An assessment of mangrove diseases and pests in South Africa

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Abstract

Mangroves are critically important components of coastal ecosystems. However, their survival is globally threatened, mostly due to impacts resulting from human activities. Reports of mangrove deaths associated with pathogens and insect pests have emerged during the past few years. In South Africa, mangrove species are under pressure from both environmental and anthropogenic disturbances, potentially making them more susceptible to diseases. We present the most detailed evaluation of possible biotic causes of mangrove decline in South Africa to date. Surveys covering the entire distribution range of mangroves in the country were conducted. Qualitative and quantitative data from siltation of pneumatophores, stand density, diameter at breast height and the presence of wood-boring beetles were correlated with disease incidence and severity to elucidate the possible relationships with mangrove health. Phylogenetic analyses were performed to determine the taxonomic placement of fungi isolated from symptomatic trees. Of five true mangrove species and two mangrove associates examined, only *Avicennia marina* showed signs and symptoms of branch and stem cankers, die-back, wood-boring insects and leaf galls. *Barringtonia racemosa* showed symptoms of fruit and leaf disease and *Hibiscus tiliaceus* was observed with herbivory by leaf-feeding beetles. Using a multivariate approach, the presence of beetles and high pneumatophore siltation appeared to be associated with the observed die-back and canker levels of *A. marina*. Four main fungal groups were recovered from symptomatic trees. The results suggest that natural and anthropogenic stressors exerted on the mangrove trees lead to the colonization of an array of opportunistic pests and diseases.

Introduction

Mangrove trees provide crucial environmental services including habitat for birds, fish and invertebrates. They are responsible for coastal protection from hurricanes, floods, sea level rise, wave action and erosion (Kathiresan and Bingham, 2001; Sherman *et al*., 2001). Mangrove systems are recognized as one of the most fragile ecosystems, hence they are amongst the most threatened ecosystems globally (Taylor *et al*., 2003; Martinuzzi *et al*.,

2009). This is mainly due to increasing urban and industrial development (Abuodha and Kairo, 2001; Ellison and Farnsworth, 2001; Spalding *et al*., 2010). In addition, abiotic factors such as hurricanes, lightning strikes, salinity and flooding are also reported to be affecting the health of mangroves (Smith *et al*., 1994; Kathiresan and Bingham, 2001). These phenomena can expose the trees to a variety of opportunistic pests that are important agents of tree decline (Seifert *et al*., 1993; Gilbert, 2002; Hulcr and Dunn, 2011).

An increasing concern regarding mangrove health has prompted several studies to determine possible diseases of these trees caused by fungi and other microbes (Tattar *et al*., 1994; Gilbert *et al*., 2002; Ukoima *et al*., 2009; Sakayaroj *et al*., 2012). However, despite the direct benefits provided by mangroves globally, studies regarding biological factors associated with the decline of these trees remain limited (Osorio *et al*., 2014). Although information regarding tree decay and mortality caused by microorganisms remains poorly understood, there are some reports of possible biotic causes of tree disease and death. For example, in the Gambia (Africa) high levels of infestation of an unidentified gall-inducing fungus was reported as the cause of mortality of *Rhizophora* species (Teas and McEwan, 1982). Another case of microorganisms involved with mangrove decay globally was reported in Australia where a *Halophytophthora* sp. (reported as *Phytophthora* sp.) was associated with the mortality of *Avicennia marina* trees along the Queensland coast (Pegg *et al*., 1980; Ho and Jong, 1990).

In South Africa, six species of mangroves, belonging to four families, are restricted to swamps and riverine areas along the coast of the Kwazulu-Natal (KZN) and Eastern Cape (EC) Provinces. Mangrove species in the country include *A. marina* (Forssk.) Vierth. (Acanthaceae, white mangrove), *Bruguiera gymnorrhiza* (L.) Savigny (Rhizohophoraceae, black mangrove), *Ceriops tagal* (Perr.) C.B Rob. (Rhizophoraceae, Yellow/Indian mangrove), *Lumnitzera racemosa* Willd. (Combretaceae, Tonga mangrove), *Rhizophora mucronata* Lam. (Rhizophoraceae, red mangrove) and *Xylocarpus granatum* König (Meliaceae, Mangrove Mahogany). The height of these trees varies depending on the species and area where they grow, however, *A. marina* generally reaches heights of up to 10 m tall, *B. gymnorrhiza* and *R. mucronata* usually reach 10–15 m, while *C. tagal*, *L. racemosa* and *X. granatum* do not exceed 5 m in height (Steinke, 1995, 1999; Rajkaran and Adams, 2011; South African National Biodiversity Institute – SANBI 2016). Additionally, the mangrove associates *Barringtonia racemosa* (L.) Roxb. (Lecythidaceae; fresh water mangrove) and *Hibiscus tiliaceus* (Malvaceae; Wild cotton tree) occur along riverine and coastal areas of the Indian Ocean of South Africa (Coates and Coates, 2002; SANBI 2016). Studies related to mangrove health in the country have focused mostly on the effects of abiotic factors and human activities, such as flooding, drought, livestock browsing and wood harvesting (Breen and Hill, 1969; Adams *et al*., 2004; Hoppe-Speer *et al*., 2011, 2013, 2015).

There are few reports of mangrove deaths in South Africa. These include the die-back of *A. marina* and *B. gymnorrhiza* in the Beachwood Mangrove Nature Reserve, where the reported disease was likely influenced by increased anthropogenic disturbances (Demetriades, 2009). Likewise, mortality of mangrove trees near the Durban coast (N. Pammenter and J. Buzzard, personal communication) as well as in the Isimangaliso Wetland Park (J. Adams and C. Fox personal communication). The only report of a fungal pathogen affecting mangroves in South Africa was of *Pseudocercospora mapelanensis* J.A. Osorio &

Jol. Roux, which causes a fruit and leaf disease of the mangrove associate, *B. racemosa* (Lecythidaceae) (Osorio *et al*., 2015).

The aim of this study was to survey mangrove trees in South Africa for the occurrence of pest and disease problems. Where found, pests and pathogens possibly involved in disease development were identified using Deoxyribonucleic acid (DNA) sequence data and morphology. Furthermore, disease incidence and severity data were collected using transects located along the distribution range of mangrove species. On-site environmental proxies that could help explain mangrove health were also correlated with disease incidence and severity of *A. marina*: specifically, the siltation of pneumatophores, stand density at a site, the diameter at breast height (DBH) and the presence of wood-boring beetles.

Methods

Disease survey and sample collection

All major mangrove areas in the country, from Kosi Bay in the north of the KZN Province to East London in the EC Province, were visited and mangrove trees visually examined for the presence of diseases on all mangrove species. The sites included from north to south, in the KZN Province: Kosi Bay, where all six South African mangrove species occur together; St. Lucia, with dense stands of *A. marina* and *B. gymnorrhiza*; Mhlathuze (Richards Bay), where *A. marina*, *B. gymnorrhiza* and *R. mucronata* occur; Mlalazi Nature Reserve (Mtunzini), with *A. marina* and *B. gymnorrhiza*; Beachwood Nature Reserve (Durban North) with dense stands of *A. marina* and *B. gymnorrhiza*, and Reunion Park (Isipingo) in Durban, with *A. marina*, *B. gymnorrhiza* and *R. mucronata*. In the EC Province, sites included Mgazana estuary (near Port St. Johns), with stands of *A. marina*, *B. racemosa* and *R. mucronata* and Wavecrest and Nahoon Estuary Nature Reserve (East London) with mostly *A. marina* trees and a few individuals of *B. gymnorrhiza* and *R. mucronata* (Figure 1). These sites were chosen to represent the entire distribution range of mangroves and all species present in South Africa. Where present at these sites, and where pest damage was observed on them, the mangrove associates *B. racemosa* and *H. tiliaceus* were also investigated.

Surveys were haphazardly conducted at all sites, depending on accessibility at the time of the visits. Sites were visited several times during 2011–2013 and the areas where diseases were observed were visited multiple times to obtain sufficient material for analyses. Samples collected consisted of leaf, branch and stem sections showing leaf spots, branch and stem bleeding, cankers and insect activity. The time spent at each site during the surveys depended on the size of the site, accessibility, tides and the disease symptoms observed. Care was taken to investigate trees representing each species present at a site and to evaluate trees of different ages. An undetermined number of trees of each mangrove species growing at every sampling site were visually examined. The leaves and branches of taller trees were visually examined by using a FUJINON 7 × 28 M Binoculars. The plant material sampled was processed on the day of collection, where possible, or alternatively transported to the laboratory facilities of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, for isolations and analyses.

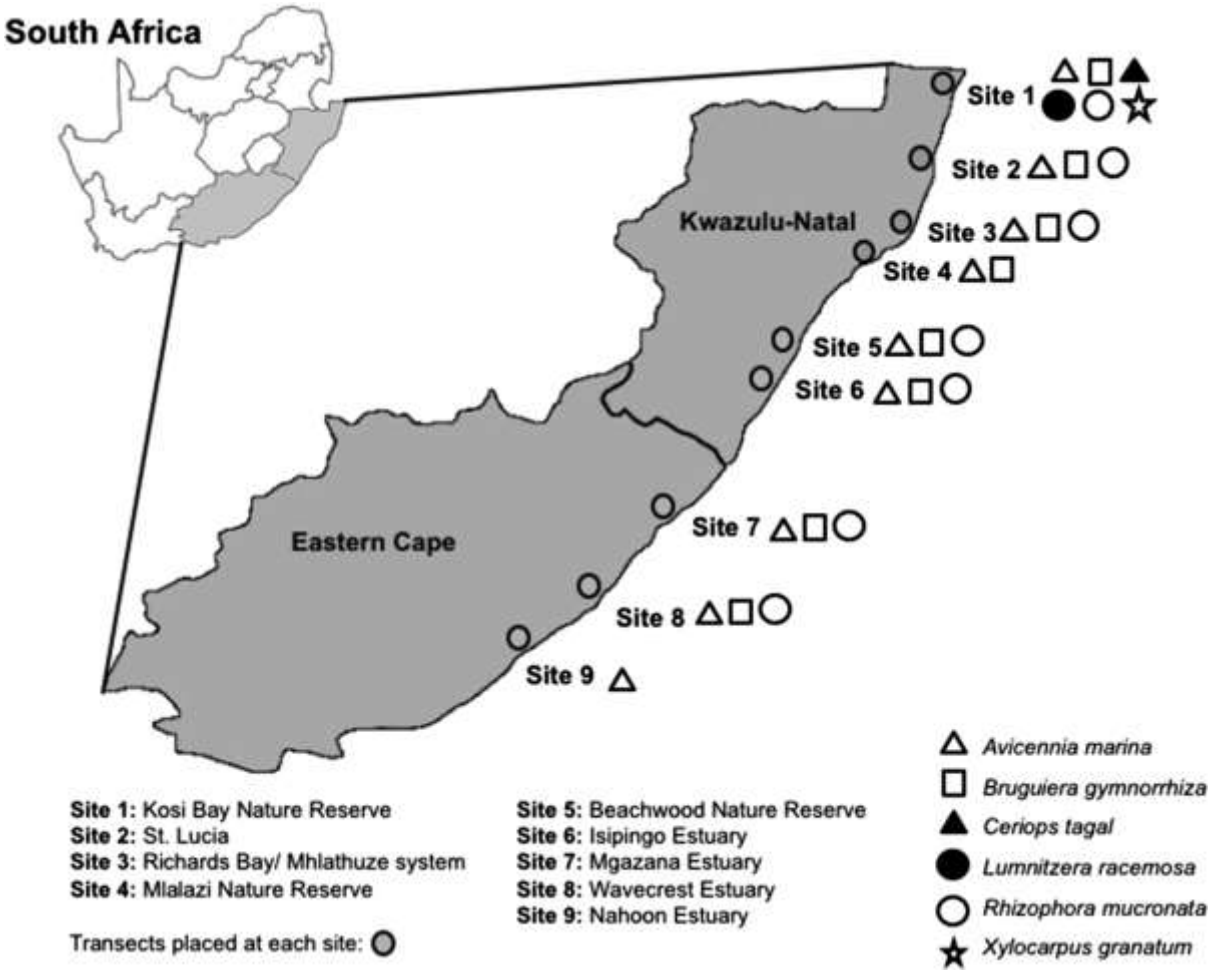

Figure 1. Map of the eastern coast of KZN and EC provinces showing the distribution range of mangrove species (triangle colours). Sampling sites used in this study are indicated by the site number. Transects per site $n = 4$.

