A serious canker disease caused by *Immersiporthe knoxdaviesiana* gen. et sp. nov. (Cryphonectriaceae) on native *Rapanea melanophloeos* in South Africa

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Abstract

Recent disease surveys in the Western Cape province of South Africa have revealed a previously unknown and serious stem canker disease on native *Rapanea melanophloeos* (Myrsinaceae, Ericales) trees. Cankers commonly result in the death of branches or entire stems. Fruiting structures typical of fungi in the Cryphonectriaceae were observed on the surfaces of cankers. In this study, the fungus was identified and its pathogenicity to *R. melanophloeos* was tested. Multigene phylogenetic analyses based on DNA sequences of the partial LSU gene, ITS region of the nuclear ribosomal DNA gene and two regions of the β-tubulin (BT) gene, showed that the fungus represents a formerly undescribed genus and species in the Cryphonectriaceae. The fungus was also morphologically distinct from other genera in this family. Inoculation trials showed that the fungus described here as *Immersiporthe knoxdaviesiana* gen. et sp. nov. is an aggressive pathogen of *R. melanophloeos* trees.

Keywords:

canker pathogens; Cape beech; Myrsinaceae; tree death; Western Cape

Introduction:

The Cryphonectriaceae accommodates fungal genera in the *Cryphonectria–Endothia* complex and includes many important tree pathogens (Gryzenhout *et al.*, 2006a). Subsequent to the description of this family, several new genera and species were described and it currently accommodates 14 genera (Gryzenhout *et al.*, 2006b,c, 2009, 2010; Nakabonge *et al.*, 2006; Cheewangkoon *et al.*, 2009; Begoude *et al.*, 2010; Chen *et al.*, 2011; Vermeulen *et al.*, 2011). The family includes the notorious chestnut blight pathogen, *Cryphonectria parasitica* (Anagnostakis, 1987, 1992; Heiniger & Rigling, 1994), and species of *Chrysoporthe* that cause stem cankers and death of *Eucalyptus* trees (Wingfield, 2003; Gryzenhout *et al.*, 2009).

Five genera of Cryphonectriaceae have been reported from the African continent, including *Aurifilum* (Begoude *et al.*, 2010), *Celoporthe* (Nakabonge *et al.*, 2006), *Chrysoporthe* (Gryzenhout *et al.*, 2004, 2009), *Holocryphia* (Gryzenhout *et al.*, 2006b) and *Latruncellus* (Vermeulen *et al.*, 2011). *Aurifilum marmelostoma* is known only from Cameroon where it causes cankers on native *Terminalia ivorensis* (Combretaceae, Myrtales) and non-native

T. mantaly (Begoude *et al.*, 2010). *Celoporthe dispersa* is a pathogen of native *Heteropyxis canescens* (Heteropyxidaceae, Myrtales) and *Syzygium cordatum* (Myrtaceae, Myrtales) as well as non-native *Tibouchina granulosa* (Melastomataceae, Myrtales) in southern Africa (Nakabonge *et al.*, 2006; Vermeulen *et al.*, 2011). Five species of *Chrysoporthe*, *Chr. austroafricana*, *Chr. cubensis*, *Chr. deuterocubensis*, *Chr. syzygiicola* and *Chr. zambiensis* are known in Africa where they occur on trees in the Myrtales including native *Syzygium* spp. (Myrtaceae), introduced *Ti. granulosa* (Melastomataceae) and *Eucalyptus* spp. (Myrtaceae) (Gryzenhout *et al.*, 2009; Chungu *et al.*, 2010). In Africa, *Holocryphia eucalypti* is associated with cankers on non-native *Eucalyptus* trees in South Africa, Swaziland and Uganda (Van der Westhuizen *et al.*, 1993; Gryzenhout *et al.*, 2003; Roux & Nakabonge, 2010; Vermeulen *et al.*, 2011). *Latruncellus aurorae* was most recently described causing cankers on native *Galpinia transvaalica* (Lythraceae, Myrtales) in Swaziland (Vermeulen *et al.*, 2011).

