A new species in the Mycosphaerellaceae from Cecidomyiidae leaf galls on *Avicennia marina* in South Africa

J. Alexander Osorio[1, 2], Riikka Linnakoski[3, 4], Michael J. Wingfield[1], Z. Wilhelm de Beer[1] & Jolanda Roux[5]

[1]Department of Biochemistry, Genetics and Microbiology, DST/NRF Centre of Excellence in Plant Health Biotechnology (CPHB), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, Gauteng, South Africa

[2]Facultad de Ciencias Básicas y Tecnologías, Programa de Biología, Universidad del Quindío, Armenia, Colombia

[3]Natural Resources Institute Finland (Luke), Latokartanonkaari 9, 00790, Helsinki, Finland

[4]Department of Forest Sciences, University of Helsinki, Latokartanonkaari 7, 00014, Helsinki, Finland

[5]Department of Plant and Soil Sciences, DST/NRF Centre of Excellence in Tree Health Biotechnology (CTHB), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, Gauteng, South Africa

*Correspondence to J. Alexander Osorio. e-mail: osorio.romero17@gmail.com

Abstract

During studies to investigate the health of mangrove trees in South Africa, high numbers of *Avicennia marina* were found with leaf galls caused by unidentified adults and larvae of midges (Cecidomyiidae). Fungal fruiting structures were commonly observed on the abaxial areas of the galls. To determine the identity of the fungi associated with the gall midges, phylogenetic analyses using multigene sequence data were used. The nuclear large subunit (LSU), internal transcribed spacer (ITS), and a portion of the actin gene region (ACT), were amplified and analyzed. The results revealed that the fungal fruiting structures represent a new taxon in the Mycosphaerellaceae described here as *Zasmidium mangrovei* sp. nov. This is the first report of a species in the Mycosphaerellaceae associated with cecidomyiid leaf galls on *A. marina*.

Keywords: 1 novel taxon; Angiosperms; Capnodiales; Mangroves; White mangrove

Introduction

Mangroves are considered amongst the most productive ecosystems globally. They are important for carbon sequestration, vital for several food chains, anthropogenic uses and the protection of coast lines against storm damage (Ellison 2015). Mangrove trees grow along coastal areas of 123 countries in tropical and sub-tropical zones (Spalding et al. 2010). They are well known for their ability to cope with harsh environments due to morphological and

physiological adaptations, such as glands that allow them to accumulate and excrete salt, enabling plants to survive under these conditions (Tomlinson 1986; Wang et al. 2010).

In South Africa, six species of mangrove trees grow along the coastline of the Eastern Cape and KwaZulu-Natal Provinces (Steinke 1999). Of these, *Avicennia marina* (Forssk.) Vierh. (white mangrove) is the most prevalent species. As in other regions, mangrove ecosystems are under significant threat due to anthropogenic activities (Spalding et al. 2010; Sippo et al. 2018). There are also increasing reports of pests and diseases affecting mangrove trees (Jenoh et al. 2016; Osorio et al. 2017b; Piątek and Yorou 2018; Sánchez et al. 2018). Recent surveys considering the health of mangrove trees in South Africa showed that of all the mangrove species in the country, *A. marina* is most severely challenged by pests and pathogens (Osorio et al. 2017b). These surveys also showed that the leaves of *A. marina* were commonly colonized by different types of galls. The most common of these had a characteristic flat shape, caused by cecidomyiid midges (Diptera: Cecidomyiidae). Interestingly, fungal fruiting structures were commonly observed sporulating in these galls formations (Osorio et al. 2017b).

Leaf galls are abnormal plant tissue growths, formed as a response to stimuli commonly caused by various microorganisms (Rohfritsch and Shorthouse 1982; Maia et al. 2008; Carneiro et al. 2009) and/or arthropod attack (Espirito-Santo and Fernandes 2007). This growth can be due to increase in cell volume (hypertrophy) and/or cell number (hyperplasia). Plant galls usually give rise to a complex microhabitat where predators, parasitoids, tenants and successors become established (Maia 2001). Fungi are regularly found within insect galls (Rohfritsch 2008; Lawson et al. 2014; Washburn and Bael 2017), but little is known regarding their role in the biology of the causal insects.

Despite increasing interest in mangrove trees, the microbiota of these trees remains a topic that is relatively poorly studied worldwide. In this regard, there have been very few studies considering the diversity of mangrove fungi in South Africa (Kohlmeyer and Kohlmeyer 1971; Steinke and Jones; 1993; Steinke and Hyde 1997; Osorio et al. 2016a). The most recent investigations, including phylogenetic analyses for isolated fungi, have contributed substantially to the knowledge of fungal communities associated with mangroves globally (de Souza Sebastianes et al. 2013; Abdel-Wahab et al. 2014, 2018, 2020) and in South Africa (Osorio et al. 2015, 2016b, 2017a). The aim of this study was to elucidate the identity of fungi associated with leaf galls reported on *A. marina*. Multigene sequence analyses of the nuclear large subunit (LSU), internal transcribed spacer (ITS) regions, and a portion of the actin gene region (ACT) were conducted, accompanied by morphological comparisons.

Materials and methods

Sampling

Avicennia marina leaves with galls and with obvious fungal fruiting bodies on them were collected at eight localities in South Africa. These included Kosi Bay (26°54′23.91"S, 32°52′26.18"E), St. Lucia (28°22′44.87"S, 32°25′22.67"E), Mlalazi Nature Reserve at Mtunzini (28°57′15.39"S, 31°46′23.03"E), Beachwood at eThekweni/Durban (29°48′19.01"S, 31°02′28.90"E) and Isipingo (29°59′32.10"S, 30°56′59.72"E) in the KwaZulu-Natal Province, South Africa. In the Eastern Cape Province samples were obtained from Mgazana estuary (31°41′44.97"S, 29°24′32.24"E), Nahoon (32°58′54.13"S, 27°56′38.36"E) and Wavecrest (32°34′49.19"S, 28°31′24.33"E) (Fig. 1). Sampling was

conducted from March to November 2013. The sample size was standardized; 20 leaves in total of *A. marina* per site (one leaf per tree) with different levels of gall formation were collected and placed in paper bags and transported to the laboratory facilities of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, for processing. All sampling was carried out with the permission of the pertinent conservation authorities (Ezemvelo KZN Wildlife; Eastern Cape Parks & Tourism Agency, permits no OP3728, OP 4776, OP 1457, ECPTA—RA 00,119).