Fungal isolations

Symptomatic plant material was examined for the presence of fungal fruiting bodies and insect activity using a Nikon (SMZ 745) dissection microscope. Where present, spore drops and mycelial strands were transferred directly to sterile 2 per cent malt extract agar (MEA 20 g malt + 17 g agar in 1 l of distilled water) (Biolab, Midrand, South Africa) amended with 0.4 g of streptomycin sulphate (Sigma-Aldrich, Saint Louis, MO, USA). Thereafter, lesions were surface-sterilized with 70 per cent ethanol and ~1 mm sections were removed from the leading edges of cankers and stains observed, and transferred to 2 per cent water agar and MEA medium and incubated at 25°C. The isolations were examined periodically for the presence of mycelial growth, using a dissecting microscope. Single hyphal-tips of mycelium growing directly from the plant material, or spore drops, were transferred to 2 per cent MEA. Purified cultures were incubated at 25°C.

Fungal identification

Fungal cultures obtained from symptomatic plant material were grouped based on texture and colour and used for DNA extraction. All isolates were barcoded (Schoch *et al.,* 2012)

using the Internal Transcribed Spacers regions (ITS1 and ITS2) and the 5.8 S ribosomal RNA to corroborate the taxonomic placement of the fungi isolated. Thereafter, the β-tubulin (BT) gene region was amplified to confirm the species identity of dominant fungi and putative pathogens.

Prior to DNA extraction, cultures were grown on MEA for between 10 and 15 days, after which the mycelium was freeze-dried and then pulverized by using 2 or 3 mm metal beads (depending on size of beads) previously sterilized and placed in a Mixer Mill type MM 301 RetschR tissue lyser (Retsch, Haan, Germany) for 3 min at a frequency of 30 cycles per second. The DNA was extracted using the method described by Raeder and Broda (1985). The quality and quantity of DNA was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA), and then diluted to achieve a concentration of 20 ng/µl and stored at −20°C to be used in the polymerase chain reaction (PCR).

The ITS locus was amplified using primers ITS1 and ITS4 (White *et al*., 1990) and the BT locus was amplified using the primers Bt2a and Bt2b (Glass and Donaldson, 1995). The reaction mixture and PCR thermal cycling protocols were completed as described by Osorio *et al*. (2014), with a modification of the annealing temperature for the BT gene region at 54°C. To evaluate the success of the PCR, 5 µl of the PCR product from each reaction were stained with GelRedTM nucleic acid gel stain (Biotium, Hayward, CA, USA) and separated on a 1 per cent agarose gel for 20 min at 90 Volts and viewed with a Gel Doc EZ Imager (Bio-Rad Laboratories Inc.). The PCR products were cleaned using Sephadex G-50 columns (Sigma-Aldrich, Sweden) following the manufacturer's protocol. Pure PCR products (2 µl) were added to each sequencing reaction, with a Big-Dye terminator cycle sequencing kit (PE Applied Biosystems, California, USA) and using the same primers and annealing temperatures that were used in the initial PCR. The sequencing product was cleaned using the steps mentioned above, placed in a drier to evaporate excess water, and submitted for sequencing to the DNA Sequencing Facility of the Faculty of Natural and Agricultural Sciences at the University of Pretoria. Pure cultures used for identification were deposited in the culture collection (CMW) of the Tree Protection Co-operative Program (TPCP) at FABI, University of Pretoria.

Phylogenetic analyses

Consensus sequences of the ITS gene region were assembled with CLC Main Workbench 6.7.1 and then compared with available databases utilizing the Blast-Algorithm on the BLAST homepage (http: //blast.ncbi.nih.gov/blast.cgi) and selecting the highly similar sequences ‘megablast’ option. Sequences of closely related sister taxa to those obtained from mangroves were selected as outgroups in the individual sequence data sets. The data matrices were aligned online using MAFFT 7 (Katoh and Standley, 2013) and edited manually for alignment errors with MEGA 6 (Tamura *et al*., 2013).

Phylogenetic analyses for Maximum Likelihood (ML) were performed with the program PhyML version 3.0 (Guindon and Gascuel, 2003) using the best fit substitution models determined by jModeltest 0.1.1 and the Akaike information criterion (Darriba *et al*., 2012). The confidence levels were estimated with 1000 bootstrap replicates. The Bayesian

Inference (BI) analysis was performed for each individual data set, based on a Markov Chain Monte Carlo (MCMC) algorithm using MrBayes 3.2 (Ronquist *et al*., 2012). Two independent runs were performed simultaneously for 3 million generations at every 100th generation. Burn-in values were determined with Tracer 1.4 (http: //tree.bio.ed.ac.uk/software/tracer) and the first 25 per cent sampled trees representing the burn-in were discarded. Phylogenetic trees obtained from BI and ML analyses were viewed in MEGA 5 (Tamura *et al*., 2011).

Insect collection

Where insect activity was observed, plant material was sampled by cutting portions of stems and branches with visible lesions, gummosis and frass exudation. Because few beetles were collected directly from wood samples, a trapping methodology was used to obtain a higher number of individuals to identify beetles and to isolate potential pathogens from these insects. The insects were collected by using aerial traps baited with 96 per cent ethanol. Six traps each were placed at Beachwood, Isipingo, Mtunzini and St. Lucia in KZN Province and Mgazana in the EC Province. Paper towel was used in the traps to collect living or freshly dead insects. The beetles were separated into different groups based on morphology and fresh beetles were used for fungal isolations. Some of the collected beetles from galleries and traps were squashed while others were left intact and plated directly onto the surfaces of 2 per cent MEA plates amended with Cycloheximide antibiotic, used for the selective isolation of Ophiostoma species (Harrington, 1981). Fungi were further purified following the steps for fungal isolation, mentioned previously. Adults of leaf-feeding insects were caught directly from the surfaces of leaves and placed in Falcon™ 50 ml Conical Centrifuge Tubes containing 70 per cent ethanol.

Insect identification

Insects were examined using a Nikon (SMZ 745) dissection microscope and grouped into morpho-groups for further identification. A maximum of five individuals were deposited in 1.8 ml CryoTubesTM Vials with 70 per cent ethanol and sent to either Dr Roger Beaver (Thailand), for the identification of ambrosia and bark beetles or Dr Elizabeth Grobbelaar, Agricultural Research Council, South Africa, for the identification of the phytophagous beetles. Likewise, fresh leaves with the presence of galls were sent to Prof. Stefan Neser (South Africa) and photographs of flies inside flat galls of different stages were sent to Dr Robin Adair (Australia).

Pathogenicity tests

Four isolates of a fungus commonly isolated from symptomatic *A. marina* trees were used for pathogenicity trials. Approximately 6-day-old cultures, grown on 2 per cent MEA at 24°C were used to inoculate *A. marina* trees in the field. A sterile cork-borer with a 7-mm internal diameter was used to remove the bark from selected trees to expose the cambium for inoculation. Plugs were cut from pure cultures, or un-inoculated agar in the case of the controls, with a sterilized 7 mm cork borer. One branch per tree, ~11–13 mm in diameter, was inoculated with the selected fungi or control agar plug, with 15 different trees inoculated per isolate. The control MEA plugs were also inoculated into branches of 15

individual trees. The inoculated wounds were sealed with masking tape to reduce contamination and to prevent desiccation of the inoculum.

Lesion lengths, both in the bark and cambium, were measured 6 weeks after inoculation. Five portions of each inoculated branch were retained for fungal isolation and identification to investigate whether the lesions had been caused by the inoculated fungus.

All data were tested for normality and their variances tested for homogeneity using a Shapiro–Wilk test (SPSS Version 17, SPSS Statistics for Windows, SPSS Inc., Chicago, IL, USA). Following the rejection of normality (Shapiro–Wilk $P < 0.05$), non-parametric Kruskal–Wallis analyses of variance (*H*-tests) were used to determine whether the four isolates induced larger bark and cambium lesions compared with the controls. Individual Mann–Whitney *U*-tests were then performed for each isolate against the control. Results were presented in Boxplots also created in SPSS.

Evaluation of disease incidence and severity

To determine the incidence and severity of stem and branch cankers and die-back of *A. marina* in South Africa, four transects of 25 m each were laid out perpendicular to the water source (a lagoon, estuary or canal) at nine sampling sites. At each site, a total of 104 individual trees, with Circumference at Breast Height (CBH) between 10 and 120 cm and height between 2 and 15 m, were evaluated (26 per transect). First, canker and die-back incidence were recorded for each tree by evaluating the tree from the basal area at soil level to the canopy for a period of no longer than 60 s per tree. This entailed a standard present (1) or absent (0) classification system for each tree. Then, to score the severity of canker and tree die-back at a site, a modified Cobb table was used, entailing a categorical classification system ranging from 0, being healthy trees, to 5 for trees displaying severe canker and die-back symptoms (Table 1).

Table 1. Scoring criteria for evaluation of disease severity.

