. marina, B. gymnorrhiza, L. racemosa, R. mucronata*. Collectors J.A Osorio & Jol. Roux. **Holotype** (PREM 61305, ex-holotype cultures CMW41365 = CBS 140738), **Paratypes** (ex-paratype cultures CMW42481 = CBS 140740, CMW42487 = CBS 140740).

Habitat: Asymptomatic branches of *A. marina, B. gymnorrhiza, L. racemosa* and *R. mucronata*

Known distribution in South Africa: Beachwood, Isipingo, Kosi Bay, Mtunzini, St. Lucia, Richards Bay (KwaZulu-Natal Province, South Africa), Mgazana, Nahoon and Wavecrest (Eastern Cape Province, South Africa).

Notes: *Neofusicoccum mangroviorum* is phylogenetically closely related to *N. luteum*, but differing in three bases in the ITS, four bases in the *tef*1-α, two bases in the *tub*2 and three bases in the *rpb*2 sequences. In addition, there is an insertion of one base on the ITS and *tef*1-α sequences in *N. mangroviorum*. Morphologically, conidiomata in *N. mangroviorum* are considerably larger (up to 300 µm) compared to those of *N. luteum* (up to 150 µm). Conidiogenous cells (5–8.5 µm) and conidia (17–18 µm) are shorter in *N. mangroviorum* than the conidiogenous cells (8–16 µm) and conidia (16.5–22.5 µm) formed in *N. luteum.*