Rapanea melanophloeos (Myrsinaceae, Ericales), commonly known as Cape beech, is a dense, evergreen tree (Van Wyk & Van Wyk, 1997). The natural range of this tree is from Cape Town in the south, to Zambia in the north in Southern Africa (Van Wyk & Van Wyk, 1997) and it is also a popular ornamental in gardens (Coates Palgrave, 1977; Van Wyk & Van Wyk, 1997). There are no reports of serious disease problems on *R. melanophloeos*.

During recent tree health surveys in the Western Cape province of South Africa, a serious stem disease was observed on *R. melanophloeos* in a botanical garden. Infections resulted in cankers on branches and stems of trees, often leading to tree death. Fruiting structures typical of fungi in the Cryphonectriaceae were observed on the surfaces of cankers. The aim of this study was to identify the causal agent of this disease based on phylogenetic analysis as well as morphological characteristics of the fungus, and by conducting pathogenicity tests on healthy *R. melanophloeos*.

Materials and methods

Disease symptoms, samples and isolations

Rapanea melanophloeos trees (Fig. 1a) growing in the Harold Porter National Botanical Garden (18°55′56″E and 34°20′99″S), Western Cape Province, South Africa were first observed to have serious cankers on their branches and stems in December 2009. Surveys were subsequently conducted in February and March of 2011 when the disease problem appeared to have increased considerably. Disease symptoms included dying branches (Fig. 1a), cracked bark (Fig. 1b), and cankers that often girdled infected stems and branches (Fig. 1c). Cankers commonly originated at branch attachment points on trees (Fig. 1c) and usually had orange cirrhi exuding from their surfaces (Fig. 1d,e). Branch and stem sections proximal to the cankers were typically dead or dying (Fig. 1a,b,c). The disease was widespread on *R. melanophloeos* trees throughout the garden.