Scale	Symptom description	Score
0	No visible symptoms	0 None
1	Some gummosis and cankers, small cracking on the main stem or branches. Die-back starting at the branch tips	1–25% of branches/stem affected
2	Gummosis, cankers, cracking on branches and some areas of stem Die-back progressing down the branches	26–50% of branches/stem affected
3	Gummosis, cankers, cracking on many branches and large area of the stem Die-back on branches leaf loss of most parts of the crown	51–75% of branches/stem affected
4	Gummosis, cankers, cracking on branches and stem, crown canker or collar rot, dead tree Die-back on branches progressing down, high crown defoliation	75–100% of branches/stem affected
5	Strong gummosis symptoms, canker on a large areas of branches and stem, cracking on most branches/entire stem, crown canker or collar rot, dead tree Die-back on branches progressing down and reaching the main stem, defoliation of the entire crown, or dead tree	100% of branches/stem affected

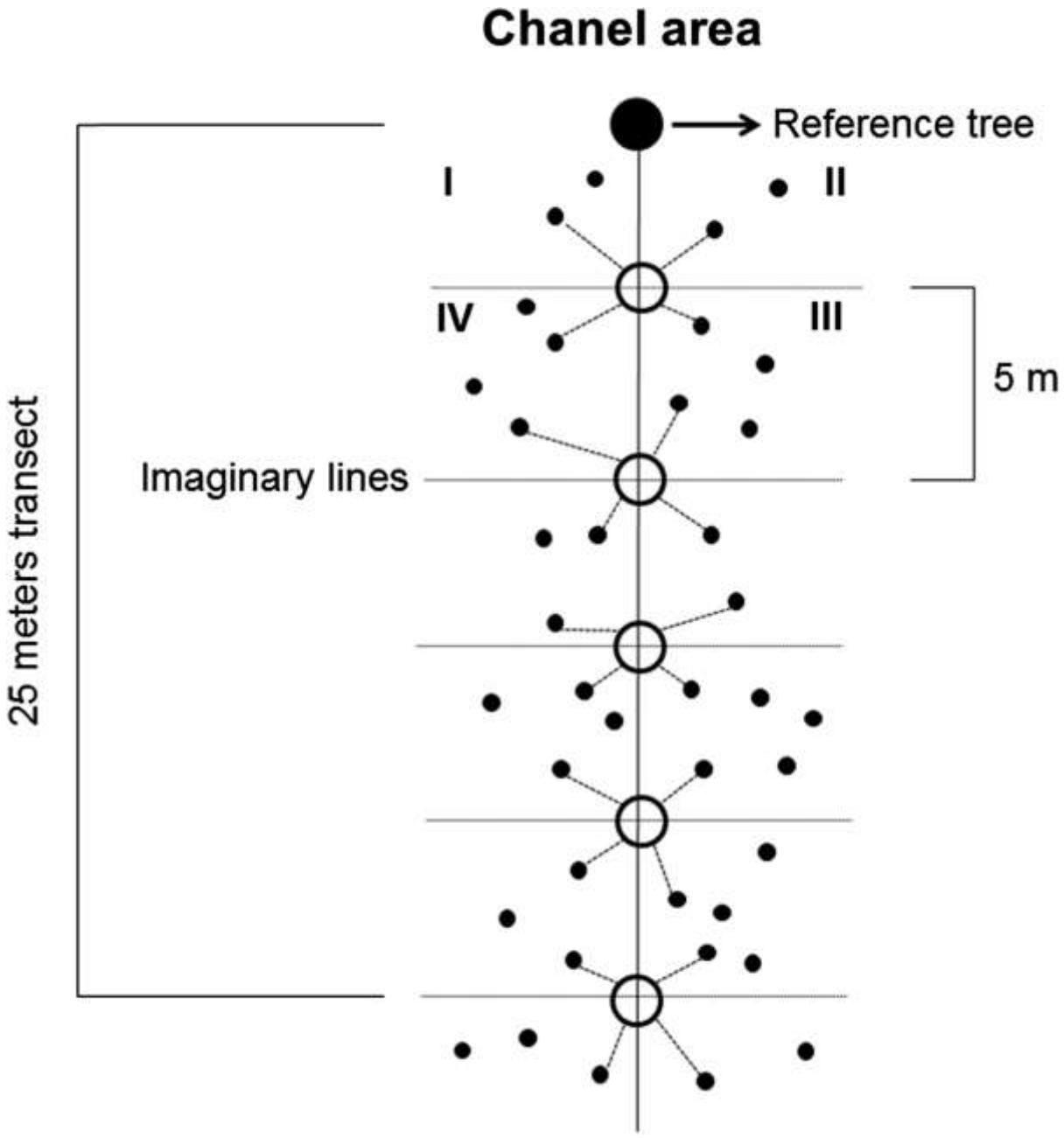

Figure 2. Graphic design of transects laid out in estuaries along the east coast of KZN and EC Provinces. Sample points along a transect with the nearest trees in each quarter (I, II, III and IV). Black circle indicates the reference tree, while the white circles indicate the main trees every 5 m across 25 m line.

Quantitative and qualitative data were collected across the sites in order to test whether there was a relationship between canker and die-back severity. Canker and die-back were also tested individually to determine the relationship with (1) pneumatophore siltation of *A. marina* individuals, (2) the stand density of the *A. marina* population at a site, (3) the DBH and (4) the presence of wood-boring beetles. Pneumatophore siltation was chosen since these plant organs are crucial to uphold mangrove physiological integrity, but are often negatively affected (e.g. oxygen starvation) due to impacts caused by natural events e.g. floods and siltation, as well as anthropogenic activities such as harvesting and livestock (Breen and Hill, 1969; Lugo and Snedaker, 1974; Ellison, 1998). Pneumatophores were scored based on three categories, considering the density around the trees: 0 = all

pneumatophores were covered in mud, 1 = all or the majority of pneumatophores were visible, 2 = only a few pneumatophores were visible. For the stand density variable, a point-centred quarter method was used every 5 m in per transect. This consisted of placing an imaginary quadrat, divided into four quarters or quadrants, selecting the main tree, and then calculating the nearest neighbour tree (Figure 2). DBH was calculated as CBH/π, where CBH is the circumference at breast height and π is Pi. Wood-boring beetles were scored as either present (wood-boring beetle activity was observed, such as circular holes in the stems/branches, often accompanied with sap bleeding from these wounds), or absent (no indication of present or past beetle activity).

To depict the canker and die-back incidence and severity patterns of *A*. *marina* across the nine sampling sites, a line plot and stacked bar charts were drawn in Excel 2010 (Microsoft, Inc., CA). Non-parametric Spearman rank correlations were used to ascertain whether there was any relationship between the percentage canker and die-back incidence across sites (SPSS Version 17). Lastly, to correlate the categories of canker and die-back (see Table 1) with the chosen environmental proxies (stand density, DBH, categories of pneumatophore siltation and wood-boring beetle presence or absence), a Principle Component Analysis (PCA) in CANOCO 5 (ter Braak and Šmilauer, 2012) was used.

Results

Disease survey and sample collection

During the assessment process, no disease symptoms or pest activity was observed on *C. tagal*, *L. racemosa* and *X. granatum*. Small circular patches of dead *B. gymnorrhiza* were observed at Beachwood and Isipingo (eThekwini, Durban). Trees in these patches had been dead for longer than a year and the material was inordinately old for possible pathogen isolation. It was concluded that the mortality was most likely caused by lightning strikes due to the shape of the patches and the fact that all trees seemed to have died at the same time. Signs and symptoms of insect infestation and disease were commonly observed on *A. marina* at Beachwood, Isipingo, Mtunzini, Mgazana, Richards Bay, St. Lucia, Nahoon, Wavecrest and at Kosi Bay. Leaves of *A. marina,* at all sites, were colonized by gall forming insects, resulting in two types of galls: flat or irregular (Figure 3A–C). The flat galls were often associated with leaf spot symptoms. Disease symptoms on *A. marina* trees regularly included canker, die-back and stem/branch bleeding (Figure 3D–F). On mangrove associates, a fungal disease and insect defoliator were observed. A fruit and leaf disease was found on *B. racemosa* in the Mapelane and Richards Bay areas. Adult leaf beetles were found feeding on the foliage of *H. tiliaceus* at Beachwood and Isipingo (Durban) (Figure 3G–I).

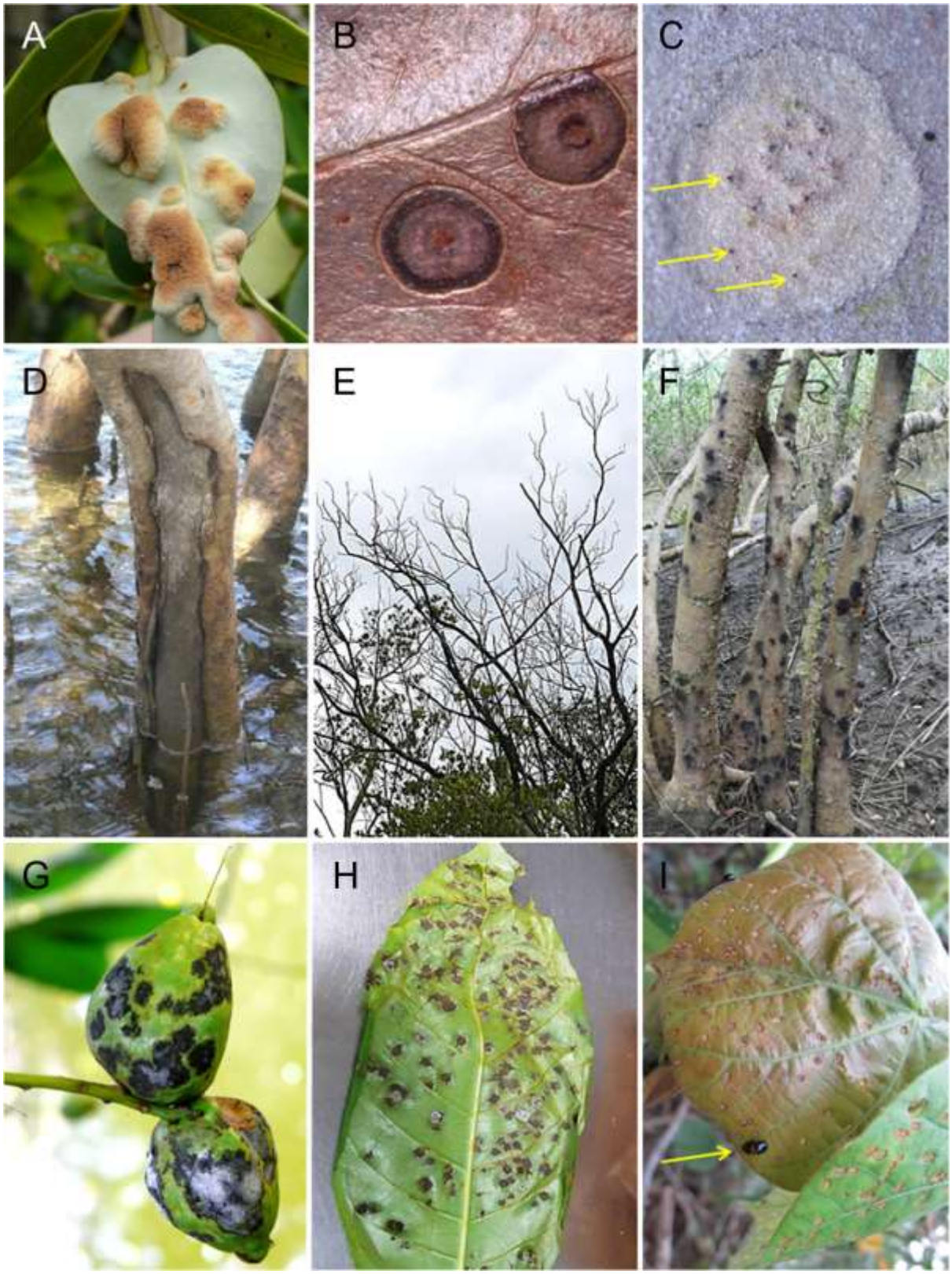

Figure 3. Pests associated with *Avicennia marina*, *B. racemosa* and *Hibiscus tiliaceus* (A–I). Gall formation on leaves of *A. marina* (A–C), notice (arrow) the fruiting structures of *Mycosphaerella* sp., forming in the galls (C). Disease symptoms found on *A. marina*, canker (D), die-back (E) stem bleeding by wood-boring beetles attack (F). Fruit and leaf disease on *B. racemosa* (G–H). Defoliation of *H. tiliaceus* caused by the leaf-feeding beetle *Nisotra* sp. (arrow) (I).

