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ORIGINAL PAPER

Pseudocercospora mapelanensis sp. nov., associated with a fruit and leaf disease of *Barringtonia racemosa* in South Africa

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Abstract *Barringtonia racemosa* (Lecythidaceae) is a widely distributed mangrove associate in coastal areas of Africa, Asia and Australia. During routine disease surveys along the east coast of the KwaZulu-Natal Province in South Africa, *B. racemosa* trees were observed with symptoms of leaf and fruit spot. A novel species of *Pseudocercospora* was commonly associated with these symptoms. This fungus is described as *Pseudocercospora mapelanensis* sp. nov., based on multi-gene sequence analyses for the ACT, ITS, LSU and TEF gene regions, as well as morphological characteristics.

Keywords *Capnodiales* · *Lecythidaceae* · *Mangroves* · *Mycosphaerellaceae*

Introduction

Mangroves, including true mangroves and mangrove associates (Tomlinson 1986; Ellison and Farnsworth 2001), are adapted to survive in saline-rich water and anoxic soils. They are found along tropical and sub-tropical coastlines where they provide many environmental and economic benefits (Spalding et al. 2010). Mangroves are, however, frequently threatened by anthropogenic activities and/or environmental factors (Spalding et al. 2010). Despite their importance, there have been relatively few studies to consider the impact of microbial diseases on these trees (Osorio et al. 2014).

Barringtonia racemosa (fresh water mangrove) is a mangrove associate that belongs to the Lecythidaceae, found along canals and rivers of coastal areas of Africa, Asia and Australia (Chantaranothai 1995; USDA-ARS 2014). Although several fungal species have been reported from *Barringtonia* species globally, nothing is known regarding fungal pathogens of *B. racemosa* in South Africa. During a survey of the health of mangrove species in this country, a fruit, floral and leaf disease was observed on *B. racemosa* trees in the Zululand region. The aim of this study was to describe the symptoms of the disease and to identify its causal agent.

Materials and methods

Disease observations and sample collection

In February 2011, a disease affecting the flowers, fruits and leaves of *Barringtonia racemosa* was observed in the Mapelane area of the Kwazulu-Natal Province in South Africa. In order to identify the fungus most closely associated with the disease symptoms, infected fruits, floral sepals and leaves were collected from several sites, placed in paper bags and transported to the laboratory for further study. During 2012 and 2013, additional areas where *B. racemosa* is known to occur were also surveyed for the occurrence of the disease, and additional samples were collected for analyses.

Cultures

A single fungus was found sporulating profusely on infected green leaf and fruit tissue. Fungal structures were transferred directly from plant material, mounted on microscope slides

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and examined under a Zeiss Axioskop microscope (Carl Zeiss, Germany). Images were obtained by using an Axiocam digital camera connected to the microscope. To isolate this fungus, stromatic tissue was removed from symptomatic plant material and transferred to 500 μl autoclaved Sabax water. Of this, 100 μl were spread onto 2 % malt extract agar (MEA, Biolab Malt Extract, 20 g Biolab Agar amended with 0.4 g of streptomycin to suppress bacterial growth, in 1 L distilled water) and incubated overnight. Germinating conidia were transferred to fresh 2 % MEA. Pure cultures obtained from single spore isolates were deposited in the culture collection (CMW) of the Tree Protection Co-operative Programme (TPCP) at FABI, University of Pretoria. Duplicate cultures were deposited in the CBS-KNAW Fungal Biodiversity Centre's culture collection (Centraalbureau voor Schimmelcultures) in Utrecht, The Netherlands.

Pathogen identification

DNA extraction, PCR amplification and sequencing

To extract genomic DNA, mycelium from colonies of three isolates, no older than 4 weeks of age, were placed into 1.5 mL sterile Eppendorf tubes and freeze dried. Isolates for identification were selected based on plant part and collection site, so as to represent all symptom types and regions, as well as multiple trees. DNA was extracted using the phenol-Chloroform method described by Raeder and Broda (1985). After DNA was obtained, a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE) was used to measure DNA concentrations, and to calculate the volumes to be used in PCR reactions.

Four gene regions were sequenced for all isolates. A portion of the mitochondrial large subunit (LSU) was amplified using primers LR5 (Vilgalys and Hester 1990) and LR0R (Moncalvo et al. 1993). The internally transcribed spacer (ITS) regions (ITS1, ITS2) were amplified using the primers ITS1 and ITS4 (White et al. 1990). A portion of the translation elongation factor 1-α (TEF) was amplified using the primers EF1F and EF2R (Jacobs et al. 2004). A portion of the actin (ACT) gene was amplified using the primers ACT-512F and ACT-783R (Carbone and Kohn 1999).

For all gene regions, each reaction contained 2.5 μL of PCR buffer, 2 μL dNTP, 1 μL of each primer, 0.3 μL of fast *Taq* polymerase and 3 μL of DNA (60 ng/μl). Sterile Sabax water was added to obtain a final volume of 25 μL for each reaction. Reactions were run using the following thermal cycling conditions: initial denaturation at 94 °C for 4 min followed by a denaturation step of ten cycles consisting of 94 °C for 20 s, annealing at 55 °C (ITS and TEF), 48 °C (LSU) and 61 °C (ACT) for 48 s, and elongation at 72 °C for 45 s, followed by a further 25 cycles of 94 °C for 20 s, followed by an annealing step with temperatures as previously indicated for each gene region (55 °C, 48 °C, 61 °C), for 40 s with a time increase of 5 s per cycle, and then 45 s at 72 °C. This was concluded by a final step of 72 °C for 10 min. An aliquot of 5 μl of each PCR product was separated by gel electrophoresis at 90 V for 20 min in a 2 % agarose gel in 5 % TAE Buffer (40 mM Tris, 40 mM acetate, 2 mM EDTA, pH 8.0) to evaluate the success of the reactions using GelRedTM nucleic acid gel stain (Biotium, Hayward, CA, USA). PCR products were cleaned using Sephadex G-50 columns, following the instructions provided by the manufacturers (Sigma Aldrich, Sweden).