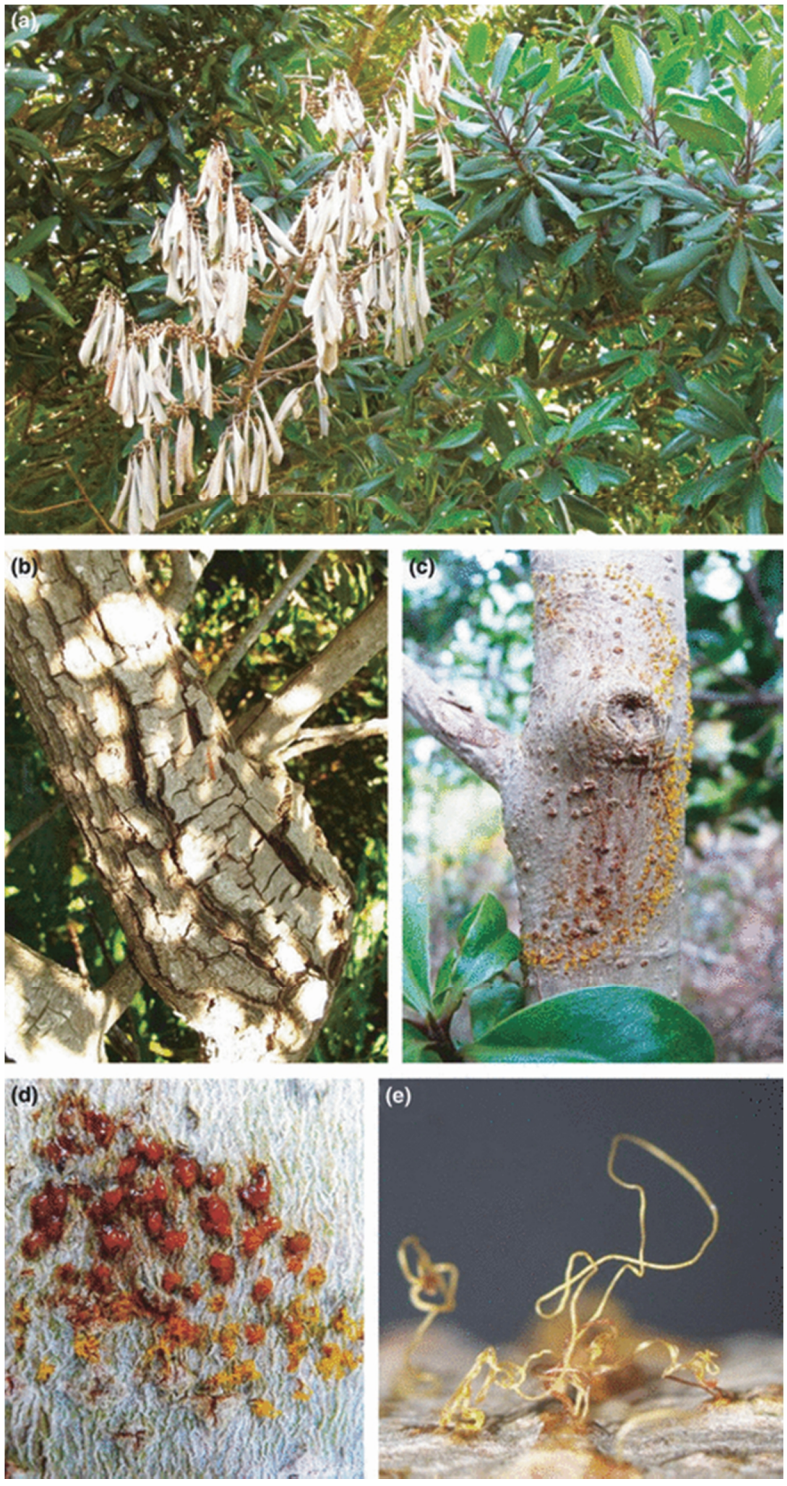

Figure 1. *Rapanea melanophloeos* and symptoms associated with infection by *Immersiporthe knoxdaviesiana*. (a) Native *R. melanophloeos* tree in the Western Cape province of South Africa, with dying branches caused by *I. knoxdaviesiana*; (b) bark of a *R. melanophloeos* tree showing cracks after infection by *I. knoxdaviesiana*; (c) typical stem canker and spores oozing from infected tissue; (d) fruiting structures with oozing spores on a stem lesion; (e) fruiting structures with oozing spores in the form of long golden-coloured cirrhi.

Sections of diseased bark containing fruiting structures were removed from trees and transported to the laboratory for morphological assessment and fungal isolation. Single tendrils of spores from fruiting bodies or spore masses from within fruiting bodies were transferred to 2% malt extract agar (MEA; Biolab, Merck; 20 g Biolab malt extract, 20 g Biolab agar, 1 L water) and incubated at 25°C in the dark. Single hyphal tips were transferred to 2% MEA to obtain pure cultures. One isolate was obtained from each of 33 diseased trees. Representative cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative isolates were also deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands (Table 1). Original bark specimens bearing fungal fruiting structures that could be connected to representative isolates were deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

DNA extraction, PCR and sequence reactions

Representative isolates, collected from different trees at different sites in the Garden (Table 1) were randomly selected and used for DNA sequence comparisons. Isolates were grown on 2% MEA at 25°C for 1 week prior to DNA extraction. Actively growing mycelium for each isolate was scraped from the surface of MEA media with a sterile scalpel and transferred to 1·5 mL Eppendorf tubes. DNA was extracted from mycelium using the methods described by Myburg *et al.* (1999). Samples were treated with 3 μL RNase (1 mg mL^{-1}) and left for 12 h at room temperature to degrade any RNA present. DNA was separated using electrophoresis on a 1% agarose gel, stained with GelRed (Biotium; 3 μL DNA extraction product with 2 μL GelRed), and visualized under UV light.

The conserved nuclear large subunit (LSU) ribosomal DNA, the β-tubulin gene regions 1 (BT1) and 2 (BT2) and the internal transcribed spacer (ITS) regions including the 5·8S gene of the ribosomal DNA operon were amplified and sequenced by methods described by Chen *et al.* (2011). Nucleotide sequences were edited in mega 4 (Tamura *et al.*, 2007). All sequences obtained in this study were deposited in GenBank (Table 1).