Fungal identification

Of 200 portions of plant material collected from symptoms on *A. marina* trees, four main fungal groups were isolated. Based on BLAST results of the ITS genome region these included *Eutypella* sp. (Diatrypaceae), *Lasiodiplodia* sp. (Botryosphaeriaceae) and *Cyphellophora* sp. (Cyphellophoraceae). Only two isolates of putative fungal pathogens were obtained from the crushed beetles. These resembled species in the genus *Cyphellophora*.

Representative isolates from each fungal group were included in ITS data sets for each genus (Table 2). The resulting alignments comprised 49 sequences in the Chaetothyriales with *Knufia cryptophialidica* and *Knufia endospore* included as outgroup, 34 sequences in the Diatrypaceae using *Xylaria curta* and *Xylaria hypoxylon* as outgroup, 21 and 20 sequences for the ITS and BT, respectively, in *Lasiodiplodia* with *Neofusicoccum cordaticola* and *Neofusicoccum parvum* used as outgroup; and 44 sequences in the Mycosphaerellaceae data set, with *Cladosporium bruhnei* and *Cladosporium herbarum* as outgroup. The final fragments obtained after editing the alignments were ~500–660 b.p. long.

Table 2. Information of representative fungal isolates obtained from diseased mangrove trees in South Africa.

Fungal species	Isolate numbers	Host	Plant tissue	Location	Genbank accession numbers	
					ITS	BT
Cyphellophora sp.	CMW41258	*Avicennia marina*	Stem canker	Isipingo	KY290842	*
Cyphellophora sp.	CMW41259	*A. marina*	Stem canker	Mgazana	KY290843	*
Cyphellophora sp.	CMW41260	*A. marina*	Branch die-back	Mgazana	KY290844	*
Cyphellophora sp.	CMW41261	*A. marina*	Beetle	Durban	KY290845	*
Cyphellophora sp.	CMW41262	*A. marina*	Beetle	St. Lucia	KY290846	*
Eutypella sp.	Mt-isolate1 - DNA	*A. marina*	Stem canker	Mapelane	KY290847	KY290862
Eutypella sp.	Mt-isolate2 - DNA	*A. marina*	Stem canker	Richards Bay	KY290848	KY290863
Eutypella sp.	Mt-isolate3 - DNA	*A. marina*	Branch canker	Richards Bay	KY290849	KY290864
Eutypella sp.	Mt-isolate4 - DNA	*A. marina*	Stem canker	Mtunzini	KY290850	KY290865
Eutypella sp.	Mt-isolate5 - DNA	*A. marina*	Stem canker	Mtunzini	KY290851	KY290866
Lasiodiplodia theobromae	Bw-Lasio1 - DNA	*A. marina*	Branch die-back	Beachwood	KY290852	*
L. theobromae	Is-Lasio2 - DNA	*A. marina*	Branch canker	Isipingo	KY290853	*
L. theobromae	Is-Lasio3 - DNA	*A. marina*	Stem canker	Isipingo	KY290854	*
L. theobromae	Mt-Lasio4 - DNA	*A. marina*	Stem canker	Mtunzini	KY290855	*
L. theobromae	Mt-Lasio5 - DNA	*A. marina*	Stem canker	Mtunzini	KY290856	*

Fungal species	Isolate numbers	Host	Plant tissue	Location	Genbank accession numbers	
					ITS	BT
Mycosphaerella sp.	CMW41457	*A. marina*	Leaf galls	Mtunzini	KY290857	*
Mycosphaerella sp.	CMW41458	*A. marina*	Leaf galls	Durban	KY290858	*
Mycosphaerella sp.	CMW41459	*A. marina*	Leaf galls	Mgazana	KY290859	*
Mycosphaerella sp.	CMW42048	*A. marina*	Leaf galls	Mtunzini	KY290860	*
Mycosphaerella sp.	CMW41472	*A. marina*	Leaf galls	St. Lucia	KY290861	*
Pseudocercospora mapelanensis	CMW40579	*Barringtonia racemosa*	Leaf spots	Mapelane	KM203116	*
P. mapelanensis	CMW40580	*B. racemosa*	Leaf spots	Mapelane	KM203117	*
P. mapelanensis	CMW40581	*B. racemosa*	Leaf spots	Mapelane	KM203118	*

* = Gene region not used in the phylogenetic analyses.

The causal agent of the leaf and fruit disease of *B. racemosa* was identified as the recently described *P. mapelanensis* sp. nov. J.A. Osorio & Jol. Roux, based on multi-gene sequence data analyses of the ribosomal Large Sub Unit, the ITS, Translation Elongation Factor and Actin (Osorio *et al.,* 2015). This disease was observed on trees near Mapelane Nature Reserve and Richards Bay.

Based on the inferred phylogenetic tree, with 38 isolates in the Mycosphaerellaceae, isolates obtained from flat insect galls on *A. marina* were close to *Periconiella* and *Mycosphaerella* (Mycosphaerellaceae). However, additional multi-gene analyses will be required to clarify the placement of the fungus (Figure 4).

Three fungal groups were recovered from branch die-back, canker and discolouration around beetle galleries on *A. marina*. The most common species (30 isolates, obtained from a total of 20 trees and two squashed beetles) grouped close to the genus *Cyphellophora* (Cyphellophoraceae, Chaetothyriales). The phylogenetic analysis completed for the Chaetothyriales comprised 27 ingroup taxa within five genera for the ITS locus. The subsequent phylogenetic trees did not indicate a clear taxonomic placement of the mangrove isolates and suggest that these isolates correspond to a new taxon in the Chaetothyriales (Figure 5). This fungus was obtained from Beachwood, Isipingo, Mtunzini, Richards Bay in KZN Province and Wavecrest, Mgazana in the EC Province.

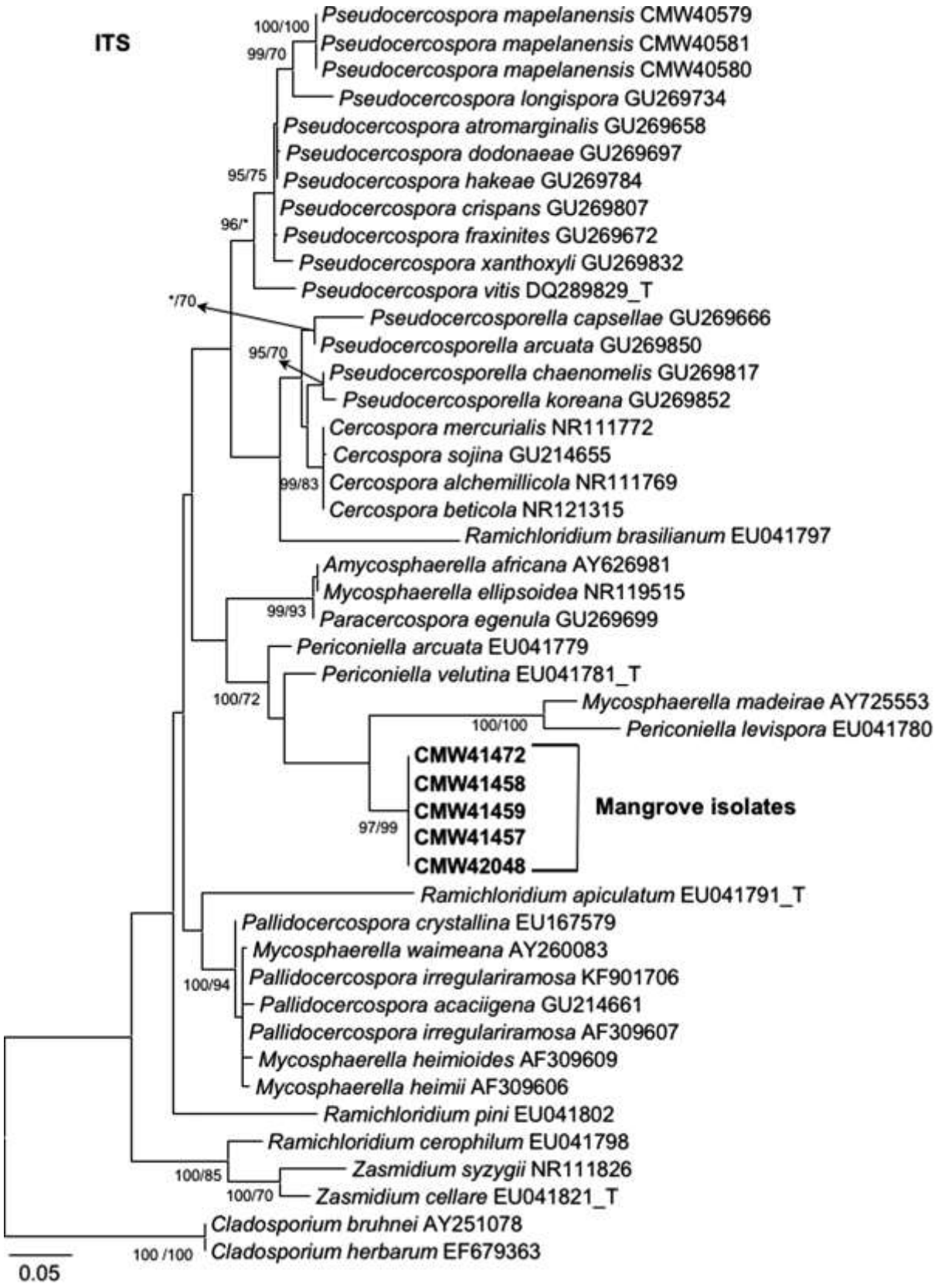

Figure 4. Phylogram obtained from (BI) and (ML) analyses of the ITS data set. This analysis provides evidence of isolates obtained from leaf-galls of *A. marina* (Bold font) are grouping close to but distinct to *Mycosphaerella* (sexual estate) and *Periconiella* (asexual state). The ITS analysis suggests that isolates from *A. marina* represents a new taxon within the Mycosphaerellaceae. BI posterior probabilities ≥95% and Bootstrap support values >70% are indicated near the nodes.