Purified PCR products were used as template DNA for sequencing reactions, using a Big-Dye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, California, USA) and the same primers and annealing temperatures that were used in the PCR. The final products were also cleaned in Sephadex G-50 columns. Sequencing was carried out on an ABI PRISM 3100 DNA sequencer (Applied Biosystems).

Morphology

Colony morphology and microscopic features of the isolated fungus were examined directly from plant material and from cultures growing on 2 % MEA. Mounts of the fungus were prepared in 85 % lactic acid on microscope slides for detailed observations of structures such as stromata, conidiophores and conidia. Where structures were dry, these were mounted in 2 % KOH. Characters such as size of conidiophores and conidiogenous cells as well as pigmentation of the conidia were used for description of the species based on morphology. Fifty measurements were made for each relevant morphological characteristic and the standard deviation (S.D.) was calculated for measurements of the stromata, conidia and conidiophores. Minimum and maximum dimensions are given in parentheses.

Colony colors (surface and reverse) were assessed after 30 days on MEA, using the color charts of Rayner (1970). Growth of cultures was assessed by measuring single conidial cultures after 15, 30 and 60 days maintained at a temperature of 25 °C. Culture characteristics were determined from cultures grown under natural day/night conditions.

Phylogenetic analyses

Sequences of the isolates obtained from *B. racemosa* were assembled using CLC Main Workbench 6.7.1 (http://www.clcbio.com/genomics/), and compared with published sequences using a Blast search in the GenBank (http://blast.ncbi.nlm.nih.gov/blast.cgi) data base. DNA sequences for closely related species, previously published, were retrieved from GenBank and combined into data sets with the *B. racemosa* sequences (Table 1). The data matrices were aligned

Table 1 Isolates included in phylogenetic analyses

Species	Isolate No[a]	Collector	Host	Country	GenBank accession numbers[b]			
					LSU	ITS	TEF-1-α	ACT
Cercospora capsici	CBS118712	P. Tyler	Unknown	Fiji	GU214653			
C. sojina	CPC12322	H.D. Shin	*Glycine soja*	South Korea	GU253861			
Cladosporium cladosporioides	CBS10921				EU019262			
C. herbarum	AFTOL-ID 1591				DQ678074			
Mycosphaerella africana	CMW4945	P.W Crous	*Eucalyptus viminalis*	South Africa	DQ246257			
M. ellipsoidea	CMW4934	Unknown	*Eucalyptus* sp.	South Africa	DQ246253			
M. madeirae	CBS112895	S. Denman	*E. globulus*	Portugal	DQ204756			
M. laricina	CBS326.52	E. Müller	*Larix decidua*	Switzerland	GU253693			
Pallidocercospora acaciigena	CPC3838	M.J. Wingfield	*Acacia mangium*	Venezuela	GU214661			
Pal. crystallina	CMW3033	M.J. Wingfield	*E. bicostata*	South Africa	DQ204747			
Pal. heimii	CMW4942	P.W. Crous	*Eucalyptus* sp.	Madagascar	DQ204751			
Pal. heimioides	CMW3046	M.J. Wingfield	*Eucalyptus* sp.	Indonesia	DQ204753			
Pal. irregulariramosa	CMW4943	M.J. Wingfield	*E. saligna*	South Africa	DQ204754			
Pal. konae	CBS120748	W. Himamam	*E. camaldulensis*	Thailand	GU253852			
Passalora eucalypti	CBS111318	P.W. Crous	*E. saligna*	Brazil	GU253860	GU269845	GU384558	GU320548
Pseudocercospora abelmoschi	CPC14478	H.D. Shin	*Hibiscus syriacus*	South Korea	GU253696	GU269647	GU384365	GU320355
P. acericola	CBS122279	R. Kirschner	*Acer albopurpurascens*	Taiwan	GU253699	GU269650	GU384368	GU320358
P. ampelopsis	CPC11680	H.D. Shin	*Ampelopsis brevipenduncula*	South Korea	GU253846	GU269830	GU384542	GU320534
P. angolensis	CBS14953	M.C. Pretorius	*Citrus* sp.	Zimbabwe	JQ324941	JQ324975	GU384548	JQ325010
P. arecacearum	CBS118406	C.F. Hill	*Rhopalostylis sapidis*	New Zealand	GU253704	GU269655	GU384373	GU320363
P. atromarginalis	CBS114640	C.F. Hill	*Solanum* sp.	New Zealand	GU253706	GU269658	GU384376	GU320365
P. chengtuensis	CPC10696	H.D. Shin	*Lycium chinense*	South Korea	JQ324942	GU269673	GU384390	GU320379
P. cladosporioides	CBS117482	P.W. Crous	*Olea europaea*	Tunisia	JQ324944	GU269678	GU384395	GU320383
P. coprosmae	CBS114639	C.F. Hill	*Coprosma robusta*	New Zealand	JQ324946	GU269680	GU384397	GU320386
P. crispans	CPC14883	P.W. Crous	*Eucalyptus* sp.	South Africa	GU253825	GU269807	GU384518	GU320510
P. cruenta	CPC10846	H. Booker	*Vigna* sp.	Trinidad	GU269688	GU269688	GU384404	JQ325012
P. cydoniae	CPC10678	H.D. Shin	*Chaenomeles speciosa*	South Korea	GU253732	GU269691	GU384407	GU320396
P. dodonaeae	CBS114647	C.F. Hill	*Dodonaea viscosa*	New Zealand	JQ324948	GU269697	GU384413	JQ325013
P. dovyalidis	CPC13771	P.W. Crous	*Dovyalis zeyheri*	South Africa	GU253818	GU269800	GU384513	GU320503
P. eucalyptorum	CPC10916	P.W. Crous	*Eucalyptus* sp.	South Africa	GU253788			
P. eustomatis	CBS110822	G. Dal Bello	*Eustroma grandiflorum*	Argentina	GU253744	GU269705	GU384421	GU320409
P. fori	CMW9095	G.C. Hunter	*E. grandis*	South Africa	DQ204748	AF468869	DQ211664	DQ147618
P. fraxinites	CPC10743	H.D. Shin	*Fomtanesia phillyraeoides*	South Korea	GU253720	GU269672	GU384389	GU320378
P. fuligena	CPC12296	Z. Mersha	*Lycopersicon* sp.	Thailand	JQ324953	GU269711	GU384427	GU320415
P. hakeae	CBS112226	P.W. Crous & B. Summerell	*Grevillea* sp.	Australia	GU253805	GU269784	GU384495	JQ325017
P. humulicola	CPC10049	H.D. Shin	*Humulus scandens*	South Korea	JQ324955	GU269724	JQ324996	JQ325018
P. mapelanensis	**CMW40579**	**J.A. Osorio & J. Roux**	***B. racemosa***	**South Africa**	**KM203119**	**KM203116**	**KM203122**	**KM203125**
P. mapelanensis	**CMW40580**	**J.A. Osorio & J. Roux**	***B. racemosa***	**South Africa**	**KM203120**	**KM203117**	**KM203123**	**KM203126**