Phylogenetic analysis

To determine the phylogenetic placement of the *R. melanophloeos* isolates, nucleotide sequences of the LSU gene region, the 5·8S rDNA and the exon regions of the BT gene (including partial exon 4, exon 5, partial exon 6 and partial exon 7) of previously described genera/species in the Cryphonectriaceae (Gryzenhout *et al.*, 2009, 2010; Begoude *et al.*, 2010; Chen *et al.*, 2011; Vermeulen *et al.*, 2011) were compared to those of the isolates collected for the current study following methods of Chen *et al.* (2011). The aligned data sets of Chen *et al.* (2011) were used as templates for these analyses. *Togninia minima*, *To. fraxinopennsylvanica* and *Phaeoacremonium aleophilum* were used as out-groups for analysis using sequence data from the LSU gene region (Gryzenhout *et al.*, 2009; Chen *et al.*, 2011). Two isolates of *Diaporthe ambigua* were selected as out-groups for phylogenetic analyses of the 5·8S gene and BT exon regions (Chen *et al.*, 2011). Before performing combined analyses for the 5·8S gene and BT exon regions in paup* (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford, 2002), the

Table 1 Isolates sequenced and used in phylogenetic analyses and pathogenicity tests in this study

Identity	Isolate no.[a]	Other no.[a]	Host	Location	Collector	GenBank[c] LSU	GenBank ITS	GenBank BT1	GenBank BT2
Cryphonectria macrospora	CMW10914		*Castanopsis cuspidata*	Japan	T. Kobayashi	**JQ862749**	AY697942	AY697973	AY697974
C. decipiens	CMW10436	CBS165.30	*Quercus suber*	Portugal	B. d'Oliviera	**JQ862750**	AF452117	AF525703	AF525710
Holocryphia eucalypti	CMW7033	CBS115842	*Eucalyptus grandis*	KwaZulu/Natal, South Africa	M. Venter	**JQ862751**	**JQ862761**	**JQ862781**	**JQ862771**
H. eucalypti	CMW7035		*E. saligna*	KwaZulu/Natal, South Africa	M. Venter	**JQ862752**	**JQ862762**	**JQ862782**	**JQ862772**
H. eucalypti	CMW7037	CBS119477	*E. delegatensis*	New South Wales, Australia	K. Old	**JQ862753**	**JQ862763**	**JQ862783**	**JQ862773**
H. eucalypti	CMW7038		*E. globulus*	Western Australia, Australia	M.J. Wingfield	**JQ862754**	**JQ862764**	**JQ862784**	**JQ862774**
Immersiporthe knoxdaviesiana	CMW37314[b]	CBS132862	*Rapanea melanophloeos*	Western Cape, South Africa	M.J. Wingfield & J. Roux	**JQ862755**	**JQ862765**	**JQ862785**	**JQ862775**
I. knoxdaviesiana	CMW37315[b]	CBS132863	*R. melanophloeos*	Western Cape, South Africa	M.J. Wingfield & J. Roux	**JQ862756**	**JQ862766**	**JQ862786**	**JQ862776**
I. knoxdaviesiana	CMW37316[b]		*R. melanophloeos*	Western Cape, South Africa	M.J. Wingfield & J. Roux	**JQ862757**	**JQ862767**	**JQ862787**	**JQ862777**
I. knoxdaviesiana	CMW37317[b]		*R. melanophloeos*	Western Cape, South Africa	M.J. Wingfield & J. Roux	**JQ862758**	**JQ862768**	**JQ862788**	**JQ862778**
I. knoxdaviesiana	CMW37318	CBS132864	*R. melanophloeos*	Western Cape, South Africa	J. Roux, S.F. Chen & F. Roets	**JQ862759**	**JQ862769**	**JQ862789**	**JQ862779**
I. knoxdaviesiana	CMW37319		*R. melanophloeos*	Western Cape, South Africa	J. Roux, S.F. Chen & F. Roets	**JQ862760**	**JQ862770**	**JQ862790**	**JQ862780**

[a]Designation of isolates and culture collections: CMW = Tree Protection Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.

[b]Isolates used in field pathogenicity trials.