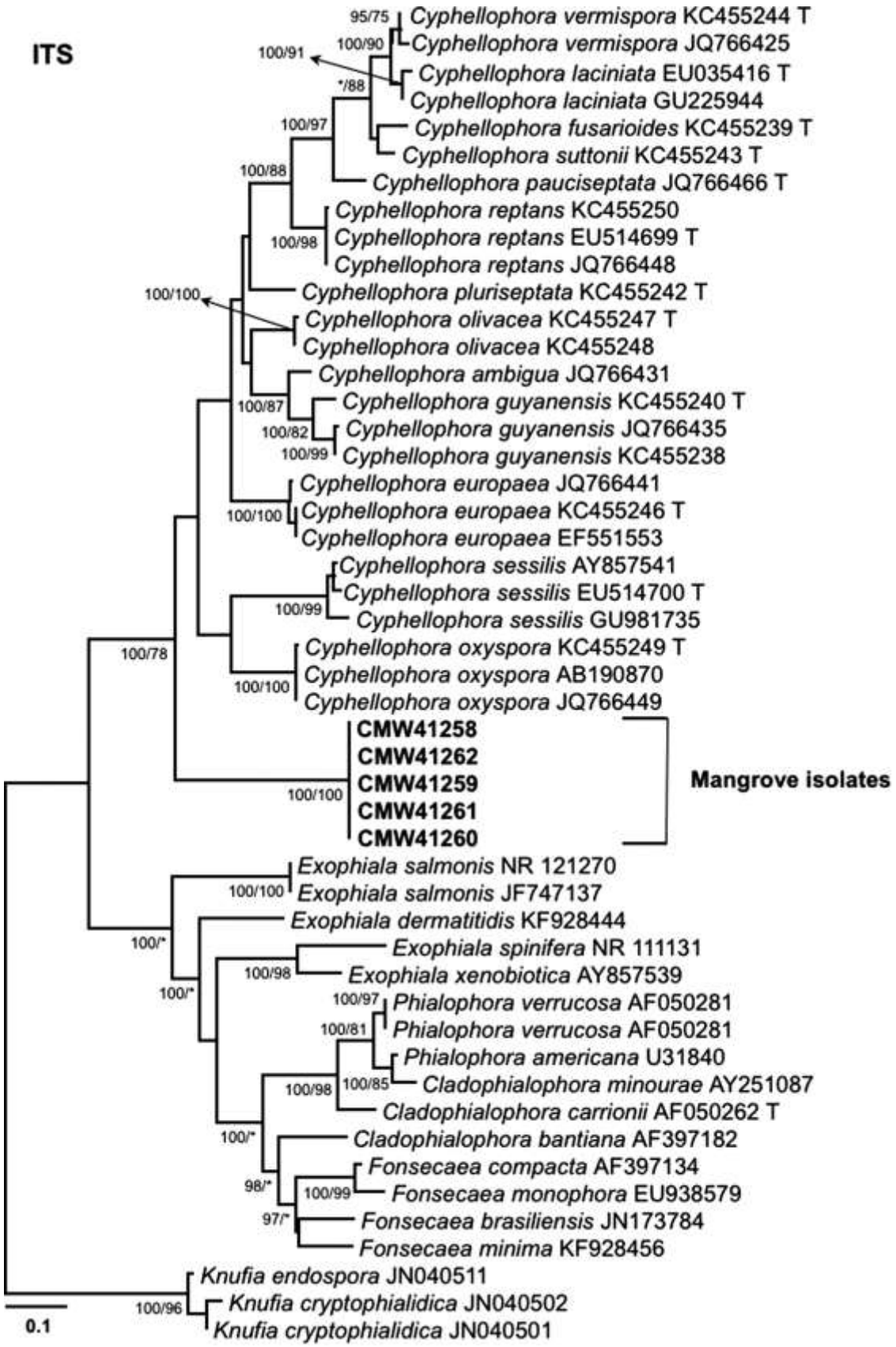

Figure 5. Phylogram obtained from (BI) and (ML) analyses of the ITS data set. This analysis provides evidence of isolates obtained from *A. marina* grouping close to *Cyphellophora* (Chaetothyriales). The ITS analysis suggests that these isolates represent an undescribed species. BI posterior probabilities ≥95% and Bootstrap support values >70% are indicated near the nodes. * = posterior probabilities <95% and bootstrap support values <70%.

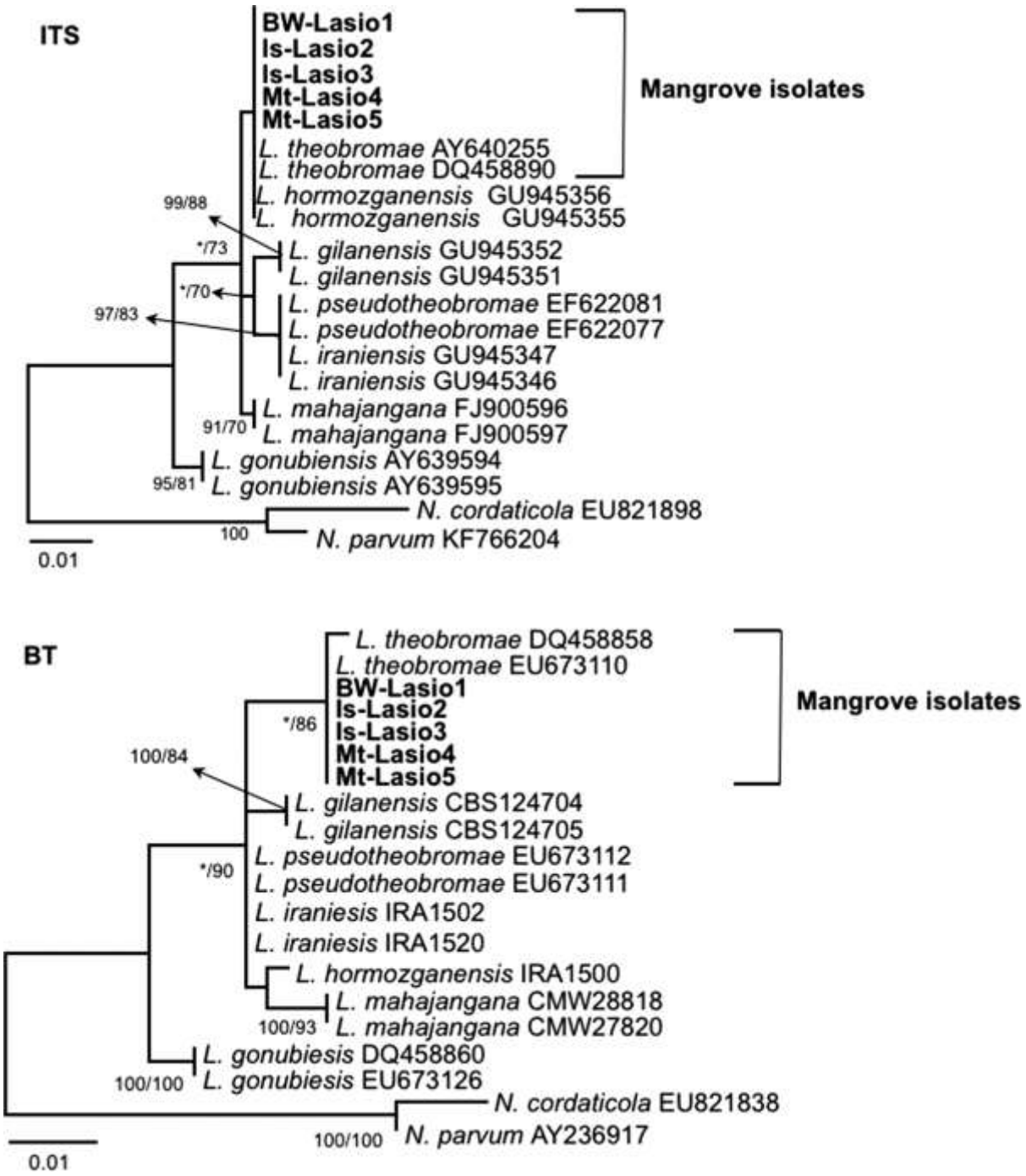

Figure 6. Phylogram obtained from (BI) and (ML) analyses of the ITS and BT data sets. These analyses indicate that isolates obtained from *A. marina* (Bold font) correspond to *Lasiodiplodia theobromae*. The BT tree indicates that isolates from *A. marina* belong to *L. theobromae.* Bootstrap support values >70% are indicated near the nodes. * = posterior probabilities <95%.

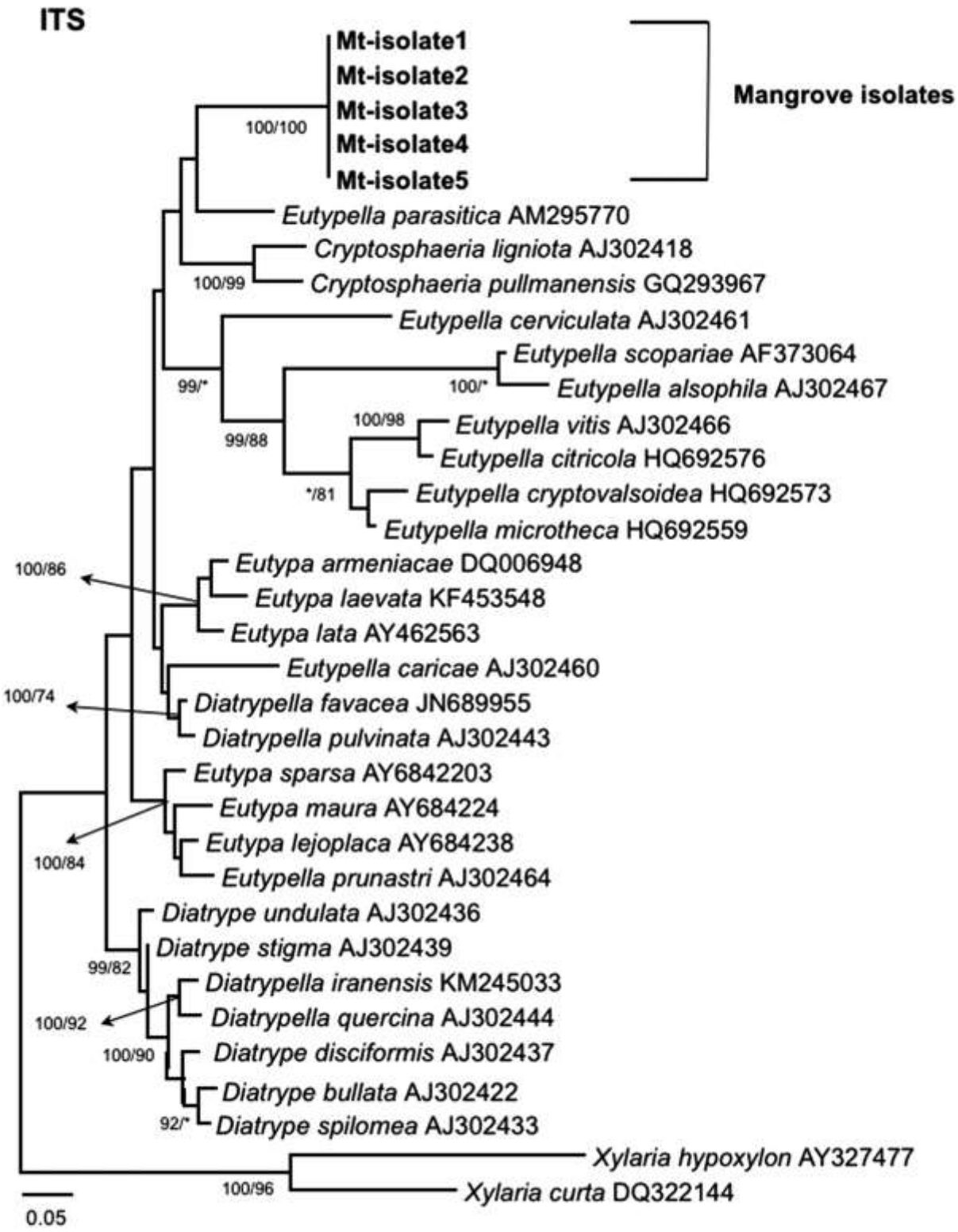

Figure 7. Phylogram obtained from (BI) and (ML) analyses of the ITS data set. This analysis indicates that isolates obtained from *A. marina* (Bold font) group close to *Eutypella parasitica*. The ITS analysis suggest that isolates from *A. marina* represent a new taxon within Diatrypaceae. * = posterior probabilities <95% and bootstrap support values <70%.