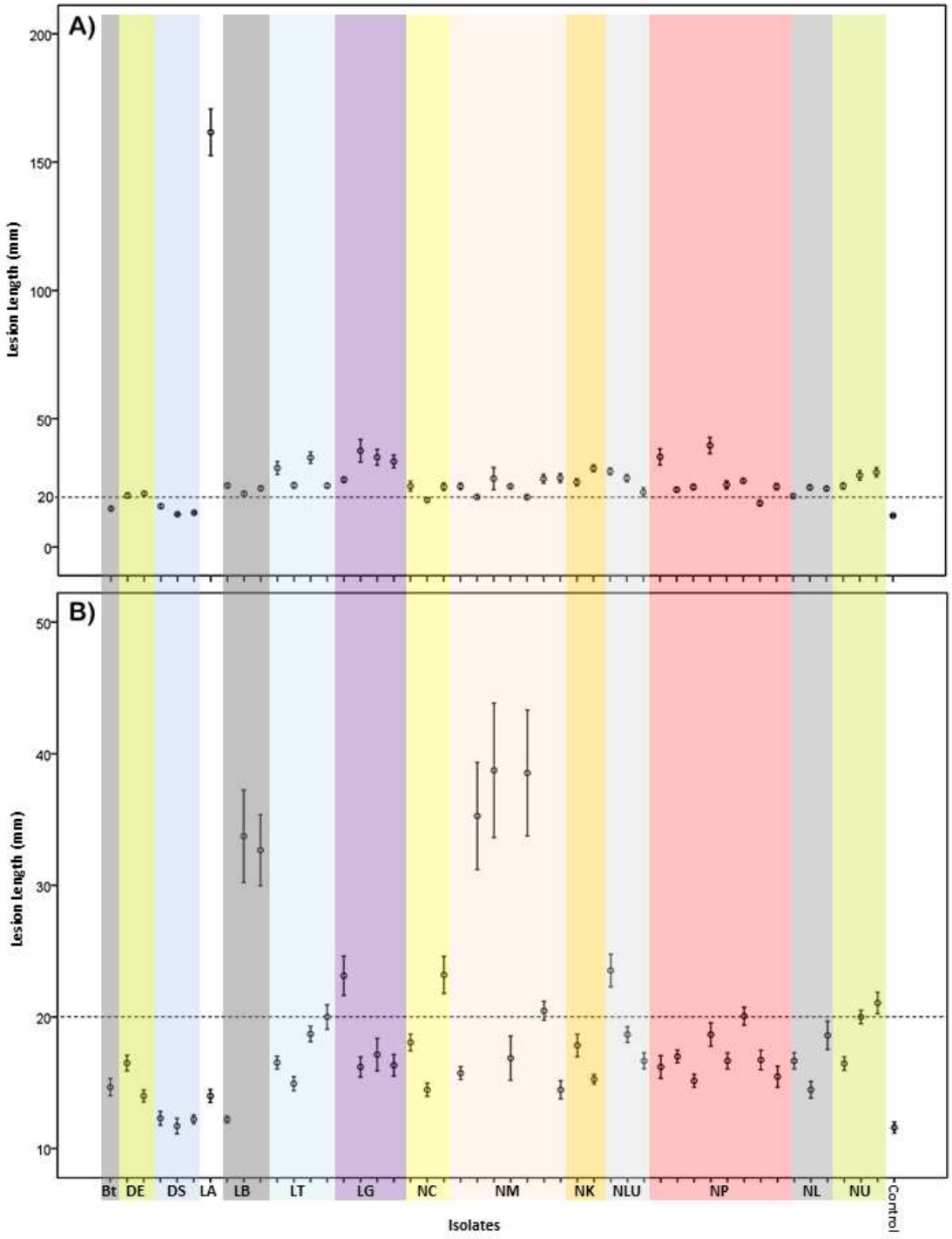

Fig 14 – Mean lesion lengths (millimeters ±1SE) from field-based inoculated branches of *A. marina* (**panel A**) and *B. racemosa* (**panel B**) after six weeks with 47 *A. marina* and *B. gymnorrhiza* associated *Botryosphaeriaceae* species, each line on the X-axis represents a different isolate of **Bt**: *Botryosphaeria* sp., **DE**: *Diplodia estuarina*, **DS**: *D. sapinea*, **LA**: *Lasiodiplodia avicenniae*, **LB**: *L. bruguierae*, **NC**: *Neofusicoccum cryptoaustrale*, **NK**: *N. kwambonambiense*, **NL**: *N. lumnitzerae,* **NLU**: *N. luteum*, **NM**: *N. mangroviorum*, **NP**: *N. parvum*,: **NU**: *N. umdonicola*. Within each treatment (n=15).

Pathogenicity tests

Most isolates produced larger lesions on branches of both *A. marina* and *B. gymnorrhiza* than the controls (Fig. 14). Furthermore**,** between the two mangrove species, *A. marina* appeared to be more susceptible to infection and branch damage by the inoculated *Botryosphaeriaceae* species than *B*. *gymnorrhiza*.

On branches of *A. marina*, the majority of fungal isolates produced lesions that were 100 % longer than those of the controls (20-mm trend line, Fig. 14). In contrast, on *B*. *gymnorrhiza* the majority of inoculated isolates showed low levels of aggressiveness. Lesion sizes on *A. marina* ranged between 11 and 223 mm and on *B. gymnorrhiza* between 9 and 98 mm. One isolate (CMW41467 = *Lasiodiplodia avicenniae* sp. nov.) was highly aggressive on *A. marina*, but had little effect on *B. gymnorrhiza*. The paired Wilcoxon signed-rank tests showed that three isolates of *D. sapinea* (CMW41234, CMW41235, CMW41362) produced lesions that were not significantly different to the controls on either of the tested mangrove species. For *B. gymnorrhiza*, the greatest average lesion lengths were associated with two isolates of *L. bruguierae* sp. nov. (CMW41470, CMW41614) and three isolates of *N. mangroviorum* sp. nov. (CMW41217, CMW41365, CMW42481). All inoculated fungi were successfully re-isolated from the lesions and their identities confirmed based on sequence data, thus fulfilling Koch's postulates.

Botryosphaeriaceae diversity and distribution

A total of 14 species of *Botryosphaeriaceae* were recovered in this study. *Neofusicoccum* was the most common genus (129 isolates from 123 trees), followed by *Lasiodiplodia* (23 isolates from 23 trees) and the least common genera were *Diplodia* (six isolates from six trees) and *Botryosphaeria* (one isolate from one tree) (Table 1). The species-accumulation curves (Fig. 15) reached an asymptote around 14 species, indicating that the sampling effort had been sufficient to obtain most of the *Botryosphaeriaceae* community associated with the mangrove species across the sampling region. However, the species accumulation-curves constructed for each of the six mangrove species (Fig. 16) did not reach an asymptote for *C. tagal* and *R. mucronata.*