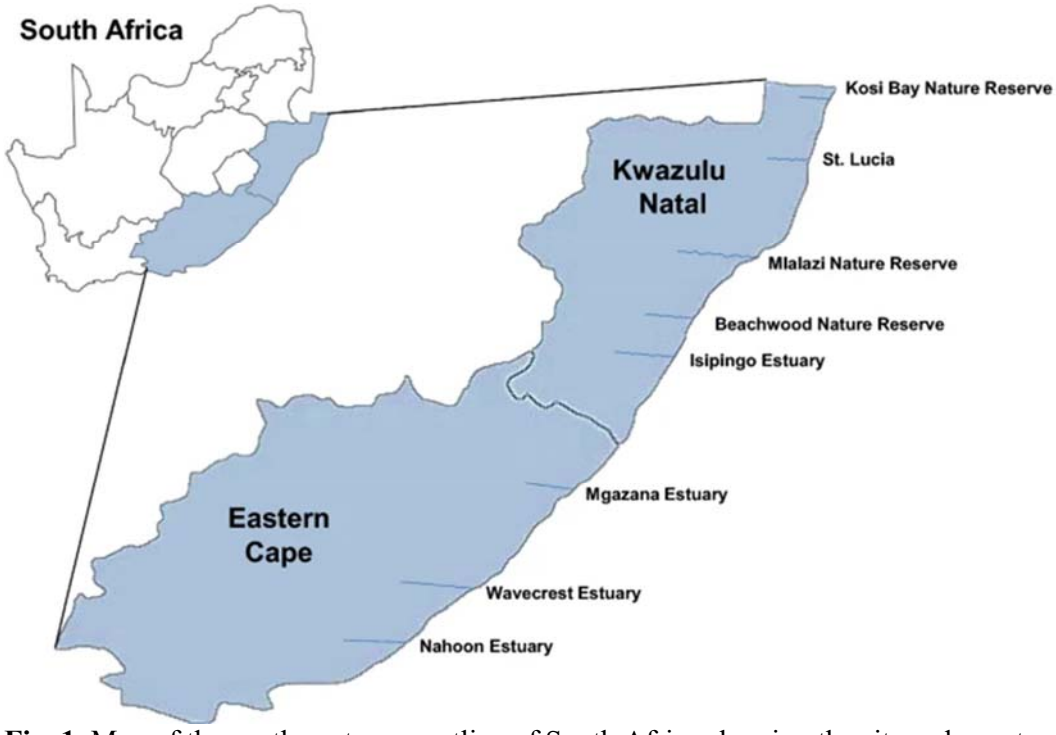

Fig. 1. Map of the south-eastern coastline of South Africa showing the sites where stands of *Avicennia marina* were sampled for midge leaf-galls

Fungal isolations

Galls were meticulously screened for fungal structures using a Nikon SMZ 745 dissection microscope. Isolation of fungi was achieved by transferring spores directly from spore masses within the pseudothecial cavities formed on the abaxial (lower) surfaces of leaf galls and/or by ascospores shot from the ascomata as described by Crous (1998), onto 2% malt extract agar (MEA; 15 g agar and 20 g malt extract l^{-1}) (Biolab). Single germinating spores were transferred onto a new Petri dish containing 2% MEA, with 0.4 g streptomycin sulfate l^{-1} (Sigma-Aldrich, USA) and incubated at 25 °C. The obtained colonies were purified and used for DNA extractions and subjected to molecular identification.

Pure cultures were selected to be deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, and duplicate cultures of the novel species were deposited in the culture collection of the CBS-KNAW Collections, Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands (Table 1). The type specimens were deposited in the fungarium of the National Collection of Fungi (PREM, Pretoria, South Africa).

Table 1 Information of representative fungal isolates obtained from *Avicennia marina* leaf-galls in South Africa

Fungal species	Isolate (CMW) No	Host	Plant tissue	Location in South Africa	GenBank Accession numbers		
					LSU	ITS	ACT
Zasmidium mangrovei sp. nov	CMW41457	*A. marina*	Leaf galls	Kosi Bay	MW046211	KY290857	MW052503
Zasmidium mangrovei sp. nov	CMW41458	*A. marina*	Leaf galls	St. Lucia	MW046212	KY290861	MW052504
Zasmidium mangrovei sp. nov	CMW41472 (T) CBS142048	*A. marina*	Leaf galls	Mtunzini	MW046214	KY290860	MW052506
Zasmidium mangrovei sp. nov	CMW41459	*A. marina*	Leaf galls	Durban	MW046213	KY290858	MW052505
Zasmidium mangrovei sp. nov	CMW42048	*A. marina*	Leaf galls	Mgazana	MW046215	KY290859	MW052507

CMW culture collection of the Tree Protection Co-operative Programme (TPCP) at FABI, University of Pretoria

(*T*) Type species

Fungal identification

DNA extraction, PCR and sequencing

To extract genomic DNA, three-week-old fungal tissue was placed into 2 mL sterile Eppendorf tubes for freeze-drying. The mycelium was powdered using a Mixer Mill type MM 301 Retsch^{R} tissue lyser (Retsch, Germany) with a frequency of 30 cycles per second for 3 min. The methodology described by Raeder and Broda (1985) was used to extract DNA, and the resulting DNA was suspended in 50 µL ddH_2O, after which 5 µL RNAse were added and the samples incubated at 37 °C for 60 min. To measure the quality and quantity of the extracted DNA, a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) was used.

Three loci were used for identification of fungi obtained from leaf galls on *A. marina*. Initial identification was based on sequencing of the ITS, including the 5.8S rDNA regions one and two. Based on results from the ITS sequences, the LSU and ACT gene regions were sequenced for species level identification.

The ITS region was amplified with the primer pair ITS1/ITS4 (White et al. 1990). The LSU region was amplified using primers LR5 (Vilgalys and Hester 1990) and LR0R (Moncalvo et al. 1993), and the partial actin (ACT) gene region was amplified using the primers ACT-512F and ACT-783R (Carbone and Kohn 1999). PCR reaction mixtures consisted of 25 µL containing 2 µL of DNA (20 ng), 2.5 µL 10 × PCR reaction buffer (containing MgCl2), 2.5 µL dNTP (5 mM), 0.5 µL of each primer (10 mM), 0.2 µL Fast start Taq DNA Polymerase (Roche Applied Science, Mannheim, Germany) and 0.5 µL MgCl2 (25 mM). To adjust the reaction mixtures to 25 µL, ddH_2O was added.

PCR reactions were performed using the following thermal cycling conditions: initial denaturation at 94 °C for 4 min followed by ten cycles consisting of 94 °C for 20 s, 53 °C (LSU) and 55 °C (ITS) for 48 s, 72 °C for 45 s, followed by a further 25 cycles of 94 °C for 20 s, 61 °C (ACT) and 55 °C (ITS) for 40 s with a time increase of 5 s each cycle, and 72 °C for 45 s. This was concluded with a final elongation step at 72 °C for 10 min. An aliquot of 5 µL of each PCR product was stained with GelRed™ nucleic acid gel stain (Biotium, USA), separated on a 1% agarose gel for 20 min at 90 V and viewed with the Gel Doc EZ Imager (Bio-Rad Laboratories Inc.).

PCR products were cleaned using Sephadex G-50 columns (Sigma Aldrich, Sweden). Both strands of each gene region were sequenced with the BigDye terminator cycle sequencing kit (PE Applied Biosystems, USA) using the same primers used for the initial amplification.

Phylogenetic analyses

Sequences were checked manually and contigs generated from the forward and reverse sequences for each isolate and each locus using CLCBio 7.6.1 (Cambridge, Massachusetts). Sequences obtained in this study were compared with those from GenBank (http://www.ncbi.nlm.nih.gov) using the BLASTn algorithm (Altschul et al. 1990).