Table 1 (continued)

Species	Isolate No[a]	Collector	Host	Country	GenBank accession numbers[b]			
					LSU	ITS	TEF-1-α	ACT
P. mapelanensis	**CMW40581**	**J.A. Osorio & J. Roux**	***B. racemosa***	**South Africa**	**KM203121**	**KM203118**	**KM203124**	**KM203127**
P. indonesiana	CBS122473	I.W. Buddenhagen	*Musa* sp.	Sumatra	GU253765	GU269735	GU384448	GU320437
P. kiggelariae	CPC11853	W. Gams	*Kiggelaria africana*	South Africa	GU253762	GU269730	GU384443	GU320432
P. libertiae	CBS114643	C.F. Hill	*Libertia ixioides*	New Zealand	JQ324959	GU269733	GU384446	GU320435
P. longispora	CBS122470	D.R. Jones	*Musa* sp.	Malaysia	GU253764	GU269734	GU384447	GU320436
P. luzardii	CPC2556	A.C. Alfenas	*Hancornia speciosa*	Brazil	GU214477	GU269738	GU384450	GU320440
P. musae	CBS116634	J. Carlier	*Musa* sp.	Cuba	GU253775			
P. oenotherae	CPC10290	H.D. Shin	*Oenothera odorata*	South Korea	JQ324961	GU269856	GU384567	GU320559
P. plectranthi	CPC11462	H.D. Shin	*Plectranthus* sp.	South Korea	JQ324962	GU269791	GU384501	GU320492
P. proteae	CPC15217	F. Roets	*Protea mundii*	South Africa	GU253826	GU269808	GU384519	GU320511
P. prunicola	CPC14511	H.D. Shin	*Prunus* x *yedoensis*	South Korea	GU253723	GU269676	GU384393	GU320382
P. punctata	CPC14734	P.W. Crous	*Syzygium* sp.	Madagascar	GU253791	GU269765	GU384477	GU320468
P. ranjita	CPC11141	M.J. Wingfield	*Gmelina* sp.	Indonesia	GU253810	GU269790	GU384500	GU320491
P. ravenalicola	CBS122468	M. Arzanlou & W. Gams	*Ravenala madagascariensis*	India	GU253828	GU269810	GU384521	GU320513
P. rhoina	CPC11464	H.D. Shin	*Rhus chinensis*	South Korea	JQ324966			
P. rubi	MUCC875	T. Kobayashi & C. Nakashima	*Rubus allegheniensis*	Japan	GU253795	GU269773	GU384485	GU320476
P. rumohrae	CBS117747	C.F. Hill	*Marattia salicina*	New Zealand	GU253796	GU269774	GU384486	GU320477
P. subsessilis	CBS136.94	R.F. Castaneda		Cuba	GU253832	GU269815	GU384527	GU320517
P. subtorulosa	CBS117230	R. Kirschner	*Melicope* sp.	Taiwan	GU253833			
P. tereticornis	CBS124996	A.J. Carnegie	*E. nitens*	Australia	GQ852647			
P. timorensis	MUCC 819	C. Nakashima & T. Akashi	*Ipomoea indica*	Japan	GU253840	GU269823	GU384536	GU320526
P. viticicola	MUCC777	C. Nakashima	*Vitex trifolia*	Japan	GU253845	GU269828	GU384540	GU320532
P. vitis	CPC11595	H.D. Shin	*V. vinifera*	South Korea	GU214483	GU269829	GU384541	GU320533
P. xanthocercidis	CPC11665	A.R. Wood	*Xanthocercis zambesiaca*	South Africa	JQ324971			
P. xanthoxyli	CPC10065	H.D. Shin	*Xanthoxylum ailathoides*	South Korea	GU253848	GU269832	GU384544	GU320536
Septoria cerastii	CPC12343	H.D. Shin	*Cerastium holosteoides* var. *hallasanense*	South Korea	GU253869			
S. chelidonii	CPC12337	H.D. Shin	*Chelidoniummajur* var. *asiaticum*	South Korea	GU253870			
S. crepidis	CPC12539	H.D. Shin	*Crepis japonica*	South Korea	GU253871			
S. dysentericae	CPC12328	H.D. Shin	*Inula britannica* var. *chinensis*	South Korea	GU253866			
S. erigerontis	CPC12340	H.D. Shin	*Erigeron annuus*	South Korea	GU253872			
S. eucalyptorum	CPC11282	W. Gams	*Eucalyptus* sp.	India	GU253873			
S. justiciae	CPC12509	H.D. Shin	*Justicia procumbens*	South Korea	GU253874			
S. quercicola	CBS663.94	H.A. Van der Aa	*Quercus robur*	Netherlands	GU253867			