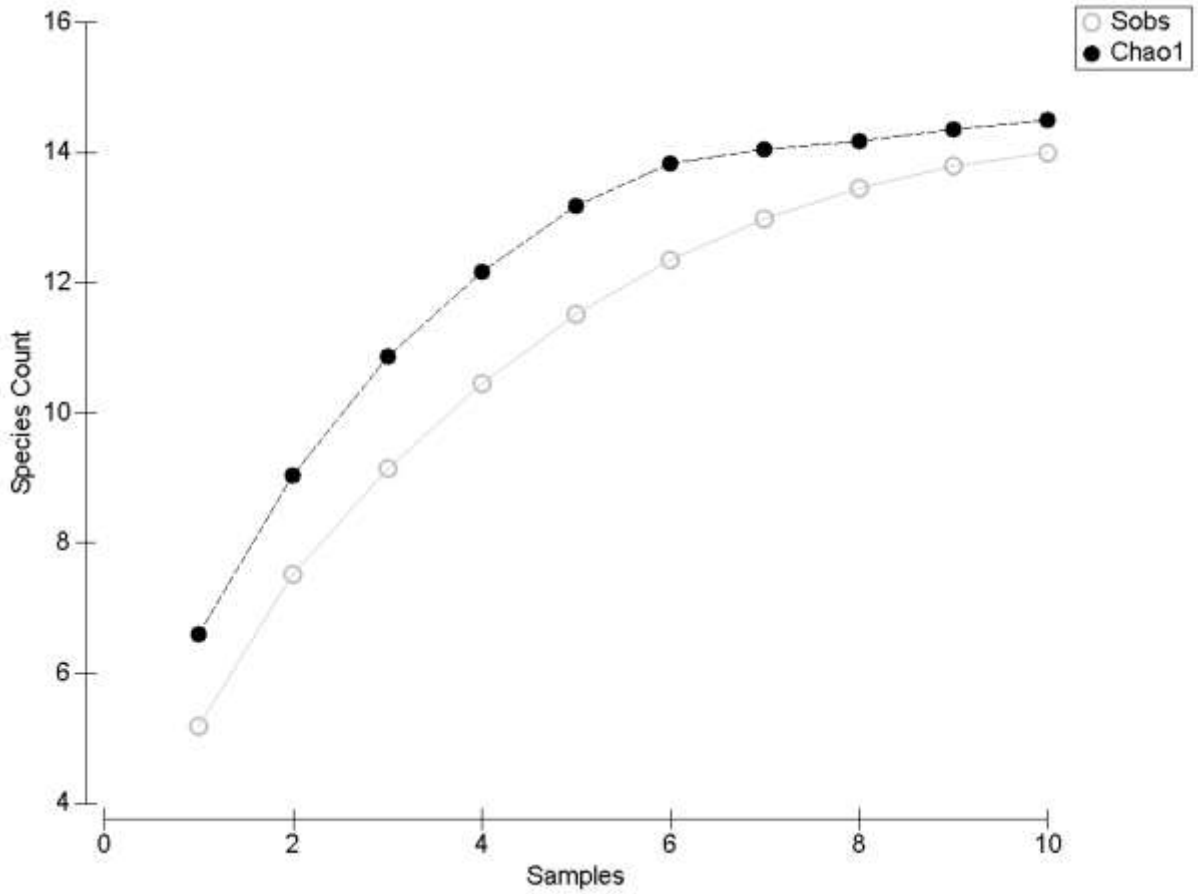

Fig 15 – Sample-based species accumulation curves for endophytic *Botryosphaeriaceae* isolated from asymptomatic branches of six mangroves species along the eastern coast of South Africa.