Data matrices for each gene region were compiled in MEGA 7 (Kumar et al. 2016). These included sequences of isolates obtained from mangroves and reference sequences from GenBank. Alignments were done online using MAFFT 7 (Katoh and Standley 2013), and

ends were trimmed in MEGA 7 (Kumar et al. 2016). All datasets were subjected to Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP) analyses.

ML analysis was performed using the online version of PhyML 3.0 (Guindon et al. 2010), with the automatic substitution model selection by SMS (Smart Model Selection) (Leford et al. 2017). The Akaike selection criterion (AIC) (Sugiura 1978) was used to determine the gamma shape and number of substitution sites (nst), and confidence levels were estimated with fast likelihood-based method (Anisimova and Gascuel 2006). MP analyses were conducted using PAUP* v. 4.0b10 (Swofford 2003). Gaps and missing data were excluded in the MP analyses. Appropriate substitution models for BI analyses were determined for each of the data sets using the MrModeltest 2.3 (Nylander 2004). Bayesian analysis was completed using the Markov Chain Monte Carlo (MCMC) algorithms (Larget and Simon 1999) in MrBayes 3.2 (Ronquist et al. 2012). The MCMC chains were run for 5 million generations. Four chains were used and trees were sampled every 100th generation. Burn-in values were manually determined, and all sampled trees having lower than the burn-in values were discarded. The phylogenetic trees were viewed in MEGA 7 (Kumar et al. 2016), and post-processed with Adobe Illustrator CC v. 2018 (Adobe, San Jose, USA).

Morphological characterization

For the description of the novel species, the morphology of microscopic features including asci, ascospores and ascomata were investigated from leaf preparations and likewise, the colony morphology was characterized. Fungal material directly from leaves was mounted on microscope slides in 85% lactic acid and examined under a Zeiss Axioskop microscope (Carl Zeiss, Germany). To obtain images an Axiocam digital camera connected to the microscope was used. Fungal structures were measured using the Axiovision 3.1 software (Carl Zeiss, Germany). Characters such as size of ascomata, size, shape and pigmentation of ascospores were determined. Fifty measurements of the length and/or width of structures (l/w) were made for each morphological character and the mean, standard deviation and 95% confidence intervals were calculated. Minimum and maximum sizes are presented in parentheses as (min–) mean −/ + Confidence interval (–max).

For the description of the novel species, colony color, size and texture were described by transferring a small portion from a pure culture (less than 1 mm approx.) into the center of 60 mm-diam Petri dishes containing 2% MEA. Three replicates per isolate were incubated at 25 °C, and assessed every seven days for one month. Colony color was defined using the color charts of Rayner (1970). A nomenclatural novelty and description was deposited in MycoBank (www.MycoBank.org) (Robert et al. 2013).

Results

Fungal isolations

A total of 160 leaves showing similar gall shapes, were sampled from 20 *A. marina* individuals per site, at eight sampling sites. All the leaves showed similar fruiting structures and all of these were associated with leaf-galls on the abaxial sides of the leaves. After evaluation of the 160 leaves, half of these (80) were selected for single spore isolations, which resulted in 100 pure colonies resembling species in the *Mycosphaerellaceae.*

Fungal identification

PCR amplification and sequencing

Because the 100 isolates obtained from leaf galls were morphologically similar based on colony colour and microscopy, 20 representative cultures were randomly selected for identification based on DNA sequencing. These isolates were selected to represent all eight sampling localities. ITS sequences were generated for all the selected cultures and were found to be 100% identical. A sub-set of eight isolates was then selected for further identification using additional loci, namely the LSU and ACT gene regions. The sequence fragments were approximately 826 bp in size for the LSU, 485 bp for the ITS, and 367 bp for ACT.

Phylogenetic analyses

Preliminary analyses based on BLAST searches of the ITS sequence data for the isolates obtained from the leaf galls on *A. marina* indicated that the isolates represented an unknown species in the Mycosphaerellaceae. The phylogenetic relationship of the mangrove isolates with currently known species was determined in a subsequent analysis of single sequence datasets of three loci (ACT, ITS and LSU) (Table 1).

The LSU sequences were used to show placement of the isolates at the family and genus level (Online Resource 1) following the recent most comprehensive re-assessment of the Mycosphaerellaceae (Videira et al. 2017). Isolates from *A. marina* grouped in a clearly distinct and novel lineage in the genus *Zasmidium* (Online Resource 1). Species level identification was further confirmed by separate analyses of data sets for the ITS and ACT gene regions, which both clearly showed that isolates from *A. marina* is distinct from other currently known species for which DNA data are available in *Zasmidium* (Figs. 2 and 3). Similar to the LSU analyses, the fungus from *A. marina* in South Africa formed a novel lineage within the genus *Zasmidium*.