[a] CBS: Centraalbureau,voor Schimmelcultures, CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CMW: Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; MUCC: Murdoch University Algal Culture Collection, Murdoch, Western Australia; CPC Collection of Pedro Crous housed at CBS

[b] Accession numbers of DNA sequences generated in the present study are printed in bold

LSU: partial 28S nrRNA gene; ITS: internal transcribed spacer regions 1 & 2 including 5.8S nrRNA gene; EF-1α: partial translation elongation factor 1-alpha gene; ACT: partial actin gene

using an online version of MAFFT v. 7 (Katoh and Standley 2013) and edited manually for alignment errors with MEGA version 6 (Tamura et al. 2013).

Phylogenetic analyses of sequence data for Maximum Parsimony (MP) were performed with the software package PAUP* 4.0b10 (Swofford 2003). Maximum parsimony (MP) genealogies for single genes were constructed with the heuristic search option (1000 random taxa additions, tree bisection and reconstruction or TBR in PAUP). Uninformative characters in each data set were excluded from the analyses, and the consistency index (CI), homoplasy index (HI), rescaled consistency index (RC), retention index (RI) and tree length (TL) were determined for the resulting trees. Partition homogeneity tests (PHT) were conducted to determine the congruence of trees obtained from the different gene regions with PAUP* 4.0b10 (Swofford 2003) to define whether data from the different gene regions could be combined.

Phylogenetic analyses of sequence data for Maximum Likelihood (ML) were performed with the program PhyML version 3.0 (Guindon and Gascuel 2003). The confidence levels were estimated with 1000 bootstrap replicates. The best fit substitution models for each of the data combinations were determined using jModeltest 0.1.1 (Posada 2008). MrBayes v. 3.2 (Ronquist et al. 2012) was implemented to perform the Bayesian inference (BI) analyses. Trees were sampled at every 100th generation for six million generations and the posterior probability values above 0.95 were accepted.

Results

Disease symptoms and sample collection

The fruit, leaf and floral sepal disease of *B. racemosa* was found in the Mapelane area on the southern border of the Isimangaliso Wetland Park, the city of Richards Bay and in Mlalazi Nature Reserve near the town of Mtunzini. Symptoms were mostly observed during summer, particularly January to April. Isolates of the fungus consistently associated with this disease were obtained from the aerial organs of ten trees from Mapelane, as well as from ten trees each in two areas in Richards Bay. Three representative isolates from fruits, leaves and sepals were selected for identification based on DNA sequence data.

The disease affecting *B. racemosa* was characterized by sooty black spots on the fruits (Fig. 1a and b); on the floral sepals (Fig. 1c and d) as well as dark stromata becoming confluent to produce large spots on the leaves (Fig. 1e and f). These spots could merge and cover the entire surface of the aerial organs affected. Leaf infection often resulted in leaf deformation (Fig. 1e). Brownish conidiophores and pale brown conidia were commonly observed under the microscope (Fig. 2). Although the disease did not result in defoliation of trees, flower and fruit abortion was common on affected trees at Mapelane and Richards Bay.

Identification

PCR amplification and sequencing

DNA was successfully extracted from the isolates and PCR and sequence products generated for all selected gene regions. Sequence fragments were approximately 367 bp in size for the ACT, 485 bp for the ITS, 826 bp for the LSU and 456 bp for the TEF-1α. All sequences used in this study have been deposited in GenBank (Table 1).

Phylogenetic analyses

Blast searches in Genbank, of sequences generated for the LSU and ITS gene regions, showed that all isolates obtained from diseased *B. racemosa* represented a species of *Pseudocercospora*. Based on these results, data sets were assembled for all gene regions sequenced, including sequence data for previously described, published species of *Pseudocercospora* (Table 1).

Sequence data sets of the ACT, ITS, LSU, and TEF-1α gene regions were analyzed individually (Table 1). A phylogenetic re-construction was conducted for the aligned LSU data set to determine generic relationships. The LSU data set comprised species from the five closest related sister genera in the Mycosphaerellaceae, based on Blast searches and published literature. These included *Cercospora* Fresen., *Mycosphaerella* Johanson., *Passalora* Fresen., *Pseudocercospora* Speg., *Septoria* Sacc., and *Pallidocercospora* Crous. *Cladosporium cladosporioides* (Fresen.) G.A. de Vries., and *Cladosporium herbarum* (Pers.) Link, were included as outgroups. The dataset for the LSU phylogenetic analyses comprised a total of 69 taxa. ML analyses resulted in 1000 trees and MP analyses in 7304 trees (Fig. 3).

The isolates obtained from *B. racemosa* grouped strongly within the genus *Pseudocercospora* in all analyses. Subsequently, a species level phylogeny was derived from individual analysis of the ACT, ITS and TEF-1α alignment of described species of *Pseudocercospora*, with *Passalora eucalypti* (Crous & Alfenas) Crous & U. Braun, included as outgroup. A total of 43 taxa were included in the analyses for each of the three gene regions. ML analyses of the ITS data set resulted in 1000 trees and MP analyses in 93,765 trees. These trees all suggested that isolates from *B. racemosa* represent an undescribed species, most closely related to, but distinct from, *P. longispora* (Fig. 3).