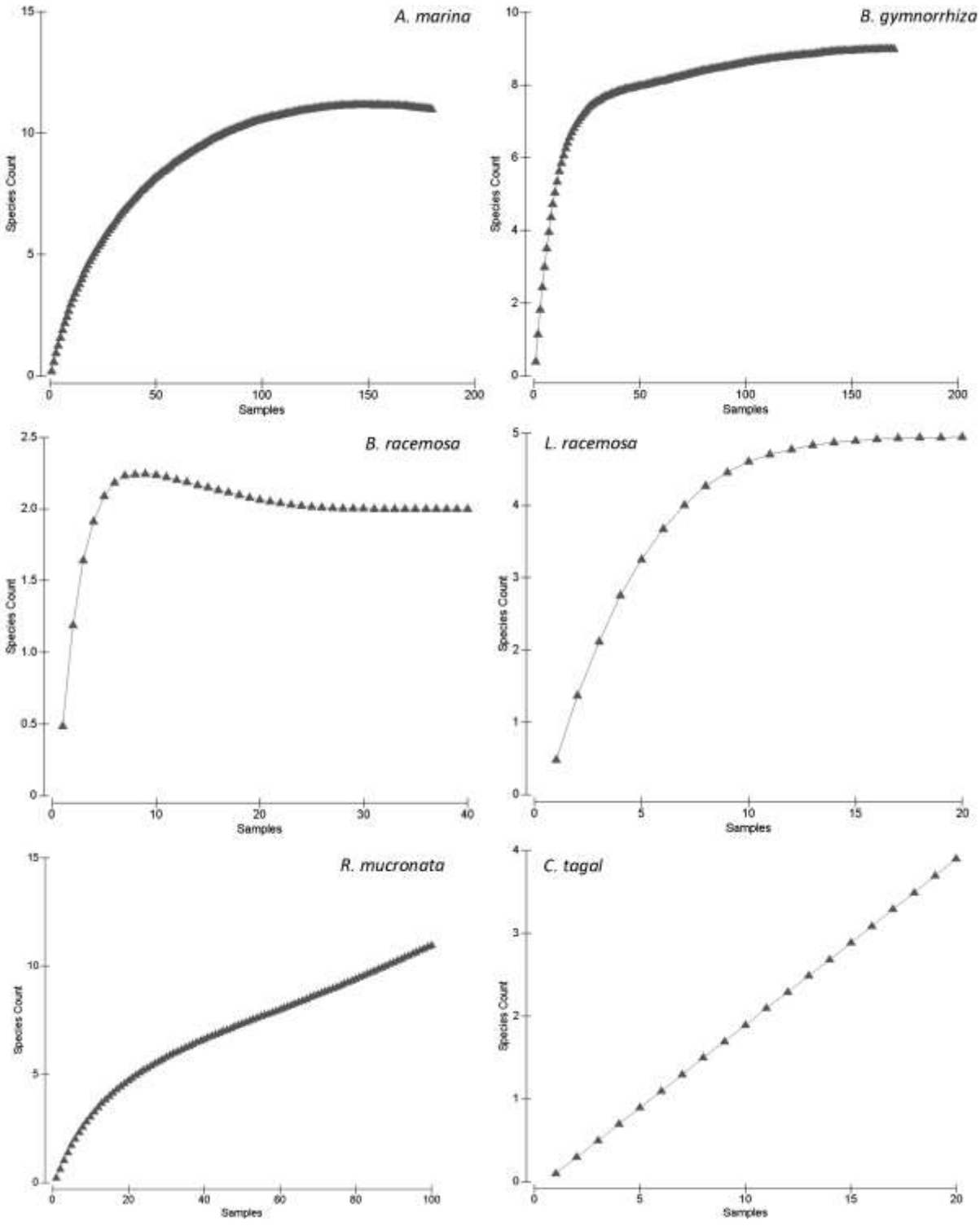

Fig 16 – Sample-based species accumulation curves for each of the six mangrove species sampled for endophytic *Botryosphaeriaceae* in South Africa.

Botryosphaeriaceae species richness ranged from two at Mapelane, where only the mangrove associate *B. racemosa* was sampled, to eight at Richards Bay where *A. marina*, *B. gymnorrhiza*, *B. racemosa* and *R. mucronata* were sampled (Fig. 17). Correspondence analysis suggested low species turnover (low gradient

lengths of both axes) between sites (Fig. 17). However, there was no spatial autocorrelation concerning the distance between sites and endophytic *Botryosphaeriaceae* assemblage composition (Spearman's Rho = -0.127; P = 0.733).