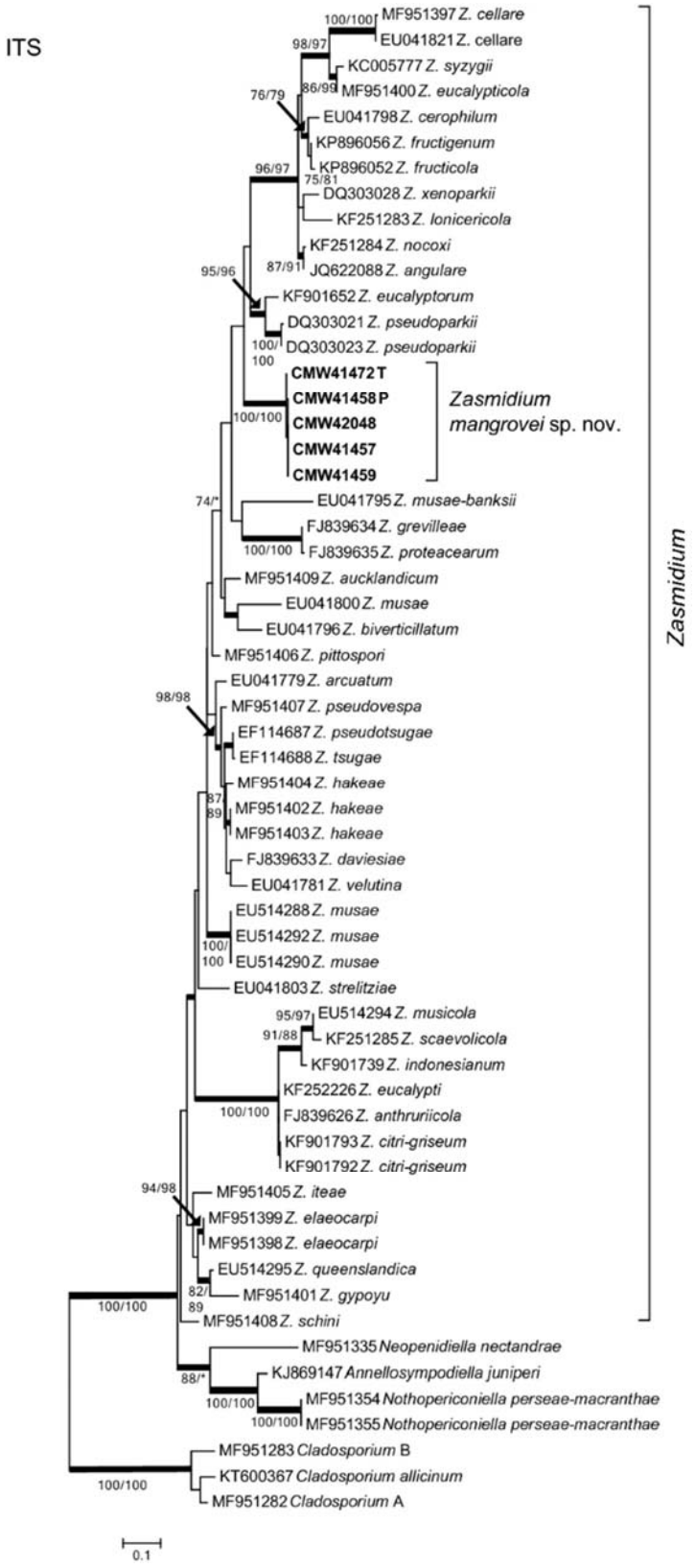

Fig. 2. Phylogram obtained from BI and ML analyses of the ITS data set. The isolates obtained from mangroves (**in bold**). The ex-type isolate of each species is indicated by T. BI posterior probabilities (PP) ≥ 90% and Bootstrap support values (BS) > 70% are indicated near the nodes as BI/ML. * = PP < 90

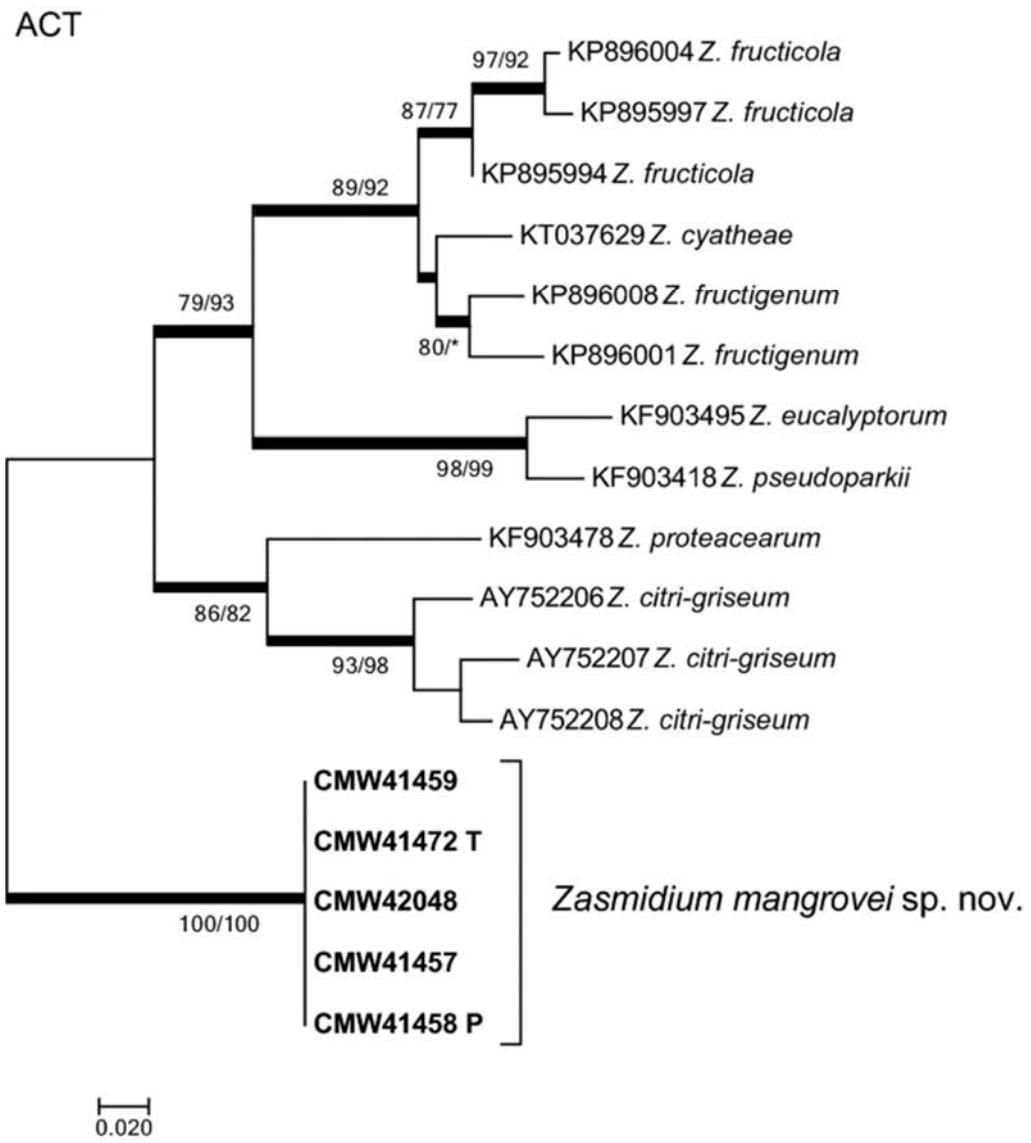

Fig. 3. Phylogram of species in the *Mycosphaerellaceae* from BI and ML analyses of the ACT gene regions. The isolates obtained from mangroves (**in bold**). The ex-type isolate of each species is indicated by T. BI posterior probabilities (PP) ≥ 90% and Bootstrap support values (BS) > 70% are indicated near the nodes as BI/ML. * = PP < 90

Taxonomy

Based on comparisons of multigene sequence data, a new species of *Zasmidium* in the Mycosphaerellaceae was found to be associated with galls of a cecidomyiid midge on *A. marina*. The sexual state of the fungus was commonly observed on galls while an asexual stage was never found.

Zasmidium mangrovei

J.A Osorio & Jol. Roux **sp. nov.** (Fig. 4). MycoBank: (MB 835,776).