Phylogenetic analyses involving sequence data from the ACT and TEF gene regions helped to clarify the placement of the species from *B. racemosa* (Fig. 4). ML analysis of the

Fig. 1 Signs and symptoms of disease caused by *Pseudocercospora mapelanensis* on *B. racemosa* (**a**, **b**). Fruit infection (**c**, **d**). Sooty black spots on floral sepals (**e**). Leaf spots on the abaxial part of the leaf (**f**). Close-up view of leaf spots

ACT gene region resulted in 1000 trees and MP analysis in 4274 trees. The ML analysis of the TEF gene region data set resulted in 1000 trees and MP analysis in 156 trees. These trees also suggested that isolates from *B. racemosa* represent an undescribed species of *Pseudocercospora,* showing *P. dodonaeae* as the closest relative in the ACT gene region and *P. xanthoxyli* as the closest relative in the TEF-1α gene region.

Taxonomy

Based on sequence comparisons for multiple gene regions, the fungus obtained from diseased *B. racemosa* in South Africa represents an undescribed species of *Pseudocercospora*. The following description based on morphological characteristics is provided:

Pseudocercospora mapelanensis J.A Osorio & Jol. Roux (Figs. 1 and 2)

MycoBank MB 809625

Diagnosis: Morphologically akin to *Pseudocercospora barringtoniicola*, but conidia shorter and wider, (19–)24–38(−45) x (4–)5–7(–10) μm, only 1–3-septate, conidiogenous loci sometimes subconspicuous, circle-like in front view, and hila of the conidia often somewhat refractive or even darkened-refractive and thus more conspicuous.

Leaf spots amphigenous, subcircular to angular-irregular, 2–18 mm diam or confluent and larger, margin indefinite or surrounded by a diffuse, paler halo, pale greenish, yellowish to ochraceous. *Caespituli* amphigenous, also on fruits, punctiform to pustulate, scattered to gregarious, dark brown.

Asexual s*tromata* (10–)20–60 μm diam, sometimes expanded or confluent and larger, to 100 μm diam or larger, brown to blackish brown, sub-globose, erumpent on leaves

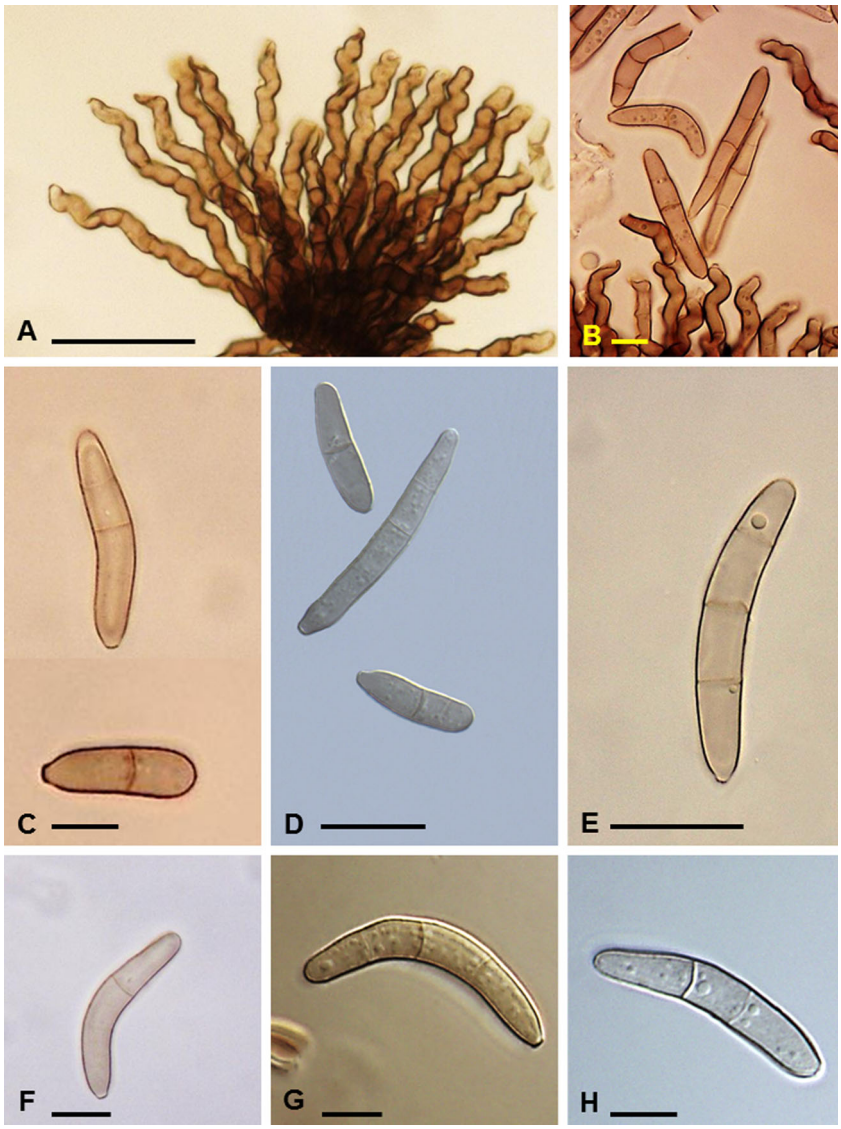

Fig. 2 Conidiophores and conidia of *Pseudocercospora mapelanensis* (**a**). Geniculate conidiophores, observed with zoom (40x) (bar = 50 μm). (**b**) Conidiophores and conidia observed with zoom (40x) (bar = 10 μm). (**c**, **d**, **e**, **f**, **g**, **h**) Pale brown conidia with truncate base, different shape, length and septa observed with zoom (100x) (bar **c**, **f**, **g**, **h** = 10 μm), (bar **d**, **e** = 20 μm)