A second fungal group (18 isolates from 15 trees), isolated from branch die-back, canker and discoloration around beetle galleries, grouped with *Lasiodiplodia theobromae* (Botryosphaeriaceae) in the ITS tree (Figure 6). The phylogenetic analysis completed for the

genus *Lasiodiplodia* included a total of seven ingroup species for the ITS and BT data sets. The resulting trees from the BT analyses confirmed that isolates obtained from *A. marina* represent *L. theobromae*. This species was obtained from Beachwood, Isipingo and Mtunzini.

The least frequent fungal group (10 isolates from 10 trees) was also isolated from die-back and discoloration around beetle galleries. These isolates clustered within the Diatrypaceae (Xylariales), with *Eutypella* as the closest related genus. Phylogenetic analysis included a total of 27 ingroup species within five genera for the ITS locus. The isolates from *A. marina* grouped together in the Diatrypaceae with *Eutypella parasitica* as the closest taxon. This analysis also suggested that the isolates obtained from mangroves correspond to an undescribed taxon in the Diatrypaceae (Figure 7). These isolates were obtained from Mtunzini.

Insect identification

Leaf-feeding beetles were collected from leaves of *H. tiliaceus* and identified as *Nisotra* sp. and *Podagrica* sp., in the Chrysomelidae. These insects were found affecting *H. tiliaceus* at Beachwood and Isipingo (eThekwini).

Ambrosia beetles were collected from diseased *A. marina* trees and bark beetles from healthy and symptomatic *A. marina* trees. The bark beetles were identified as *Hypothenemus eruditus* (Westwood) and the ambrosia beetles as *Euwallacea xanthopus* (Eichhoff*), Xyleborinus aemulus* (Wollaston) and *Xyleborus perforans* (Wollaston). Bark beetles were collected at Beachwood, Isipingo, Mtunzini, St. Lucia and Mgazana and the ambrosia beetles from branch and stem galleries at Beachwood and Isipingo (Table 3).

Table 3. Beetles collected from mangrove trees in South Africa.

Genus	Fam./Subfam.	Collection method/Host	Location
Ambrosiodmus natalensis (Schaufuss)	Scolytinae	LindGren Traps	Mtunzini, St. Lucia (KZN), Mgazana (EC)
Ambrosiodmus eichhoffi (Schreiner)	Scolytinae	LindGren Traps	KZN, Mtunzini
Coccotrypes niger (Eggers)	Scolytinae	LindGren Traps	Isipingo, St. Lucia (KZN) Mgazana (EC)
Crossotarsus externedentatus (Fairmaire)	Platypodinae	LindGren Traps	Mgazana (EC)
Diuncus haberkorni (Eggers)	Scolytinae	LindGren Traps	Mtunzini (KZN)
Eccoptopterus spinosus (Olivier)	Scolytinae	LindGren Traps	Mgazana (EC)
Euwallacea xanthopus (Eichhoff)	Scolytinae	Directly from galleries of *A. marina*	Isipingo
Hypothenemus eruditus (Westwood)	Scolytinae	Directly from galleries of *A. marina*	Beachwood, Isipingo, Mtunzini, St. Lucia (KZN) and Mgazana (EC)
Nisotra sp.	Chrysomelidae	Directly from leaves of *Hibiscus tiliaceus*	Beachwood and Isipingo (KZN)
Podagrica sp.	Chrysomelidae	Directly from leaves of *H.*	Beachwood and Isipingo (KZN)

Genus	Fam./Subfam.	Collection method/Host	Location
		tiliaceus	
Premnobius cavipennis (Eichhoff)	Scolytinae	LindGren Traps	Beachwood, St. Lucia (KZN)
Stenoscelis sp. cf	Cossoninae	LindGren Traps	Mgazana (EC)
Xyleborinus aemulus (Wollaston)	Scolytinae	LindGren Traps and directly from galleries of *A. marina*	Beachwood (KZN)
Xyleborus affinis (Eichhoff)	Scolytinae	LindGren Traps and directly from dead *Bruguiera gymnorrhiza*	Beachwood, Isipingo, Mtunzini (KZN)
Xyleborus ferrugineus (F.)	Scolytinae	LindGren Traps and directly from dead *B. gymnorrhiza*	Beachwood, Isipingo, St. Lucia (KZN)
Xyleborus perforans (Wollaston)	Scolytinae	Directly from galleries of *A. marina*	Isipingo, St. Lucia (KZZ), Mgazana (EC)
Xyleborus volvulus (F.)	Scolytinae	Directly from dead *B. gymnorrhiza*	Isipingo (KZN)
Xylopsocus capucinus (F.)	Bostrichidae	LindGren Traps	Isipingo, St. Lucia (KZN)
Xylosandrus compactus (Eichhoff)	Scolytinae	LindGren Traps	Mgazana (EC)
Xylosandrus crassiusculus (Motschulsky)	Scolytinae	LindGren Traps	Beachwood (KZN)
Undetermined genus	Colydiidae	LindGren Traps and directly from dead *B. gymnorrhiza*	Beachwood, Isipingo, Mtunzini, St. Lucia (KZZ)
Undetermined genus	Cossoninae	LindGren Traps	Mgazana (EC)
Undetermined genus	Dermestidae	LindGren Traps	Mgazana (EC)
Undetermined genus	Cerambycidae	LindGren Traps	Mgazana (EC)
Undetermined genus	Anthribidae	LindGren Traps	Mgazana (EC)

KZN = Kwazulu-Natal.

The flat leaf-galls on *A. marina* were caused by the colonization of an unidentified midge fly species (Diptera: Cecidomyiidae) according to Prof. Stefan Neser and Dr Robin Adair (personal communication). The irregular galls were caused by an undescribed eriophyid mite species (Prof. Stefan Neser, personal communication). However, it was not possible to determine the taxonomic placement of these arthropods.

Insects from traps were separated into 29 morphogroups and were confirmed to represent 24 taxa. Of these, 19 were identified to genus level while 16 were successfully identified to species level. The most common genera were *Ambrosiodmus*, Xyleborus and *Xylosandrus*, followed by *Coccotrypes*, all of them in the sub-family Scolytinae. The least common genera were *Crossotarsus* (Platypodinae), *Diuncus*, *Eccoptopterus*, *Hypothenemus*, *Premnobius*, *Xyleborinus* (all in the Scolytinae) and *Xylopsocus* (Bostrichidae). The site with the highest number of specimens and different taxonomical groups collected was Mgazana (EC) followed by Beachwood, Isipingo and St. Lucia (KZN), respectively, while Mtunzini (KZN) was the site with the least insects obtained (Table 3). *H. eruditus*, *X. aemulus*, *Xyleborus affinis*, *Xyleborus ferrugineus* and *X. perforans* were collected from both plant material and the aerial traps.

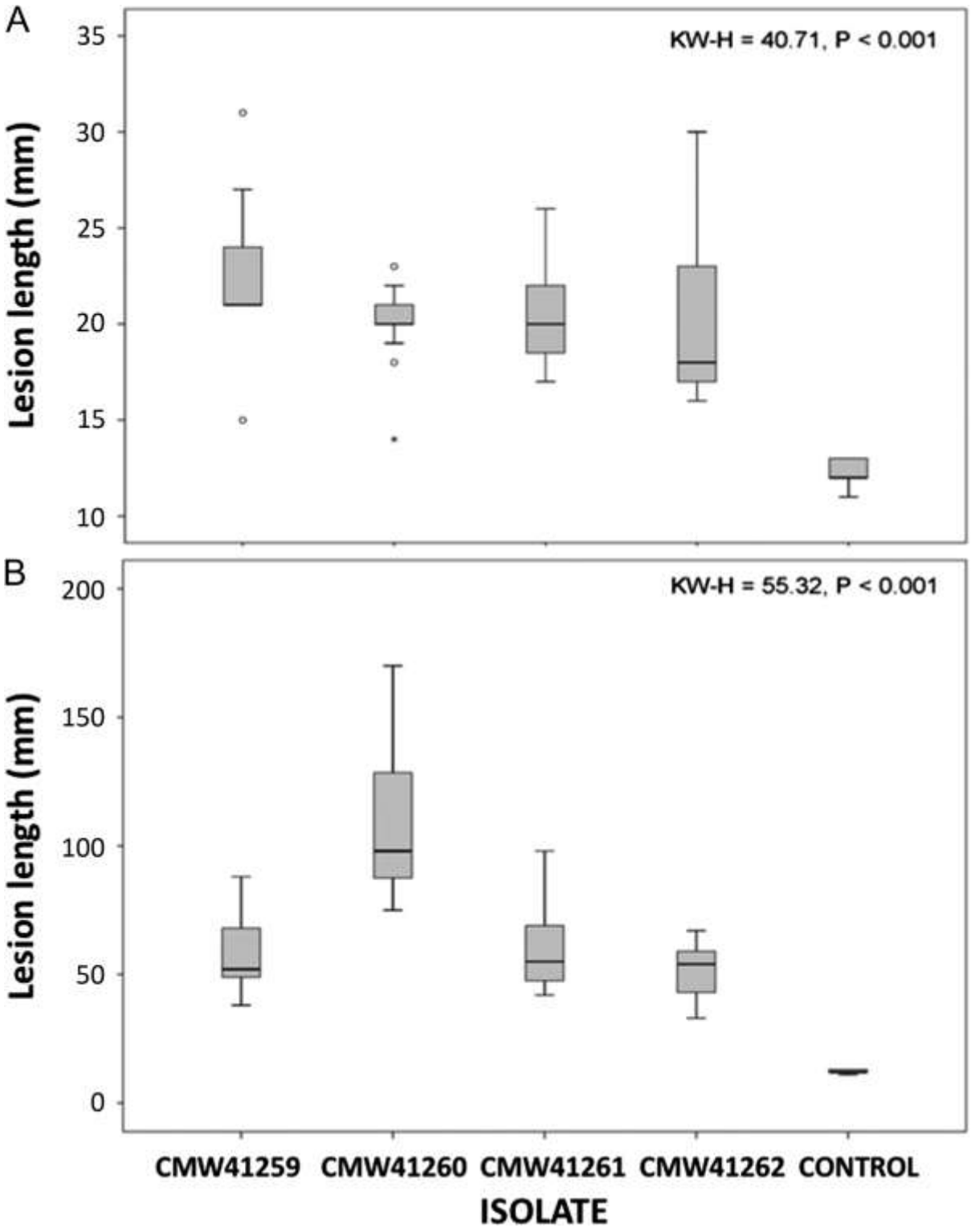

Figure 8. Boxplots of lesion lengths on *A. marina* bark (A) and cambium (B) due to inoculation by four *A. marina* associated *Cyphellophora* isolates. For both bark and cambium, all isolates significantly induced greater lesions than the control (Mann–Whitney *U*-tests; $P < 0.05$). In panel (A), circles are outliers, and the star is an extreme outlier. $N = 15$.