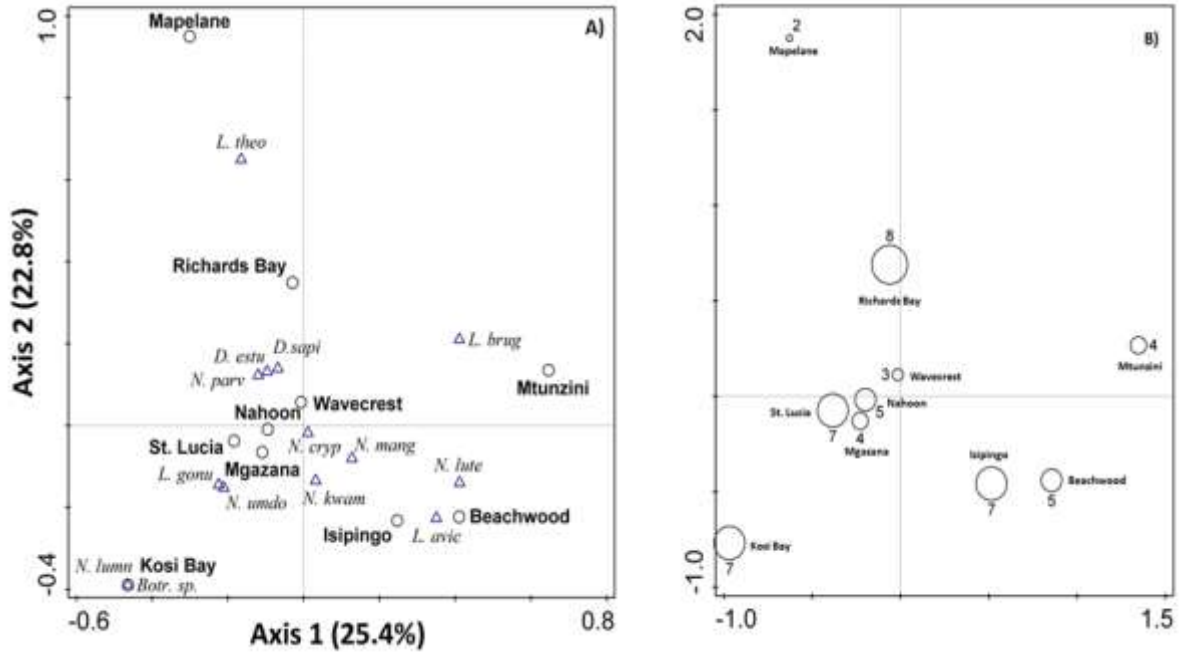

Fig 17 – Correspondence analysis (CA) depicting **A**) the *Botryosphaeriaceae* endophyte community associated with each sampled site, and **B**) the species richness of these endophytes at a site. *Botryosphaeriaceae* species names as per **Table 1**.

Endophytic *Botryosphaeriaceae* species richness ranged from two in the freshwater mangrove-associate *B. racemosa*, to nine in the true mangrove, *A. marina* (Fig. 18). From the 14 species of *Botryosphaeriaceae* isolated from the five true mangroves and one associate mangrove, *D. sapinea*, *L. avicenniae* and the *Botryosphaeria* sp. were isolated only from *A. marina*. *Lasiodiplodia bruguierae* was isolated only from *B. gymnorrhiza,* and although *L. theobromae* has been isolated from different plant species in other countries, in this study it was obtained only from *B. racemosa*. The remaining species are considered as generalists since they were found on multiple hosts (Fig. 18). Although there was high species turnover between the studied mangrove species (Axis 1 gradient length = 3.98, Ter Braak & Šmilauer 2012; Fig. 18), this pattern was particularly driven by the tight host-specificity of *Botryosphaeriaceae* species on the two mangrove species at the polar end of axis 1, *B. racemosa* and *L. racemosa*. In turn, the specificity of *D. sapinea*, *L. avicenniae* and *Botryosphaeria* sp. in *A. marina* also contributed to species turnover, especially