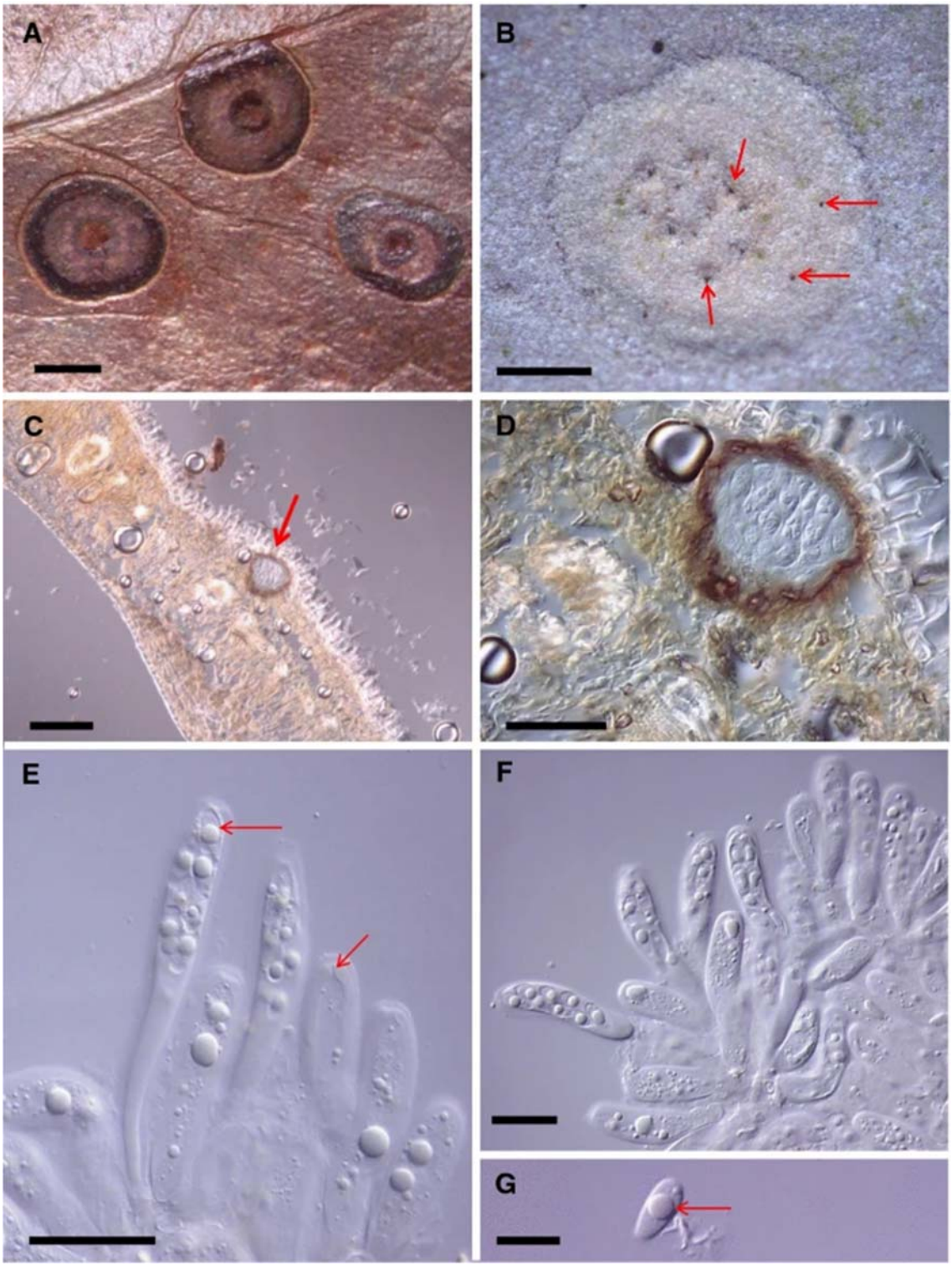

Fig. 4. Mature midge leaf-galls on *Avicennia marina* (**a–b),** showing with arrows the fruiting bodies **(b)** Vertical section through leaf-gall tissue and hypophyllous (**c–d)** Asci apex showing the apical thickening and pore, and biseriate ascospores (**e–f).** Mature ascospore showing with arrow the big guttule in the center **(g).** (Bars: A–B = 2 mm, C–D = 100 µm, E–F–G = 10 µm)

Etymology: Epithet refers to mangroves, the tree on which the novel species was found in association with cecidomyiid midge flies.

Leaf galls amphigenous, circular to subcircular (2.43–) 3.8–4.7 (–6.2) mm, becoming black (*1) on the adaxial side.

Sexual state: *Ascomata pseudothecial* immersed, hypophyllous, subepidermal, within the spongy mesophyll, erumpent, unilocular, several in the same gall but not forming stroma, globose to subglobose, rarely pyriform (56–) 68–77 (–88) µm wide. *Peridium* composed of an outer 1–3 layered of pale brown drab (9′′′d) wall cells, textura angularis, inner cells are hyaline. *Asci* clavate to clavate-cylindrical, with developed ocular chamber, aparaphysate, fasciculate, bitunicate, subsessile, arising from abaxial side, octosporic, (22–) 28–37 (–45) µm long, (7–) 8–9.5 (–10.5) µm wide. *Ascospores* aseptate when young, no mature ascospores were observed, biseriate, thin walled, ovoid with rounded ends, hyaline, smooth (without mucoid sheath), unicellular, one big guttule in the center of the spore (6.5–) 8–10 (–12) µm long, (3.8–) 4.2–5 (–5.5) µm wide, ascospore germination not seen.

Asexual state: Unknown, no spore forming was observed from the colonies growing on nutrient media, neither on leaves.

Colonies slow growing (reaching 2 mm diameter after 2 wk. and 5–6 mm diam. after 1 mo.) on MEA (Malt Extract Agar) at 25 °C. Colonies with circular to irregular blackish margins, rising centrally with a hard texture almost without aerial mycelium. Turning from greyish olive (21′′′) to greenish glaucous (33′′′f), reverse colonies black to dark blackish brown (1′′′m) after one month. Even though different nutrient media were tested for stimulation of sporulation, only vegetative hyphae was present, spore forming structures were not obtained (all cultures obtained and examined in the present study were infertile).

The type: SOUTH AFRICA, KWAZULU-NATAL PROVINCE, St. Lucia, isolated from galls formed on the leaves of *Avicennia marina*, collector J.A. Osorio, March 2013. **Holotype** PREM 41,457, living culture **Ex-holotype** CMW41472 = CBS142048.

Additional material examined: SOUTH AFRICA, KWAZULU-NATAL PROVINCE, Kosi Bay, isolated from galls formed on the leaves of *Avicennia marina*, collector J.A. Osorio, March 2013, living culture ex-paratype CMW41457; SOUTH AFRICA, KWAZULU-NATAL PROVINCE, St. Lucia, isolated from galls formed on the leaves of *Avicennia marina*, collector J.A. Osorio, March 2013, living culture ex-paratype CMW41458; SOUTH AFRICA, KWAZULU-NATAL PROVINCE, Durban, isolated from galls formed on the leaves of *Avicennia marina*, collector J.A. Osorio, March 2013, living culture ex-paratype CMW41459.

Habitat: Mangrove forest characterized by high salt concentration, siltation, anoxic soils, dominated by *A. marina* trees.

Host tree: Avicennia Marina

Substrate: Cecidomyiidae leaf galls

Known distribution in South Africa: Beachwood, Isipingo, Kosi Bay, Mlalazi Nature Reserve at Mtunzini and St. Lucia in KwaZulu-Natal Province. Mgazana estuary, Nahoon and Wavecrest in Eastern Cape Province.