[c]GenBank accession numbers in bold indicate nucleotide sequences generated in this study.

partition homogeneity test (PHT), as implemented in paup, was used to determine whether conflict existed between the data sets (Farris *et al.*, 1995; Huelsenbeck *et al.*, 1996). All the data sets were aligned using the iterative refinement method (FFT-NS-i settings) of mafft v. 5.667 with the online version (Katoh *et al.*, 2002). The alignments were further edited manually in mega 4. All alignments were deposited in TreeBASE (http://www.treebase.org).

For each of the data sets, two different phylogenetic analyses were conducted. Maximum parsimony (MP) analyses were executed in paup and Bayesian inference was determined using the Markov chain Monte Carlo (MCMC) algorithm in MrBayes v. 3.1.2 (Ronquist & Huelsenbeck, 2003) following methods previously published by Chen *et al.* (2011).

Morphology

Only asexual structures were found on the bark of diseased *R. melanophloeos* trees. For morphological evaluations, fruiting structures were located using a dissection microscope, cut from the bark, boiled for 2 min in water and sectioned (12 μm thick) using a Leica CM1100 cryostat (Setpoint Technologies) at −20°C (Gryzenhout *et al.*, 2004). To examine the conidiophores, conidiogenous cells and conidia, fruiting structures were crushed on microscope slides with 85% lactic acid. Fifty measurements for each morphologically informative character were made for the specimen selected to represent a holotype, and 20 measurements were taken for the remaining specimens. Results are presented as (minimum–) (mean−standard deviation)−(mean + standard deviation) (–maximum). Measurements were made for fruiting structures representing the smallest and largest of the anamorphic stromata. Morphological characters of specimens obtained in this study were compared with those for other genera/species in the Cryphonectriaceae (Gryzenhout *et al.*, 2009, 2010; Begoude *et al.*, 2010; Chen *et al.*, 2011; Vermeulen *et al.*, 2011).

Six representative isolates (CMW37314–CMW37319) from six different *R. melanophloeos* trees were used for studies of culture characteristics. Optimal growth conditions for each culture were determined using the methods of Chen *et al.* (2011). The entire experiment was repeated once. For the characteristics of cultures and fruiting bodies, colour designations were determined using the colour charts of Rayner (1970).

Pathogenicity tests

Four isolates (CMW37314–CMW37317) were selected for field inoculations to fulfil Koch's postulates. Prior to inoculations, these isolates were grown on 2% MEA at 25°C for 1 week. Isolates were inoculated into the branches or stems (1–2 years old, approximately 2 cm diameter) of healthy *R. melanophloeos* trees in the Harold Porter National Botanical Garden. Wounds were made with a cork borer (7 mm diameter) to expose the cambium and discs of agar of similar size were removed from the edges of cultures covered with actively growing mycelium, and placed into the wounds with the mycelium facing the cambium. Sterile MEA was inoculated into wounds to serve as negative controls. Wounds were covered with masking tape to prevent desiccation and contamination. Ten stem/branches were inoculated for each of the four isolates and 10 stem/branches were treated as negative controls. The 50 inoculated stems/branches were distributed randomly in the Garden.

Stems/branches were inoculated on 10 March 2011 and results were evaluated 6 weeks later by measuring the lesion lengths in the cambium. Reisolations were made by cutting small pieces of wood from the lesion edges and placing these on 2% MEA at 25°C. Reisolations were made from all trees inoculated as controls and from four randomly selected trees per isolate. Results were analysed in excel (2003). Single factor analysis of variance (anova) was used to define the effects of fungal isolate on lesion length. To test the significance among means, *F*-values with $P < 0{\cdot}05$ were considered significantly different. The standard errors of means of lesion length for each fungal strain and control were calculated.

Results

Laboratory observations and isolations

Fruiting structures (conidiomata) taken from the surfaces of cankers were typical of those found in the Cryphonectriaceae. These were consistently found on all cankers and isolations resulting in pure cultures could easily be made from them. Isolates on MEA in culture were white to white yellow in colour, which is also typical of species in the Cryphonectriaceae (Gryzenhout *et al.*, 2009). Thirty-three isolates were obtained, each from a different tree.