and fruits, composed of swollen hyphal cells, subglobose to slightly angular-irregular in outline, 2–6 μm diam, amphigenous, gregarious on both surfaces, denser on the abaxial surface, circular to irregular in shape, at first smaller and substomatal, later expanding or confluent and also immersed, giving rise to conidiophores. *Conidiophores* (10–)20–116(–136)×(3–)4–7(–8) μm, mostly hypophyllous, in small, loose to often large or even very large and dense fascicles, usually arising from a dark stroma, emerging through stomata or erumpent, septate, sympodial, moderately, strongly geniculate-sinuous, truncate apex, or sometimes obtusely rounded, unbranched, at first sub-hyaline to pale olivaceous, later brown, ranging from pale to medium olivaceous-brown to brown, wall smooth, thin, to about 0.5 μm, occasionally somewhat thicker near the base in older conidiophores, to about 1 μm; conidiogenous cells integrated, terminal or conidiophores reduced to conidiogenous cells, conidiogenous loci inconspicuous or subconspicuous by being somewhat refractive around the rim, in from view sometimes visible as minute circle, about 1.5–2 μm diam. *Conidia* solitary, subcylindrical, fusiform to obclavate, straight to somewhat curved, (19–) 24–38(–45)×(4–)5–7(–10) μm, 1–3-septate, subhyaline to pale brown or olivaceous-brown, smooth, thin-walled, apex obtuse and rounded, base short obconically truncate, (1.5–)2(–2.5) μm wide, hila unthickened, usually not darkened, but often refractive or slightly darkened-refractive.

Colonies slow growing (reaching 2 mm diameter after 15 days, and 5–6 mm after 30 days) on MEA (Malt Extract Agar) at 25 °C. Colonies with circular to irregular blackish margins, raising centrally, of dense cottony mycelium and hard texture. Young colonies dark, turning to greyish white or green after 1 month, reverse colony black to dark blackish brown. Despite trying different culture media types no sporulation of *P. mapelanensis* was observed in cultures.

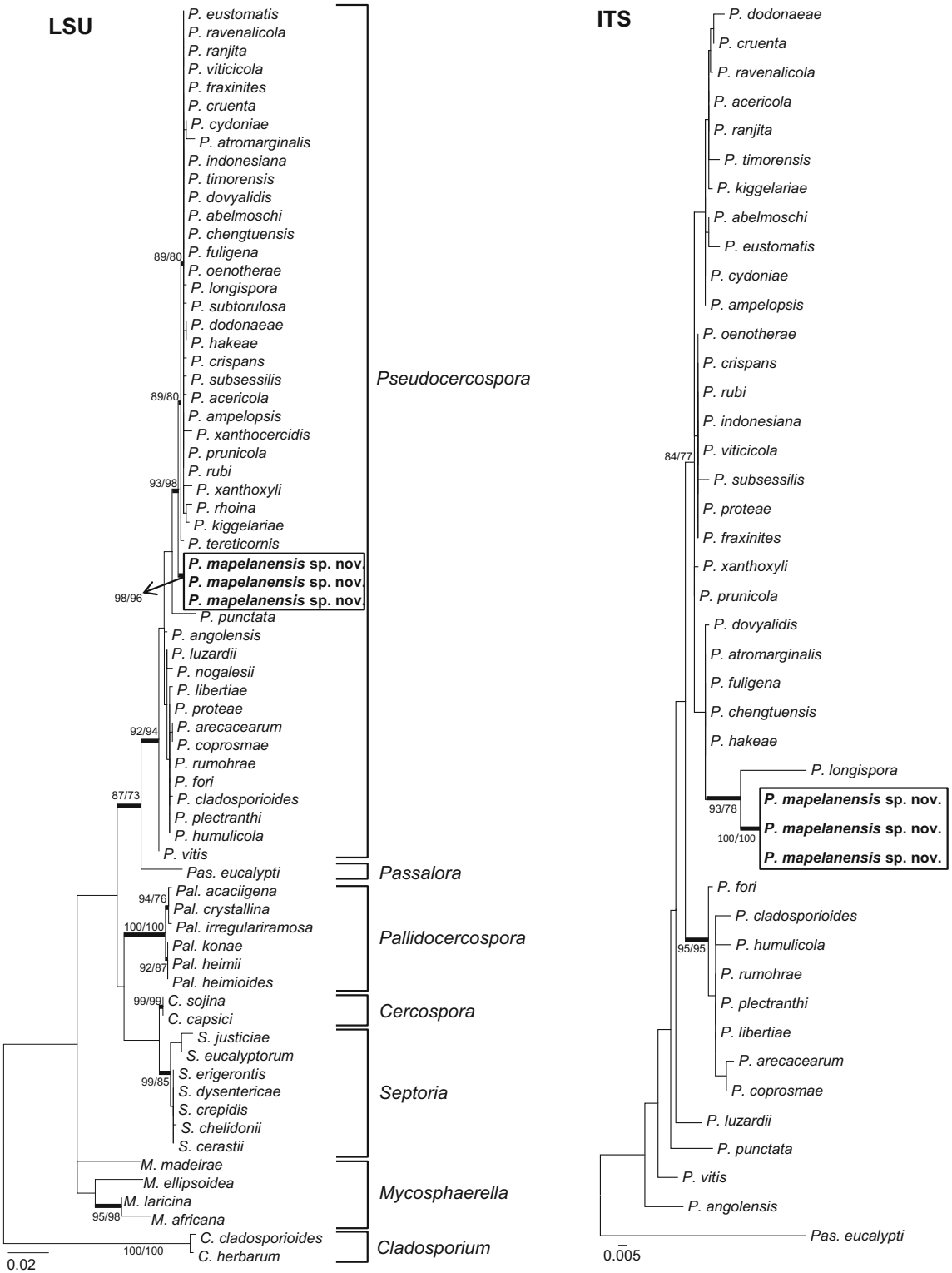

Fig. 3 Phylograms obtained from BI, ML and MP analyses of the LSU and ITS data sets. These analyses provide evidence of isolates obtained from *B. racemosa* grouping into the *Pseudocercospora* clade, indicated in the LSU tree. The ITS analyses indicate that isolates from *B. racemosa* represent an undescribed species, with *P. longispora* as the most closely related species to *P. mapelanensis* sp. nov., (BI) posterior probabilities ≥95 % are represented by thick branches. Bootstrap support values >70 % are indicated near the nodes as MP/ML. * = bootstrap support values <70 %

Etymology: Epithet refers to one of the areas in South Africa (Mapelane Nature Reserve) where the disease was first observed.