Notes Zasmidium mangrovei forms a novel lineage in *Zasmidium* with no distinct closely related species. It has aseptate ascospores that represent a distinctive trait. To the best of our knowledge *Zasmidium* species that are known to form only the sexual morph have 1-septate ascospores. The lack of septa in *Z. mangrovei* may, however, be due to the fact that no mature ascospores were found on the leaves examined. In addition, *Z. mangrovei* has wider and ovoid ascospores, with the presence of a large guttule that occupies the center of the cell, compared to, for example *Z. citri-griseum* and *Z. musae*, which have ascospores that are narrower, septate and that are straight and oblong to clavate. Likewise, ascospores of *Z. eucalyptorum* are tri- to multi-seriate, straight to slightly curved, with obtuse ends and 1-sepatate.

Discussion

This study contributes to the limited knowledge of fungi associated with mangrove trees in South Africa. A novel species was consistently isolated from the specialized Cecidomyiidae leaf gall niche on white mangrove (*A. marina*). The new species, described as *Zasmidium mangrovei,* is a member of the Mycosphaerellaceae, and this is the first study to report the occurrence of *Zasmidium* on *A. marina* leaves in South Africa. It is also the first report of this genus from mangroves.

Leaf galls provide nutrition and shelter for gall-inducing insects, and also form a niche suitable for many other organisms, including fungi. Previous studies have estimated that the global richness of leaf gall-inducing insects ranges from 21 000 to 211 000 species, with environments having greater plant richness likely harboring higher numbers (Espírito-Santo and Fernandes 2007). Despite the diversity of these insects globally, relatively little is known about them and or their interactions with fungi. Previous studies have reported a broad range of fungal families on the external surfaces of insect galls (Crous et al. 2006; Rohfritsch 2008; Lebel et al. 2012; Lawson et al. 2014). However, rather specialized fungal associates appear to line gall-midge larval chambers (Lebel et al. 2012). There are no previous reports of Mycosphaerellaceae detected from leaf galls formed on true mangrove tree species providing novelty to this study.

The Mycosphaerellaceae is a widespread and species-rich fungal family, including thousands of species and 120 phylogenetically recognized genera (Videira et al. 2017). These fungi are known from diverse habitats, and play a large variety of ecological roles. Some of these fungi are pathogens of important crop plants such as banana, citrus, and olive (Ávila et al. 2005; Chang et al. 2016; Aguilera-Cogley et al. 2017). Molecular methods have substantially advanced the problematic taxonomy of genera and species in the Mycosphaerellaceae (Crous et al. 2009; Videira et al. 2017). One of the recently reassessed genera is *Zasmidium* (Arzanlou et al. 2007; Braun et al. 2010a, 2013; Videira et al. 2017), which currently accommodates more than 40 species and the species described in this study adds to this assemblage.

Phylogenetic analyses confirmed that isolates from *A. marina* grouped in a clearly distinct lineage in the Mycosphaerellaceae, within the genus *Zasmidium*. This was confirmed by all three gene regions studied. Characteristics of the ascospores must be interpreted with caution

as only seemingly young ascospores were found on the leaves examined. The most relevant possible differences in ascospore morphology is that those of *Z. mangrovei* are ovoid and non-septate, and have large guttules that occupy the center of the cell. This is in contrast with those of for example *Z. eucalyptorum*, which aside from being narrower, and longer; have septa and are straight to slightly curved, with obtuse ends, or the septate ascosores of *Z. pseudoparkii*. Colony growth on MEA at 25 °C also differs for all the species. *Zasmidium mangrovei*, for instance, has a slow growth (reaching 2 mm diam. after 2 wk. and 5–6 mm diam. after 1 mo.) in comparison with *Z. citri-griseum* (covering the dish after 2 mo.), *Z. proteacearum* (8 mm diam after 2 wk), *Z. eucalyptorum* (25–30 mm after 3 wk.) and *Z. pseudoparkii* (23–30 mm after 3 wk).

Four other Mycosphaerellaceae species have been isolated from *Avicennia* species. These are *Mycosphaerella pneumatophorae* (Kohlmeyer and Kohlmeyer 1971), *Pseudocercospora avicenniae*, *Pseudocercospora avicenniicola* and *Rhabdospora avicenniae* (Kohlmeyer and Kohlmeyer 1971; Shivas et al. 2009; Braun et al. 2010b). *Mycosphaerella pneumatophorae* was isolated from bark, particularly pneumatophores (Kohlmeyer and Kohlmeyer 1971), *P. avicenniae* and *P. avicenniicola* occurs on leaves (Shivas et al. 2009; Braun et al. 2010b) and *R. avicenniae* was reported from dead mangrove wood (Kohlmeyer and Kohlmeyer 1971). No sequence data are available for *P. avicenniicola* and *R. avicenniae* and their identity is based only on asexual morphological descriptions. This is problematic in light of the extensive changes to the taxonomy of the Mycosphaerellaceae in recent years. *Zasmidium mangrovei*, however, cannot be compared with these species based on morphology, due to the lack of the formation of asexual structures.

Only three other *Zasmidium* species have previously been reported from South Africa. These include *Z. arcuatum* (host plant *Ischyprolepsis subieriticilliata*), *Z. strelitziae* (host plant *Strelitzia* sp.) and *Z. velutinum* (host plant *Brabejum stellatifolium*). None of these are phylogenetically closely related to *Z. mangrovei* (Videira et al. 2017).

The discovery of *Z. mangrovei* in association with midge galls over the entire range of mangrove distribution in South Africa suggests that the fungus may have a specific role in this niche. The fungus sporulated only when the galls were at their maturity and was never found sporulating on leaf parts that were not galled, indicating potential coevolution between the fungus and the insect. This is consistent with previous studies that reported symbiotic fungal associates lining the gall-midge larval chambers on Chenopodiaceae plants (Lebel et al. 2012). *Mycosphaerella nubilosa* (Cooke) Hansf. (Hansford 1956) (formerly *Mycosphaerella molleriana*) and *Zasmidium pseudovespa* (Carnegie) U. Braun, C. Nakash., Videira & Crous (Videira 2017) (formerly *M. pseudovespa*) have been isolated from wasp galls on leaves of *E. globulus* and *E. biturbinata* in Australia (Carnegie et al. 2007). Some of the gall midges are considered mycophagous (Bisset and Borkent 1988; Veenstra-Quah et al. 2007; Heath and Stireman 2010) and this may also be the case with *Z. mangrovei*. However, whether the fungus is vectored by the gall midges or is an endophyte of the host plant, remains unknown.

Despite manglicolous fungi being well documented, the majority of studies have been conducted in East and South East Asia, particularly in Malaysia, reflecting a higher sampling intensity than in other geographical regions (Alias et al. 2010; Osorio et al. 2016a). To date, approximately 2200 fungal taxa have been recorded from mangroves in tropical and subtropical regions (Jones and Tan 1987; Hyde & Jones 1988; Abdel-Wahab 2005; Vittal & Sarma 2006; Alias et al. 2010; Simões et al. 2015; Suetrong et al. 2017). The first fungal

groups (ten genera), from mangroves in South Africa were reported by Kohlmeyer and Kohlmeyer (1971). More than 20 years later, 57 different genera have been added (Singh and Steinke 1992; Steinke and Gareth Jones 1993; Steinke and Hyde 1997; Steinke 2000). Recently another eight different fungal genera were discovered (Osorio et al. 2015, 2017a, 2017b). The report of a novel fungal species from Cecidomyiidae leaf galls on *A. marina* in South Africa increases the number of fungal genera known from mangrove trees to 76 in the country.