Pathogenicity trials

For pathogenicity trials four isolates of *Cyphellophora* were selected (CMW41259, CMW41260, CMW41261, CMW41262) based on the fact that it was the fungus most commonly and consistently recovered from disease symptoms across all sites. It was also isolated from crushed beetles. The pathogenicity trials showed that isolates of the *Cyphellophora* sp. (Figure 8) produced larger lesions in both the bark and cambium compared with the controls. The *Cyphellophora* sp. was successfully re-isolated from lesions on the bark and cambium.

Evaluation of disease incidence and severity

There was no relationship between the percentage die-back and canker incidence across sites (R_S = 0.58; P > 0.05; Figure 9). By removing Nahoon as an outlying site (R_S = 0.39; P > 0.05; Figure 9), it was further clear that high levels of branch die-back are characteristic in these mangrove forests (>80 per cent present), but also that incidence of branch or stem canker varies considerably between sites (between 10 per cent and 90 per cent present). In particular, die-back incidence was the lowest at Nahoon and the highest at Richards Bay (Figure 9). In turn, the severity of canker was the highest at Isipingo, Beachwood, Mgazana and Kosi Bay, while the remaining five sites all had low incidences of severe cankers (Figure 10).

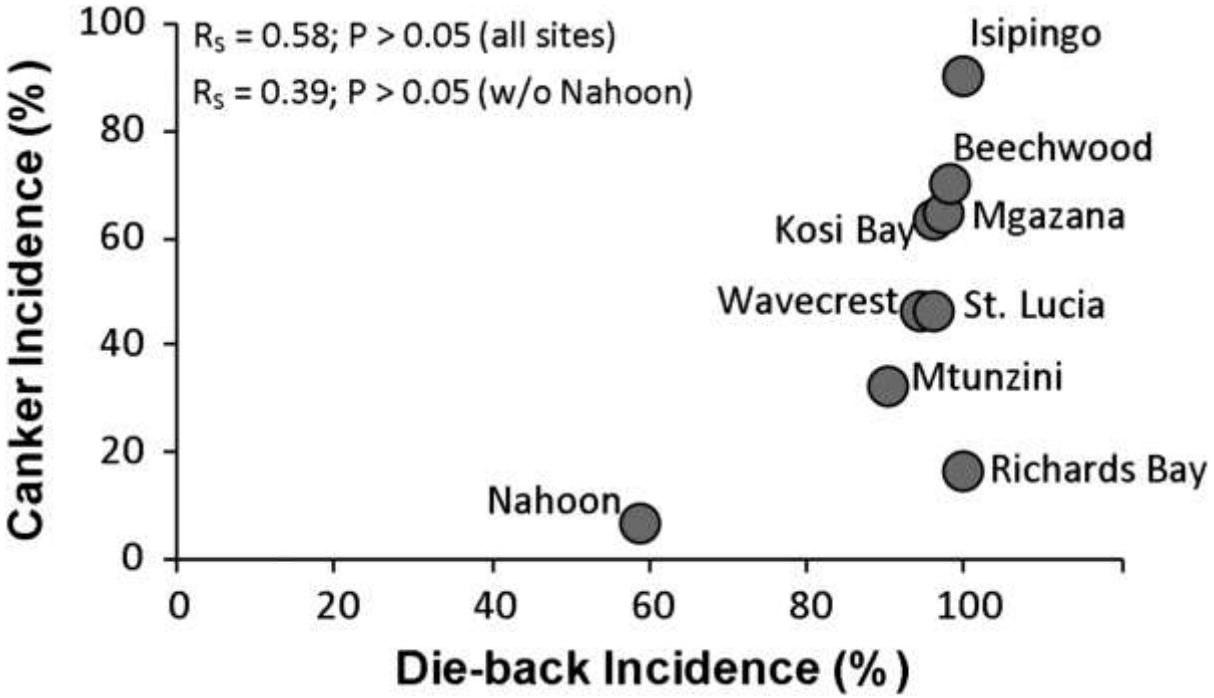

Figure 9. The relationship between % die-back and canker incidence in *A. marina* within nine sites across the northeast coast of South Africa. Sites are indicated, and the relationship assessed using a Spearman rank correlation. A Spearman rank correlation was subsequently also used to assess the relationship when an outlying site (Nahoon) was removed.

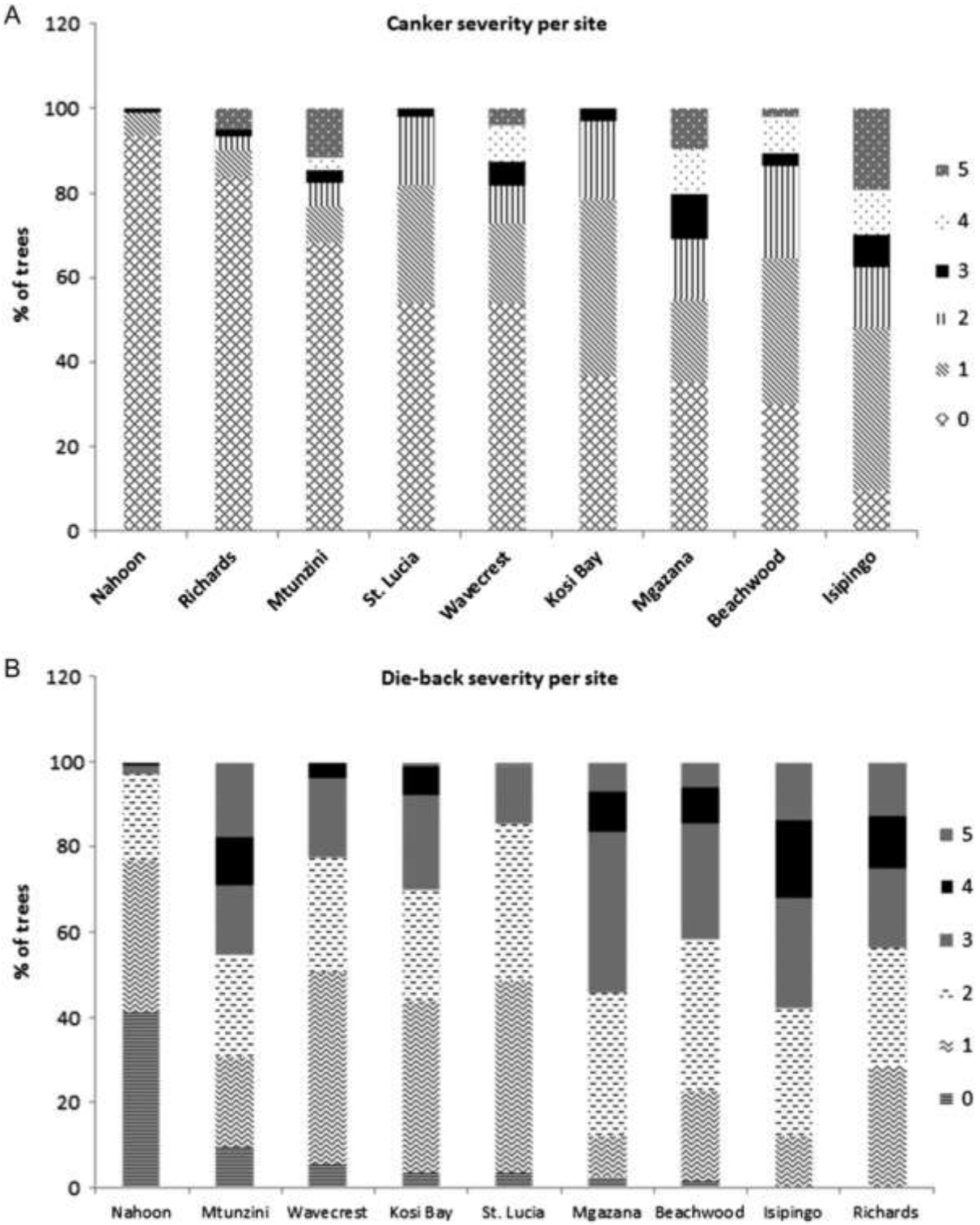

Figure 10. Stacked bar chart depicting the proportion of canker (A) and die-back (B) severity (%) found for *A. marina* individuals across nine sampling sites along the northeast coast South Africa. Values 0–5 represent the scoring criteria for evaluation of disease severity as per Table 1.

The more severe categories of tree die-back (Figure 11A) and canker (Figure 11B) were associated with beetle presence and higher pneumatophore siltation. However, these relationships appeared stronger for canker severity than for die-back. Interestingly, stand density seemed to not have any association with canker severity or die-back. Cankers also appeared to be more severe on larger trees (DBH), but not die-back.