Habitat. Symptomatic fruits, floral sepals and leaves of *Barringtonia racemosa*.

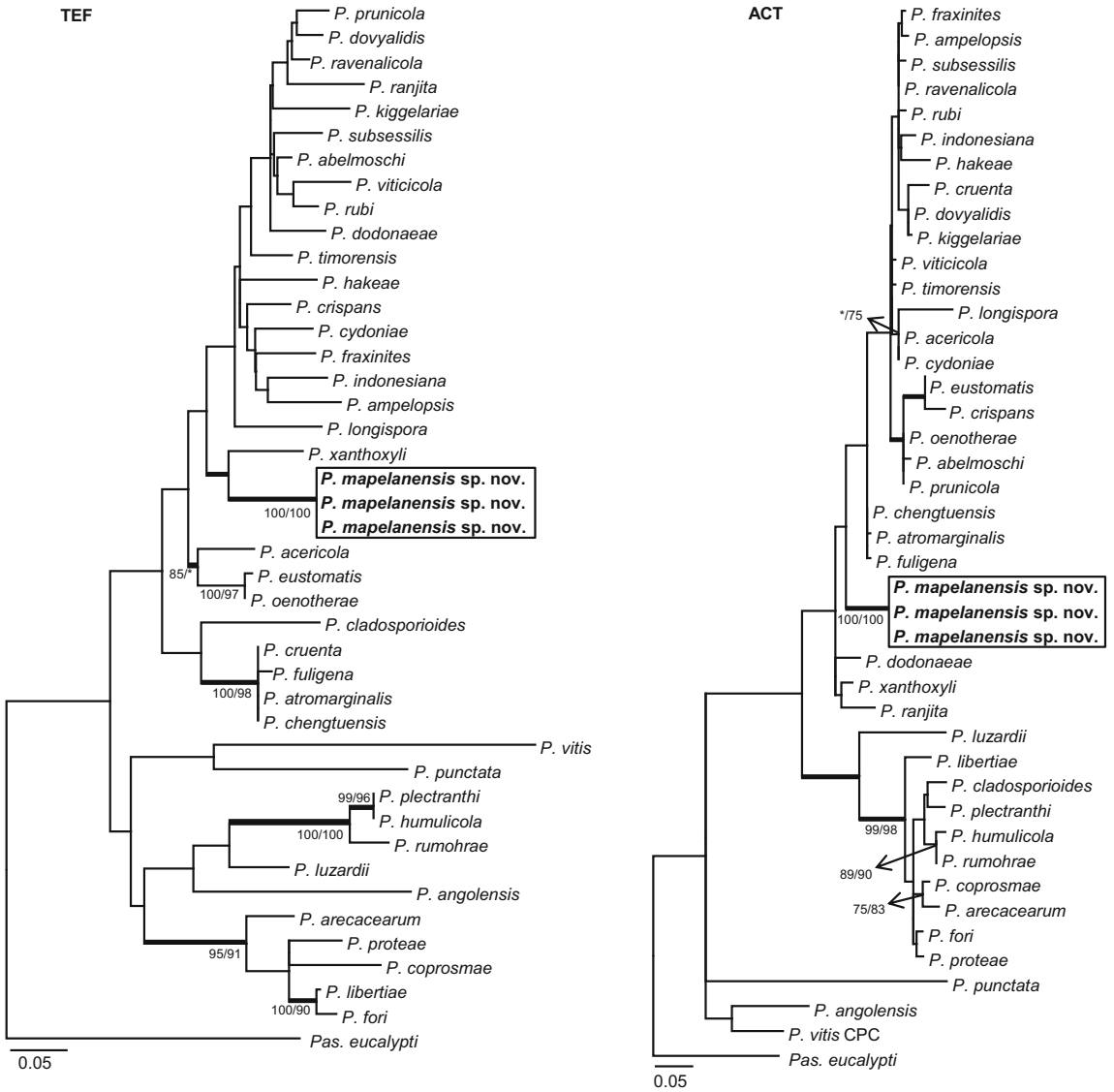

Fig. 4 Phylograms obtained from BI, ML and (MP) analyses of the TEF-1α and ACT genes. These analyses show *P. xanthoxyli* as the closest relative of *P. mapelanensis* in TEF analysis and *P. dodonaeae* as its closest relative in the ACT analysis. These analyses indicate that isolates from *B. racemosa* represent an undescribed species of *Pseudocercospora* (printed in *bold* and included in the *box*). (BI) posterior probabilities ≥95 % are represented by thick branches. Bootstrap support values >70 % are indicated near the nodes as MP/ML. * = bootstrap support values <70 %

Known distribution in South Africa: Kwazulu-Natal Province.

Specimens examined. SOUTH AFRICA, KWAZULU-NATAL PROVINCE: Mapelane Nature Reserve, from symptomatic fruits, flowers and leaves. In Herb. PREM (**Holotype** PREM 61109, Paratype PREM 61107, PREM 61108, col. J.A Osorio & Jol. Roux). Isotype HAL 2680F. Other cultures: CMW (Ex-type CMW 40581, Ex-Paratypes CMW 40579, CMW 40580). CBS (ex-type CBS 138923, Ex-Paratypes CBS 138922, CBS139544).

The following is a dichotomous key to *Pseudocercospora* species on *Barringtonia* species, adapted by Braun from the Cercosporoid hyphomycetes on *Barringtonia* spp. (Braun and Mouchacca 2000).