Acknowledgments

We thank Ezemvelo KZN Wildlife (EKZNW), the Isimangaliso Wetland Park and the Eastern Cape Parks & Tourism Agency (ECPTA) for sampling permits as well as their staff for assistance in the field. The material was collected under EKZNW permits No. OP 4776, OP 1457 and the ECPTA—RA 00119. We acknowledge fellow post-graduate students at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, who assisted us during field research.

Funding

This work was financially supported by the Department of Science and Technology (DST) and National Research Foundation (NRF) Center of Excellence in Plant Health Biotechnology (CPHB). RL received financial support from the University of Helsinki.

Contributions

JAO and JR conducted field sampling contributing to this study. JAO conducted laboratory and data analysis. RL conducted the phylogenetic analysis and produced the phylogenetic trees. All authors contributed to the writing of the manuscript and approved it for publication.

Conflict of interests

The authors declare that have no conflict of interest.

References

Abdel-Wahab MA (2005) Diversity of marine fungi from Egyptian Red Sea mangroves. Bot Mar 48:348–355

Abdel-Wahab MA, Hodhod MS, Bahkali AH, Jones EBG (2014) Marine fungi of Saudi Arabia. Bot Mar 57:323–335

Abdel-Wahab MA, Jones EBG, Bahkali AHA, El-Gorban AM (2018) Marine fungi from Red Sea mangroves in Saudi Arabia with *Fulvocentrum rubrum* sp. nov. (Torpedosporales, Ascomycota). Nova Hedwigia 108:365–377

Abdel-Wahab MA, Jones EBG, Bahkali AH (2020) Marine fungi recorded from *Avicennia marina* (Forsk.) Vierh. and their secondary product potential. Nova Hedwigia 111:357–390

Aguilera-Cogley VA, Berbegal M, Català S, Brentu FC, Armengol J, Vicent A (2017) Characterization of Mycosphaerellaceae species associated with citrus greasy spot in Panama and Spain. PLoS ONE 12(12):e0189585.https://doi.org/10.1371/journal.pone.0189585

Alias SA, Zainuddin N, Jones EBG (2010) Biodiversity of marine fungi in Malaysian mangroves. Bot Mar 53:545–554

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Anisimova M, Gascuel O (2006) Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. Systemat Biol 55:539–552

Arzanlou M, Groenewald JZ, Gams W, Braun U, Shin HD, Crous PW (2007) Phylogenetic and morphotaxonomic revision of *Ramichloridium* and allied genera. Stud Mycol 58:57–93

Ávila A, Groenewald JZ, Trapero A, Crous PW (2005) Characterisation and epitypification of *Pseudocercospora cladosporioides*, the causal organism of *Cercospora* leaf spot of olives. Mycol Res 109:881–888

Bisset J, Borkent A (1988) Ambrosia galls: the significance of fungal nutrition in the evolution of the Cecidomyiidae (Diptera). In: Hawksworth KA, Pirozynski DL (eds) Coevolution of fungi with plants and animals. Academic Press Limited, New York, pp 203–225

Braun U, Crous PW, Schubert K et al (2010) Some reallocations of *Stenella* species in *Zasmidium*. Schlechtendalia 20:99–104

Braun U, das Chargas F, Freire O, Urtiaga R (2010) New species and new records of cercosporoid hyphomycetes from brazil, New Zealand and Venezuela. Pol Bot J 55:281–291

Braun U, Nakashima C, Crous PW (2013) Cercosporoid fungi (Mycosphaerellaceae) 1. Species on other fungi. Pteridophyta Gymnospermae IMA Fungus 4:265–345

Carbone I, Kohn LM (1999) A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91:553–556

Carnegie AJ, Burgess TI, Beilharz V, Wingfield MJ (2007) New species of *Mycosphaerella* from Myrtaceae in plantations and native forests in eastern Australia. Mycologia 99:461–474

Carneiro MAA, Branco CSA, Braga CED, Almada ED, Costa MBM, Fernandes MVCGW (2009) Are gall midge species (Diptera, Cecidomyiidae) host-plant specialists? Rev Bras Entomol 53:365–378

Chang T-C, Salvucci A, Crous PW et al (2016) Comparative genomics of the Sigatoka disease complex on banana suggests a link between parallel evolutionary changes in *Pseudocercospora fijiensis* and *Pseudocercospora eumusae* and increased virulence on the banana host. PLoS Genet 12(8):e1005904. https://doi.org/10.1371/journal.pgen.1005904

Crous PW (1998) *Mycosphaerella* spp. and their anamorphs associated with leaf spot diseases of Eucalyptus. Mycologia Memoir 21:1–170

Crous PW, Slippers B, Wingfield MJ, Rheeder J, Marasas WFO, Phillips AJL, Alves A, Burgess T, Barber P, Groenewald JZ (2006) Phylogenetic lineages in the Botryosphaeriaceae. Stud Mycol 55:235–253

Crous PW, Summerell BA, Carnegie AJ, Wingfield MJ, Hunter GC et al (2009) Unravelling *Mycosphaerella*: do you believe in genera? Persoonia 23:99–118

de Souza Sebastianes FL, Romão-Dumaresq AS, Lacava PT, Harakava R, Azevedo JL, de Melo IS, Pizzirani-Kleiner AA (2013) Species diversity of culturable endophytic fungi from Brazilian mangrove forests. Curr Genet 59:153–166

Ellison J (2015) Vulnerability assessment of mangroves to climate change and sea-level rise impacts. Wetl Ecol Manag 23:115–137

Espírito-Santo MM, Wilson Fernandes G (2007) How many species of gall-inducing insects are there on earth, and where are they? Ann Entomol Soc Am 100:95–99

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithm and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Systematic Biol 59:3076–3321

Hansford CG (1956) Australian fungi III. New species and revisions (continued). Proc Linn Soc N S W 81:23–51

Heath JJ, Stireman JO III (2010) Dissecting the association between a gall midge, *Asteromyia carbonifera*, and its symbiotic fungus, *Botryosphaeria dothidea*. Entomol Exp Appl 137:36–49

Hyde KD, Jones EBG (1988) Marine mangrove fungi. P.S.Z.N.I. Marine Ecol 9:15–33

Jenoh ER, Robert EMR, Lehmann I, Kioko E, Bosire JO, Ngiasiange N, Dahdouh-Guebas F, Koedam N (2016) Wide ranging insect infestation of the pioneer mangrove *Sonneratia alba* by two insect species along the Kenyan coast. PLoS ONE. https://doi.org/10.1371/journal.pone.0154849

Jones EBG, Tan TK (1987) Observations on manglicolous fungi from Malaysia. Trans Br mycol Soc 89:390–392

Katoh K, Standley DM (2013) MAFFT Multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 30:772–780

Kohlmeyer J, Kohlmeyer E (1971) Marine fungi from tropical America and Africa. Mycologia 53:831–861