Table 2 Statistics resulting from phylogenetic analyses

			Maximum parsimony					
Data set	No. of taxa	No. of bp[a]	PIC[b]	No. of trees	Tree length	CI[c]	RI[d]	HI[e]
LSU	64	627	123	100	281	0·534	0·813	0·466
5·8S rRNA/exons of BT1,2	62	760	114 (5·8S: 6; BT: 108)	1	246	0·565	0·865	0·435

	MrBayes				
Data set	Subst[f] model	Prset statefreqpr	NST[g]	Rates	Burn-in
LSU	GTR + I+ + G	Dirichlet(1,1,1,1)	6	Invgamma	100 000
5·8S rRNA/exons of BT1,2	GTR + I + G	Dirichlet(1,1,1,1)	6	Invgamma	100 000

[a]bp = base pairs.
[b]PIC = number of parsimony informative characters.
[c]CI = consistency index.
[d]RI = retention index.
[e]HI = homoplasy index.
[f]Subst model = best fit substitution model.
[g]NST = number of substitution rate categories.

Phylogenetic analyses

The aligned LSU nucleotide sequence data set consisted of 64 taxa and 627 characters (TreeBASE: http://purl.org/phylo/treebase/phylows/study/TB2:S12585). Statistical values of both maximum parsimony and Bayesian inference analyses are provided in Table 2. The internal positions of the fungal genera differed between the MP and Bayesian inference analyses, but tree topologies for the two analyses were similar. One hundred most parsimonious trees were obtained after MP analysis, of which the first tree was saved (Fig. 2). The results of the phylogenetic analyses of LSU gene nucleotide sequence data indicated that isolates collected from *R. melanophloeos* trees formed a single consistent phylogenetic lineage in the Cryphonectriaceae, distinct from other genera (Fig. 2).