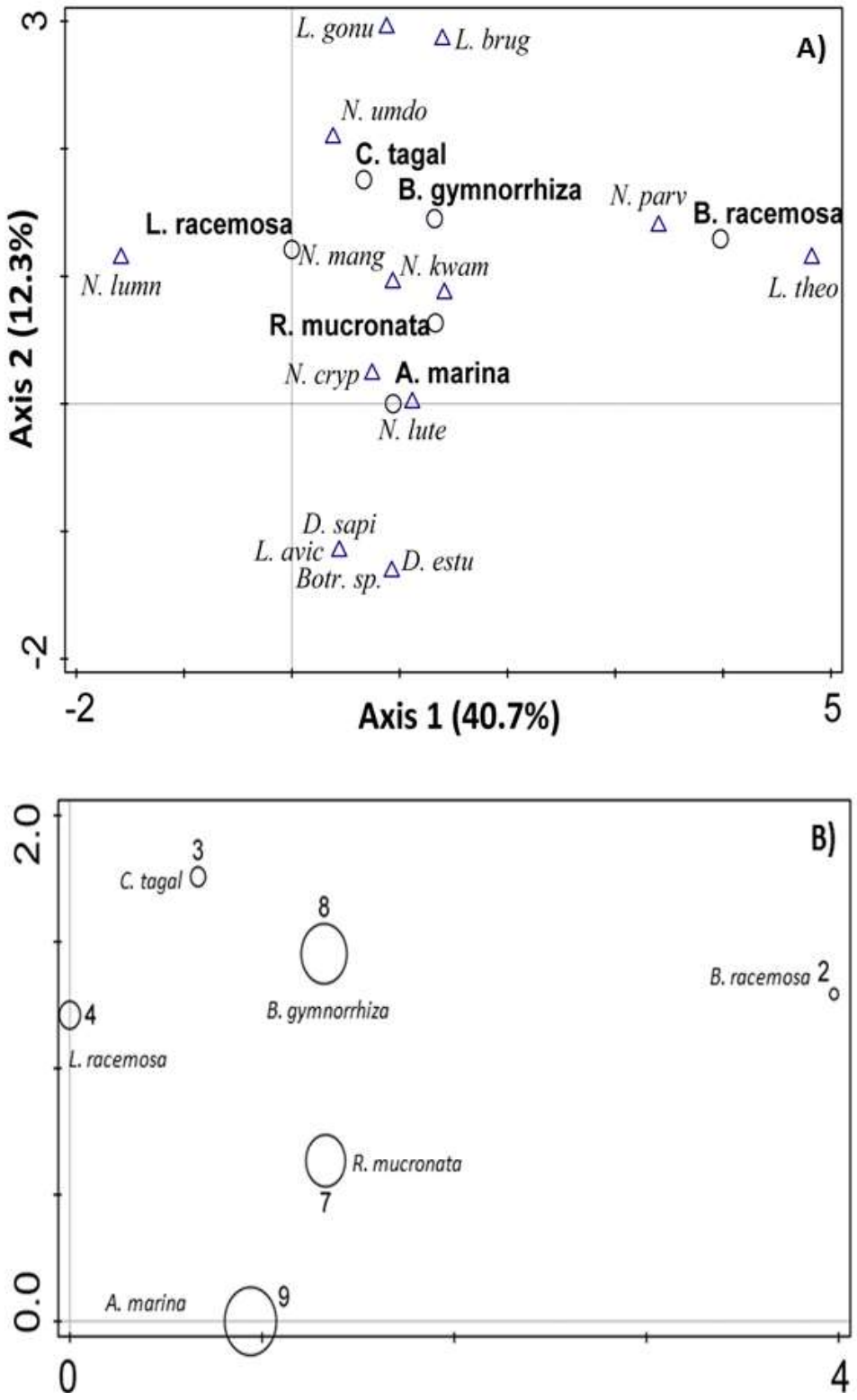

Fig 18 – Detrended correspondence analysis (DCA) depicting **A**) the *Botryosphaeriaceae* endophyte community associated with each sampled mangrove species, and **B**) the species richness of these endophytes isolated from each mangrove species. Axis 1 is most descriptive, suggesting that the difference in species community assemblage similarity is most observed between *B. racemosa* and *L. racemosa*. In turn, *A. marina* is driving further community variation on the second axis. *Botryosphaeriaceae* species names as per **Table 1**.

considering the low diversity in *C. tagal*. The presence or absence of certain mangrove species within a landscape would, thus, affect endophytic *Botryosphaeriaceae* biodiversity.

Based on percentage of occurrence, *N. cryptoaustrale* (8.1 %), *N. mangroviorum* (5.1 %) and *N. parvum* (6.04 %) were categorized as being frequent (>5 % and <10 %). The remaining species were ranked as infrequent based on the scoring criteria by Samón-Legrá *et al.* (2014). Overall, *Botryosphaeriaceae* species were isolated from 30.19 % of the total branches sampled across 10 sampling sites (Supplementary data, Table 1A).