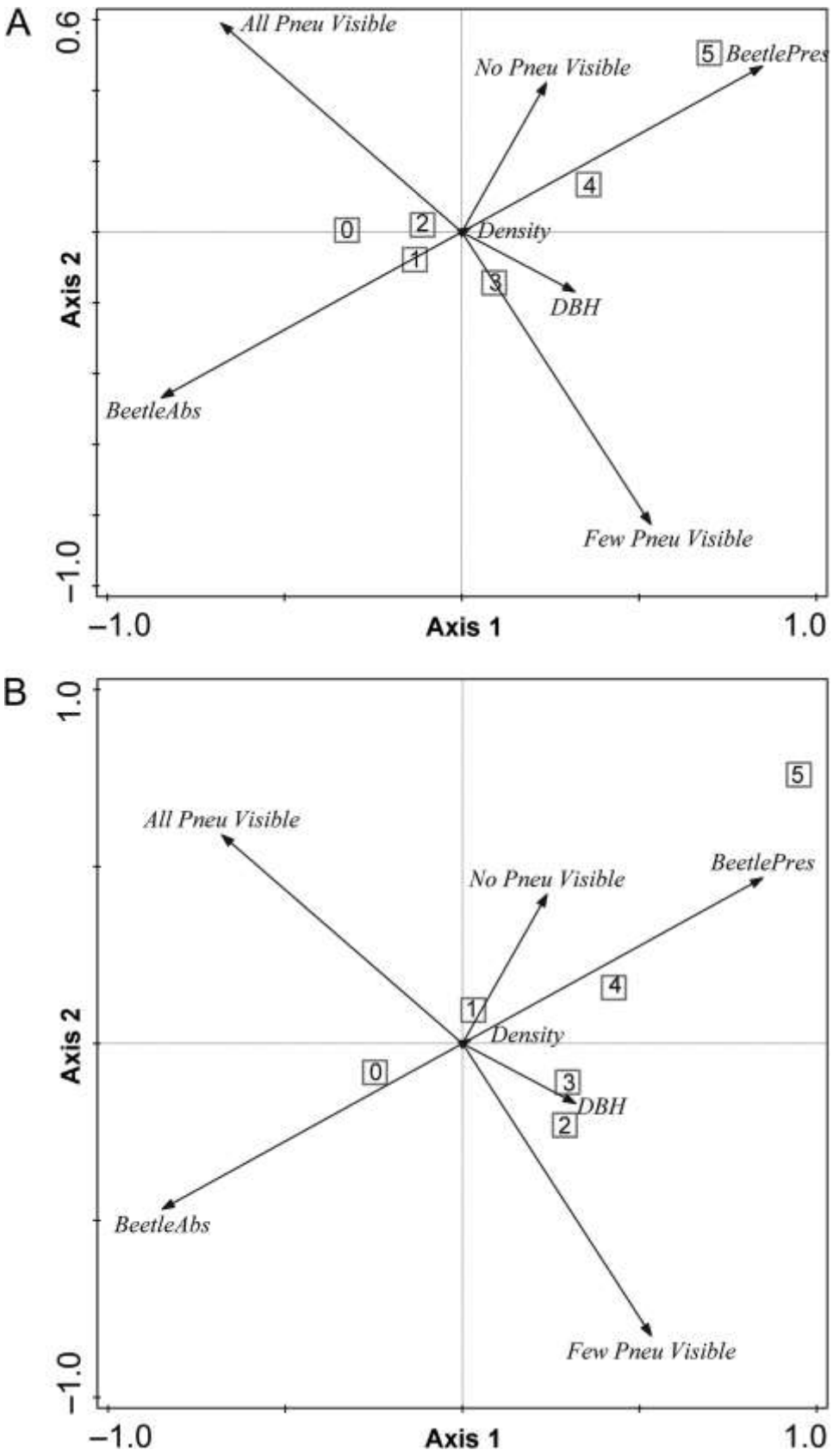

Figure 11. PCAs showing the relationships between categories of die-back severity (A) and canker severity (B) with DBH, stand density (Density), pneumatophore (Pneu) siltation and beetle presence (BeetlePres = present; BeetleAbs = absent) in nine *A. marina* populations across their distributional range in South Africa.

Discussion

This study represents the most detailed evaluation of possible biotic causes of mangrove decline in South Africa to date. The field surveys showed that no diseases caused by primary pathogens or other microbes were present on *B. gymnorrhiza*, *C. tagal*, *L. racemosa*, *R. mucronata* and *X. granatum* during the study period. The death of *B. gymnorrhiza* trees observed at two locations in eThekwini (Beachwood and Isipingo) was most likely caused by lightning strikes, which is also consistent with previous reports where similar gaps in mangrove forests were caused by electric discharges (Smith *et al*., 1994). The white mangrove, *A. marina*, however, showed concerning levels of canopy crown death, stem cankers and wood-boring insect attack at most sites. Furthermore, high incidences of leaf galls (two types) were observed on *A. marina* across the studied areas.

The disease found on fruit, flowers and leaves of *B. racemosa* in this study is caused by a novel fungal species, *P. mapelanensis*, recently described by Osorio *et al*. (2015). No new distribution or other host records for this disease were found during the survey. Studies are needed to assess the impact of this disease on flower, fruit and seed production, and thus on recruitment of this tree.

The insects obtained from mangroves, and their associates, all represent first reports from these trees in South Africa. The beetles *Nisotra* and *Podagrica* (flea beetles) belong to the Chrysomelidae, one of the largest groups of phytophagous beetles. The Chrysomelidae are specialized phytophagous insects and include economically important pest species (Jolivet and Hawkeswood, 1995). Gall midges have previously been reported from mangroves (Gonçalves *et al*., 2001; Sharma *et al*., 2003; Veldtman and McGeoch, 2003). The Cecidomyiid species on *A. marina* leaves in the current study was commonly found in association with a species of *Mycosphaerella*. Reports of Mycosphaerellaceae fungi isolated from gall forming insects are limited. Among the reports, *Mycosphaerella molleriana* (formerly *Mycosphaerella vespa*) and *Mycosphaerella pseudovespa* were isolated from wasp galls of leaves of *Eucalyptus globulus* and *Eucalyptus biturbinata* in Australia (Carnegie *et al*., 2007), but their roles, if any, remain poorly understood.

Several fungal genera were found in association with disease symptoms on *A. marina*. Beetle galleries, canopy crown die-back and stem canker were common at the study sites, with symptoms differing only in severity. Two of the putative fungal pathogen genera collected on *A. marina* could not be identified to species level. The sequencing of additional gene regions will be required to clarify the exact placement of the *Cyphellophora* sp. and *Eutypella* sp. The *Lasiodiplodia* isolates were all confirmed to represent *L. theobromae* based on comparisons of data for the ITS and BT gene regions. All three these fungal genera include pathogens of woody plants (e.g. Punithalingam, 1976; Trouillas and Gubler, 2010; Gao *et al*., 2015). The inoculations completed in the current study showed that the *Cyphellophora* sp. can produce lesions on *A. marina*, but it is more likely that these fungi are opportunistic colonizers of weakened/stressed trees. This is because they were isolated in low numbers and except for the *Cyphellophora* sp. none were consistently associated with diseased trees at all sites studied.

Signs of wood-boring beetles, residing in four different genera, were common on *A. marina* across the surveyed sites. Previous studies from other continents have also reported stem-boring beetles from mangroves (Feller, 2002), including *Coccotrypes rhizophorae* (Scolytinae) causing mortality of propagules and seedlings of *R. mucronata* in Panama (Sousa *et al*., 2003). Ambrosia beetles and other wood-boring beetles often infest stressed trees, where they find a suitable niche to complete their life-cycle (Bright and Stark, 1973; Beaver, 1987; Atkinson and Peck, 1994). There are, however, also increasing numbers of reports of ambrosia beetles and their fungal associates being the primary causes of tree-death (Ploetz *et al*., 2013), e.g. *Platypus quercivorus* and its fungal associate *Raffaelea quercivora* in Japan (Kubono and Ito, 2002; Kinuura and Kobayashi, 2006) and *Raffaelea lauricola* in the USA (Fraedrich *et al*., 2008). This suggests that wood-boring beetles associated with declining mangroves should be monitored in the future.

Although our results identified a relationship between the presence of wood-boring beetles and severe instances of canker and die-back, additional studies should be undertaken to determine the significance of this relationship. However, if we accept that the beetles collected in the study were attracted to already stressed trees, then there is more evidence that the *Cyphellophora* sp., which was the only fungus consistently isolated from the beetles, is also an opportunistic colonist.

The percentage of die-back in a site was unrelated to the percentage of canker incidence on *A. marina*. This suggests that there are most likely site-specific environmental conditions that determine disease presence and severity. Also that the presence of cankers and die-back symptoms should not be conflated. Interestingly, Nahoon (East London) was consistently scored as the least diseased mangrove forest of all the sites investigated. Nahoon represents a site that is less impacted by anthropogenic activities compared with other sites, which are more readily accessible and used by the public. For example, Isipingo, which had the highest disease incidence and severity, is an estuary that has been highly impacted by natural events, as well as large industrial and urban development in the past. This has led to Isipingo being highly polluted by heavy metals, oil spills, pesticides and other products (SSI Engineers and Environmental Consultants and Marine and Estuarine Research 2011; Pillay *et al*., 2014). Further degradation of estuaries should be avoided at all costs, especially in those sites where individuals already show high levels of canker disease.

The relationships between patterns of die-back and canker severity at a site, and DBH (proxy for tree age), pneumatophore siltation (proxy for tree physiological integrity), stand density (proxy for mangrove density in an estuary), and beetles (proxy for tree stress), revealed that only beetle presence and pneumatophore condition are particularly helpful in explaining the variation in die-back and canker severity across sites. Specifically, beetle presence appears to be correlated with die-back and the severity of cankers found on an individual. *Avicennia marina* individuals that are particularly stressed in an environment would, therefore, be more likely to show signs of wood-boring beetles. A higher beetle presence on already stressed trees would further induce die-back and finally premature tree-death.

Higher pneumatophore siltation correlated with higher levels die-back and canker severity observed on *A. marina* individuals. The integrity of pneumatophores could thus be a helpful sign to explain disease patterns across sites in South Africa. *Avicennia marina* uses the

pneumatophores (lateral roots that grow upward) to cope with the tidal changes and anaerobic substrates (Lugo and Snedaker, 1974). Pneumatophore siltation could, therefore, be directly related to the deterioration in health of an individual since the physiological integrity of a plant is compromised when they are negatively affected. For example, permanent submergence and siltation are some of the factors that explain the decrease of pneumatophores in some areas; in addition, the lack of pneumatophores is an important tree stressor and alters the health of the trees, becoming more vulnerable to opportunistic pests (Breen and Hill, 1969).

Apart from possibly *A. marina*, the health of mangroves in South Africa, associated with diseases caused by fungi, is generally good. Disease symptoms observed on *A. marina* seems to be site-associated, with higher incidence at sites likely related to increased human activities. Furthermore, beetle presence and pneumatophore integrity appear to be an important proxy for canker and die-back severity patterns in *A. marina* at the studied sites. Similar to other regions globally, the role of minimizing unnecessary stress on these sensitive ecosystems is, therefore, highlighted. Further studies are also required to elucidate the possible role of ambrosia beetles and their fungal associates in the observed deaths of *A. marina.*

Acknowledgements

We thank Ezemvelo KZN Wildlife, the Isimangaliso Wetland Park and the Eastern Cape Parks & Tourism Agency (ECPTA) for sampling permits as well as their staff members for assistance in the field. The material was collected under EKZNW permits OP 3730/2011, OP3728, OP 4776, OP 1457 and ECPTA–RA 00119. We gratefully acknowledge Dr Anusha Rajkaran (Department of Botany, Rhodes University). Special thanks are due to Dr Roger A. Beaver (Thailand) and Dr Elizabeth Grobbelaar (Agricultural Research Council–ARC, South Africa), Prof. Stefan Neser (University of Pretoria) and Dr Robin Adair for assistance with the identification of the insects. We also acknowledge the post-graduate students at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria that provided support during the often arduous field trips.

Funding

The Department of Science and Technology (DST) and National Research Foundation (NRF) Center of Excellence in Tree Health Biotechnology (CTHB).

Conflict of interest statement

None declared.

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