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–2187

Larget B, Simon DL (1999) Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. Mol Biol Evol 16:750–759

Lawson SP, Christian N, Abbot P (2014) Comparative analysis of the biodiversity of fungal endophytes in insect-induced galls and surrounding foliar tissue. Fungal Div 66:89–97

Lebel T, Peele C, Veenstra A (2012) Fungi associated with *Asphondylia* (Diptera: Cecidomyiidae) galls on *Sarcocornia quinqueflora* and *Tecticornia arbuscula* (Chenopodiaceae). Fungal Div 55:143–154

Leford V, Longueville JE, Gascuel O (2017) SMS: smart model selection in PhyML. Mol Biol Evol 34:2422–2424

Maia VC (2001) The gall midges (Diptera, Cecidomyiidae) from three restingas of Rio de Janeiro State, Brazil. Rev Bras Zool 18:583–629

Maia VC, Magenta MAG, Martins SE (2008) Ocorrência e caracterização de galhas de insetos em áreas de restinga de Bertioga (São Paulo, Brasil). Biota Neotrop 8:167–197

Moncalvo JM, Rehner SA, Vilgalys R (1993) Systematics of *Lyophyllum* section *Difformia* based on evidence from culture studies and ribosomal DNA sequences. Mycologia 85:788–794

Nylander JAA (2004) MrModeltest v2 Program distributed by the author. Evolutionary Biology Centre, Uppsala University, p 2

Osorio JA, Wingfield MJ, de Beer ZW, Roux J (2015) *Pseudocercospora mapelanensis* sp. nov., associated with a fruit and leaf disease of *Barringtonia racemosa* in South Africa. Australas Plant Pathol 44:349–359

Osorio JA, Wingfield MJ, Roux J (2016a) A review of factors associated with decline and death of mangroves, with particular reference to fungal pathogens. S Afr J Bot 103:295–301

Osorio JA, De Beer ZW, Wingfield MJ, Roux J (2016b) Ophiostomatoid fungi associated with mangroves in South Africa, including *Ophiostoma palustre* sp. nov. Antonie Van Leeuwenhoek 109:1555–1571

Osorio JA, Crous CJ, de Beer ZW, Wingfield MJ, Roux J (2017a) Endophytic *Botryosphaeriacea*e, including five new species, associated with mangrove trees in South Africa. Fungal Biol 121:361–393

Osorio JA, Crous CJ, de Beer ZW, Wingfield MJ, Roux J (2017b) An assessment of mangrove diseases and pests in South Africa. Forestry 90:343–358

Piątek M, Yorou NS (2018) *Pseudocercospora avicenniicola* on black mangrove (*Avicennia germinans*) in Benin: the first report from Africa. Forest Pathol 49:e12478. https://doi.org/10.1111/efp.12478

Raeder U, Broda P (1985) Rapid preparation of DNA from filamentous fungi. Lett Appl Microbiol 1:17–20

Rayner RW (1970) A mycological colour chart. CMI and British Mycological Society, Kew

Robert V, Vu D, Amor AB et al (2013) MycoBank gearing up for new horizons. IMA Fungus 4:371–379

Rohfritsch O (2008) Plants, gall midges, and fungi: a three component system. Entomol Exp Appl 129:208–216

Rohfritsch O, Shorthouse JD (1982) Insect galls. In: Kahls G, Schell JS (eds) Molecular biology of plant tumors. Academic Press, New York

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A et al (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systemat Biol 61:539–542

Sánchez ÁS, Melchor GIH, Cruz JMZ, González CAZ, Galarza JLS (2018) Mangrove restoration an economical alternative for generating incomes. In: Filho WL, Pociovălișteanu DM, Brito PRB, de Lima IB (eds) Towards a sustainable bioeconomy: principles challenges and perspectives. Springer, Cham

Shivas RG, Young AJ, Crous PW (2009) Pseudocercospora avicenniae. Persoonia 23:192–193

Simões MF, Antunes A, Ottoni CA, Amini MS, Alam I, Alzubaidy H, Mokhtar NA, Archer JA, Bajic VB (2015) Soil and rhizosphere associated fungi in gray Mangroves (Avicennia marina) from the Red Sea—A metagenomic approach. Genom Proteom Bioinf 13:310–320

Singh N, Steinke TO (1992) Colonization of decomposing leaves of *Bruguiera gymnorrhiza* (Rhizophoraceae) by fungi, and in vitro cellulolytic activity of the isolates. S Afr J Bot 58:525–529

Sippo JZ, Lovelock C, Santos IR, Sanders CJ, Maher DT (2018) Mangrove mortality in a changing climate: an overview. Estuar Coast Shelf S 215:241–249

Spalding M, Kainuma M, Collins L (2010) World Atlas of Mangroves. Earthscan, Washington DC, p 319

Steinke TD (1999) Mangroves in South African estuaries. In: Allanson BR, Baird D (eds) Estuaries of South Africa. Cambridge University Press, Cambridge, pp 119–140

Steinke TD (2000) Mangrove fungi on dead proproots of *Rhizophora mucronata* at three localities in South Africa. S Afr J Bot 66:91–95

Steinke TD, Gareth Jones EB (1993) Marine and mangrove fungi from the Indian Ocean coast of South Africa. S Afr J Bot 59:385–390

Steinke TD, Hyde KD (1997) *Gloniella clavitispora*, sp. nov. from *A. marina* in South Africa. Mycoscience 38:7–9

Suetrong S, Preedanon S, Klaysuban A, Gundool W, Unagul P, Sakayaroj J, Promchu W, Sangtiean T (2017) Distribution and occurrence of manglicolous marine fungi from eastern and southern Thailand. Bot Mar 60:503–514

Sugiura N (1978) Further analysis of the data by Akaike's information criterion and the finite corrections. Commun Stat Theory Methods 7:13–26

Swofford DL (2003) PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods) Version 4. Sinauer Associates, Massachusetts

Tomlinson PB (1986) The Botany of Mangroves. Cambridge University Press, p 413

Veenstra-Quah AA, Milne J, Kolesik P (2007) Taxonomy and biology of two new species of gall midge (Diptera: Cecidomyiidae) infesting *Sarcocornia quinqueflora* (Chenopodiaceae) in Australian salt marshes. Aust J Entomol 46:198–206

Videira SIR, Groenewald JZ, Nakashima C, Braun U, Barreto RW, de Wit PJGM, Crous PW (2017) Mycosphaerellaceae—Chaos or clarity? Stud Mycol 87:257–421

Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J Bacteriol 172:4238–4246

Vittal BPR, Sarma VV (2006) Diversity and ecology of fungi on mangroves of bay of Bengal region—an overview. Indian J Mar Sci 35:308–317

Wang L, Mu M, Li X, Lin P, Wang W (2010) Differentiation between true mangroves and mangrove associates based on leaf traits and salt contents. J Plant Ecol 4:292–301

Washburn G, Bael SA (2017) Fungal diversity in galls of baldcypress trees. Fungal Ecol 29:85–89

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols: a guide to methods and applications. Academic Press