Discussion

Nothing was known regarding the diversity of endophytic *Botryosphaeriaceae* on mangroves in South Africa prior to this study. We identified 14 taxa belonging to four genera based on the DNA sequence data sets for four gene regions. All these species, including five new taxa (*Diplodia estuarina* sp. nov., *Lasiodiplodia avicenniae* sp. nov., *L. bruguierae* sp. nov., *Neofusicoccum lumnitzerae* sp. nov., *N. mangroviorum* sp. nov.), eight known species (*D. sapinea, L. theobromae, L. gonubiensis, N. cryptoaustrale, N. kwambonambiense, N. luteum, N. parvum, N. umdonicola*) and a *Botryosphaeria* sp., of which the identity could not be confirmed, are reported for the first time from mangroves globally.

Botryosphaeriaceae species on mangroves in South Africa vary in their abundance, distribution and host association. *Neofusicoccum* was the most commonly isolated genus in the *Botryosphaeriaceae* and was isolated from all mangrove species at all sampling sites. This inference is consistent with several previous reports (e.g. Slippers *et al.* 2005; Pavlic *et al*. 2007; Taylor *et al.* 2009; Sakalidis *et al.* 2011, 2013) that have shown the ability of species in this genus to inhabit a wide variety of plant species and geographic areas worldwide. *Lasiodiplodia* species were isolated at eight of the 10 sampling sites, but were not recovered from the two southern most mangrove locations. This is not surprising since earlier reports (e.g. Punithalingam 1976, 1980, Burgess *et al.* 2006) have indicated the occurrence of *Lasiodiplodia* species mostly in tropical and subtropical regions. Consistent with previous studies that indicate the cosmopolitan nature of

Diplodia species (Taylor *et al.* 2005; Phillips *et al.* 2007), we found species in this genus inhabiting trees in the north (St. Lucia) and the most southern range (Nahoon) of mangrove occurrence in South Africa. This study also revealed some host association patterns. *Lasiodiplodia avicenniae*, *L. bruguierae* and *N. lumnitzerae* appeared to be relatively host specific while *D. estuarina* and *N. mangroviorum* were shown to be generalists.

Five previously undescribed *Botryosphaeriaceae* were isolated as endophytes from six species of mangroves. The identification of these new species was supported by multi gene sequence data and morphological characteristics. The gene regions commonly used to infer phylogenetic relationships within species in the *Botryosphaeriaceae* (ITS, *tef*1-α, *tub*2 and *rpb*2) made it possible to identify species with confidence. In general, taxonomic characters, such as conidial shape, color and size, are more useful to distinguish between genera than between species within genera. However, a combination between shape, color and dimension structures supported the phylogenetic separation of the new species from those previously described.

Several studies have been conducted to explore the fungal diversity of mangroves (Sarma & Hyde 2001; Sarma *et al.* 2001; Nambiar & Raveendran 2009; Alias *et al.* 2010), including the endophytic community associated with these trees, in countries such as Brazil, China, India (Suryanarayanan et al. 1998; Kumaresan & Suryanarayanan 2001; Ananda & Sridhar 2002; Xing *et al.* 2011; de Souza *et at*. 2013). Despite the fact these countries are larger and have more geographically diverse species of mangroves than South Africa, only two species in the *Botryosphaeriaceae* (one *Lasiodiplodia* sp. and one *Neofusicoccum* sp.) were reported from those studies. Likewise, Stevens & Shear (1929) reported *Botryosphaeria ribis* var. *chromogena* (= *Neofusicoccum ribis*) from the mangrove associate *Hibiscus tiliaceus* in Hawaii. It is not clear why those studies resulted in such low numbers of *Botryosphaeriaceae* when our study yielded different species from every mangrove species sampled. Several of the previous studies on mangroves, however, focused on dead and decomposing wood, while in the present study, living and asymptomatic branch material was collected. Furthermore, Kumaresan and Suryanarayanan (2001), for example, isolated endophytes from leaves and litter and Ananda and Sridhar (2002) isolated from roots, while de Souza

et al. (2013) studied the endophytic fungi of mangrove species different to those occurring in South Africa.