1. Stromata lacking or very small, only with a few substomatal cells; conidiophores in small, loose fascicles, 2–8, very long, to 185 μm; on *B. acutangulae* . . . . . *P. barringtoniae-acutangulae*

1* Stromata well-developed, about 10–100 μm diam. . . 2

2. Conidiophores to 200 μm long, pluriseptate; conidia long and narrow, filiform-acicular to narrowly obclavate-cylindrical, 20–90×1.5–5 μm, 2–8-septate, subhyaline to pale green-olivaceous; on *B. asiatica* . . *P. barringtoniigena*

2* Conidiophores shorter, usually <100 μm; conidia obclavate-subcylindrical, fusiform, shorter and broader, about 20–60×4–7(–10) μm 3

3. Conidiogenous loci and conidial hila unthickened, neither darkened nor refractive; conidia 30–60×4–6.5 μm, 3–6-septate; on *B. speciosa*..... *P. barringtoniicola*

3* Conidiogenous loci often subconspicuous, visible as minute circle in from view, conidial hila often conspicuous by being somewhat refractive or even darkened-refractive; conidia shorter, (19–)24–38×(4–)5–7(–10) μm, only 1–3-septate; on *B. racemosa*. . . . . .*P. mapelanensis*

Discussion

This study provides the first description of a disease of the mangrove associate, *B. racemosa* in South Africa. A fungus in the genus *Pseudocercospora* was consistently and intimately associated with early symptoms of the disease. It readily sporulated on green tissue and all indications are that this is the causal agent of the disease. The fungus was shown to represent a novel species for which the name *Pseudocercospora mapelanensis* has been provided.

Three species of *Pseudocercospora* have previously been described causing infections on *Barringtonia* species (Braun et al. 1999; Braun and Mouchacca 2000). These include *P. barringtoniae-acutangulae* U. Braun & Mouch., *P. barringtoniigena* U. Braun & Mouch., and *P. barringtoniicola* U. Braun & Mouch. Unfortunately neither cultures, sequences nor herbarium specimens are available for these species and they were not included in this study for comparative purposes. However, based on the published descriptions of the symptoms and micro-morphology, these species are all clearly different from *P. mapelanensis.*

Pseudocercospora barringtoniae-acutangulae was first described from *B. acutangulae* in India causing irregular to sub-circular small leaf spots and later becoming confluent to form large patches on the leaves (Braun and Mouchacca 2000). *Pseudocercospora barringtoniigena* was reported from *B. asiatica* in the South Pacific (Futuna) and it is characterized causing sub-circular and amphigenous leaf spots, and similar to *B. acutangulae* in causing large patches on the leaves when lesions become confluent (Braun and Mouchacca 2000). *Pseudocercospora barringtoniicola* was first described from *B. speciosa* in French Polynesia, Tahiti. This species forms large sub-circular to irregular spots on both sides of the leaves and the lesions become blackish during fungus fructification (Braun et al. 1999). Based on morphological descriptions provided for these fungi, they differ from the South African fungus in a number of characteristics (Table 2). Conidiophores and conidia of *P. barringtoniae-acutangulae* and *P. barringtoniigena* are larger than those of *P. mapelanensis.* Conidiophores of *P. barringtoniicola* are shorter, and conidia larger, than those of *P. mapelanensis.* Species previously described from *Barringtonia* also differ in the number of conidial septa, with *P. mapelanensis* having

Table 2 Comparison between described species of *Pseudocercospora* associated with *Avicennia marina* and *Barringtonia* species

	Pseudocercospora mapelanensis	*P. barringtoniae-acutangulae*	*P. barringtoniigena*	*P. barringtoniicola*	*P. avicenniae*
Host Species	*B. racemosa*	*B. acutangulae*	*B. asiatica*	*B. speciosa*	*Avicennia marina* (Acanthaceae)
Conidiophores (μm)	(10–)20–116(–136)×(3–)4–7(–8); pluriseptate	30–185×(3–)4–7; 2–6 septa	20–200×3–7; pluriseptate	10–50×3–6; 0–3 septa	20–90×3–5.5; NA
Conidia (μm)	(19–)24–38(–45)×(4–)5–7(–10); 1–3septa	20–70(–85)×4–4.5–7; 1–6septa	20–90×1.5–5; 2–8–septa	30–60×4–6.5; 3–6 septa	30–100×3–5; 3–12-septa
Region	South Africa (Mapelane and Richards Bay)	India (Uttar Pradesh, Gorakhpur)	South Pacific (Futuna)	French Polynesia, Tahiti, Rurutu	Australia Queensland
Reference	Current study	Braun and Mouchacca 2000	Braun and Mouchacca 2000	Braun et al. 1999	Shivas et al. 2009

between 1 and 3 septa while the other species have more than three transverse septa.

Sequence data for the *Pseudocercospora* species known to occur on *Barringtonia* are not available for comparison and the lack of cultures preclude this work. However, phylogenetic analyses showed that *P. mapelanensis* is most closely related to, but distinct from, *P. dodonaeae* Boesewinkel, *P. longispora* Arzanlou & Crous, and *P. xanthoxyli* (Cooke) Y.L. Guo & X.J. Liu. Based on multiple phenotypic characters such as conidial and conidiophores shape, dimensions and number of septa, these species can also be distinguished from *P. mapelanensis.*

It was not possible to conduct pathogenicity tests with *P. mapelanensis* in this study. This was due to the fact that the fungus does not sporulate in culture and the host trees are rare and could not be obtained for intensive study. This is consistent with many *Pseudocercospora* species and their *Mycosphaerella* sexual states that are highly host-specific and that are commonly treated as pathogens in the absence of pathogenicity tests (Crous and Braun 2003; Crous et al. 2004, 2008; Crous 2009). All indications from this study are that *P. mapelanensis* is the causal agent of the disease of *B. racemosa* and it was the only fungus found consistently on infected tissue. The disease of *B. racemosa* was often severe and although it did not cause defoliation, it appeared to result in significant levels of fruit abortion. It is probable that the pathogen is native on its host but this is a question that deserves further study.

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