The majority of isolates inoculated on *A. marina* and *B. gymnorrhiza*, produced significantly longer lesions than the control inoculations after six weeks. However, significant differences in aggressiveness were found among the fungal species and in the susceptibility of the host species. Of all fungal species examined, *L. avicenniae* (CMW41467) was the most aggressive, and of the two host species, *A. marina* was the most susceptible to most of the *Botryosphaeriaceae* isolates. Of the 14 different fungal taxa identified, eight are recognized as pathogens of other plant families. Among these, for example, *D. sapinea* is a well-known pathogen of *Pinus* species worldwide (Swart & Wingfield 1991; Smith *et al.* 1996). Although this fungus has mostly been associated with conifers, this study confirms that angiosperms can be part of the *D. sapinea* host range, as shown in previous studies (Damm *et al.* 2007; Lazzizera *et al.* 2008). However, results of the pathogenicity tests showed that *D. sapinea* is not pathogenic to *A. marina* and *B. gymnorrhiza*. Interestingly, all new species when inoculated were shown to be capable of causing lesions on the branches of either *A. marina* or *B. gymnorrhiza* or both host trees. This indicates that these fungi are potential pathogens of the test tree species.

The species richness (i.e. total number of species) of *Botryosphaeriaceae* was higher on *A. marina* and *B. gymnorrhiza* than on other mangrove species. This may be a function of the number of trees sampled from these hosts, compared to the number of trees sampled from the remaining tree species. For instance, a similar number of endophytic *Botryosphaeriaceae* was recovered from *A. marina* (nine species from nine sampling sites) and *B. gymnorrhiza* (eight species from nine sampling sites), while *R. mucronata* (sampled at five sites) harbored seven fungal species, *B. racemosa* (sampled at two sites) harbored two species, and *C. tagal* and *L. racemosa* (sampled only in Kosi Bay) harbored three and four species respectively.

Of the six mangrove species included in the study, sampling intensity to obtain *Botryosphaeriaceae* was insufficient for *C. tagal*. This was due to the fact that this tree is rare in the areas sampled. Although to a lesser extent, sampling of *R. mucronata* was also insuffient. This could be seen from the species accumulation curve that did not reach an asymptote for *R. mucronata*, despite the 100 branches sampled and seven *Botryosphaeriaceae* species isolated. Factors other than

sampling size, such as differences in plant chemical composition (Lim 2012; Naskar & Palit 2015) or the physiological adaptations and mechanisms used by mangroves for salt exclusion (Gilbert *et al.* 2002), could also have influenced fungal species richness in the various hosts.

There was no spatial autocorrelation concerning the distance between sites and endophytic *Botryosphaeriaceae* assemblage composition. This suggests that, from a regional distribution perspective, the proximity of sampling sites to one another did not determine the similarity of *Botryosphaeriaceae* communities. In turn, we found that mangrove diversity is linked to *Botryosphaeriaceae* diversity. Thus, the presence of certain mangrove species would influence the diversity patterns of *Botryosphaeriaceae* endophytes in the landscape. Specifically, *B. racemosa* and *L. racemosa* had small but rather host-specific *Botryosphaeriaceae* communities. Thus, protecting mangrove diversity in South Africa should be prioritized, since the local loss of any mangrove species could negatively affect endophytic *Botryosphaeriaceae* diversity in space and time.

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Supplementary data

Table A – Number of successful isolations out of 530 samples collected in this study

Fungus Species	Host species							
	Avicennia marina	*Bruguiera gymnorrhiza*	*Barringtonia racemosa*	*Ceriops tagal*	*Rhizophora mucronata*	*Lumnitzera racemosa*	Total	% out of 530 branches
***Botryosphaeria* sp.**	1	-	-	-	-	-	1	0.19
D. estuarina	2	-	-	-	1	-	3	0.57
D. sapinea	3	-	-	-	-	-	3	0.57
L. avicenniae	4	-	-	-	-	-	4	0.75
L. bruguierae	-	9	-	-	-	-	9	1.70
L. gonubiensis	-	4	-	1	-	-	5	0.94
L. theobromae	-	-	6	-	-	-	6	1.13
N. cryptoaustrale	17	13	-	1	10	2	43	8.11
N. kwambonambiense	-	1	-	-	1	-	2	0.38
N. lumnitzerae	-	-	-	-	-	3	3	0.57
N. luteum	2	2	-	-	1	-	5	0.94
N. mangroviorum	6	16	-	-	4	1	27	5.09
N. parvum	2	13	13	-	4	-	32	6.04
N. umdonicola	1	10	-	1	1	4	17	3.21
Total No. isolates per host	38	68	19	3	22	10	160	30.19
Total No. species per host	9	8	2	3	7	4		