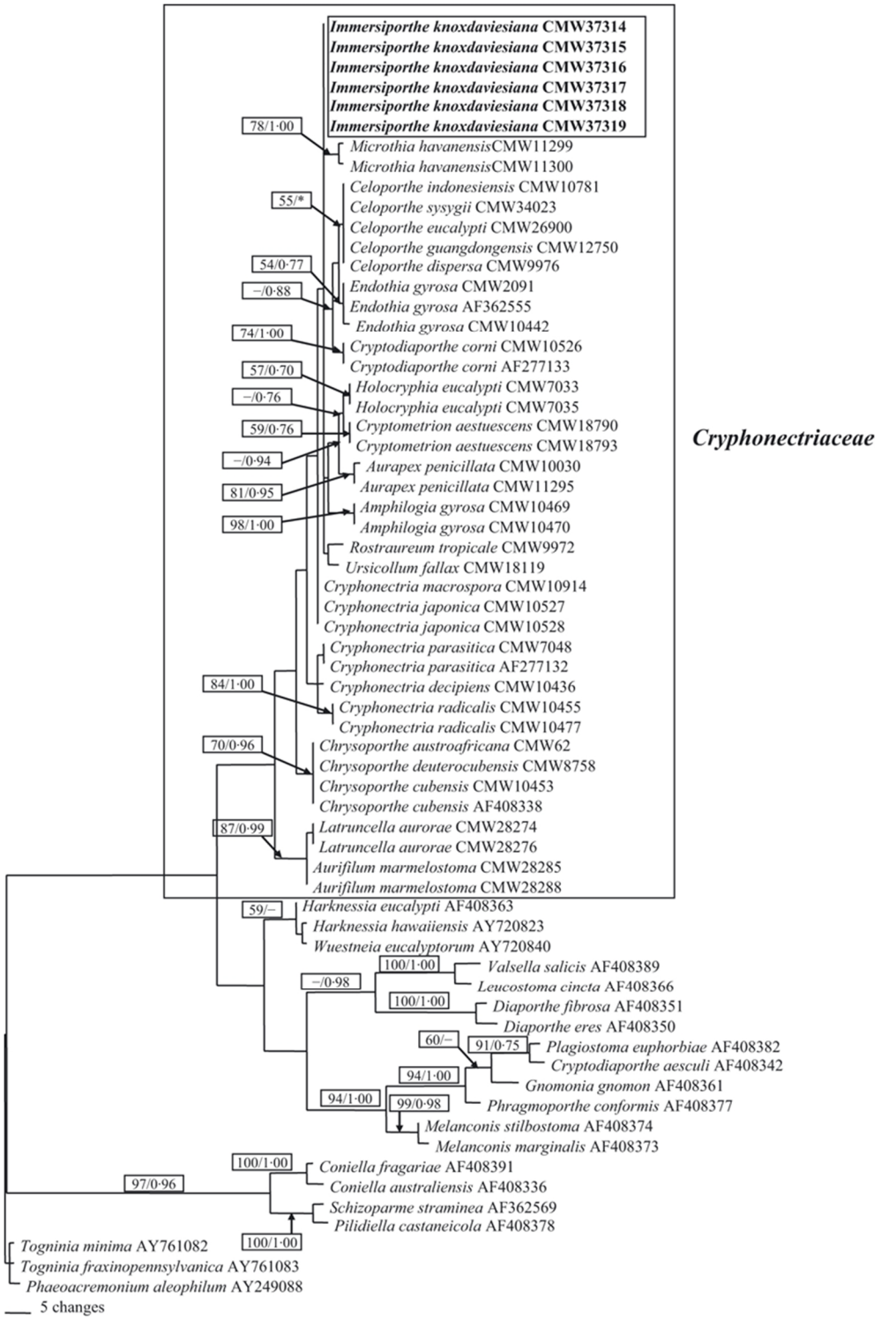

Figure 2. Cladogram based on maximum parsimony (MP) analysis of LSU DNA sequences for various genera in the Diaporthales. Bootstrap values >50% for MP and posterior probabilities >0·70 obtained from Bayesian analysis are presented at branches. Bootstrap values lower than 50%, and posterior probabilities lower than 0·70 are marked with an *, and absent analysis values are marked with –. Isolates from *Rapanea melanophloeos* are in bold.

The partition homogeneity test (PHT) for the aligned 5·8S rRNA gene and exons of the BT gene region data sets resulted in a value of $P = 0{\cdot}980$, indicating that the two data sets were congruent and could be combined for the phylogenetic analyses. The aligned sequences for the combined data sets (TreeBASE: http://purl.org/phylo/treebase/phylows/study/TB2:S12585) consisted of 62 taxa and 760 characters (Table 2). The position of genera in the Cryphonectriaceae differed slightly in relation to each other, depending on the specific phylogenetic analyses of MP and Bayesian inference, but the overall topologies were similar. Only one most parsimonious tree was retained (Fig. 3). Statistical results indicated that most of the genera of Cryphonectriaceae resided in different phylogenetic clades with high MP bootstrap support (BS) and/or high Bayesian posterior probabilities (PP; Fig. 3). Based on analysis of the combined data set, isolates from *R. melanophloeos* formed a strongly separated phylogenetic clade (MP bootstrap = 99%, Bayesian posterior probability = 1·00), distinct from all other genera in the Cryphonectriaceae (Fig. 3). The isolates from *R. melanophloeos* trees are most closely related to the genus *Microthia* (Fig. 3).

Morphology and taxonomy

Consistent with the results of the DNA sequence analyses, the fruiting structures of the unknown fungus from *R. melanophloeos* showed morphological characteristics typical of members of the Cryphonectriaceae. These comprised anamorphic fruiting structures with orange stromatic tissue (Gryzenhout *et al.*, 2006a, 2009) that turn purple in 3% KOH and yellow in lactic acid (Castlebury *et al.*, 2002) and the production of a pigment that coloured the agar yellow in culture (Gryzenhout *et al.*, 2009).

Comparisons between the anamorphic structures of the fungus from *Rapanea* and those of other species in the Cryphonectriaceae showed that they were very similar to those produced by *Microthia* and *Holocryphia*. These also produce orange pulvinate conidiomata, without conidiomatal necks and with paraphyses (Gryzenhout *et al.*, 2009, 2010; Begoude *et al.*, 2010; Vermeulen *et al.*, 2011). These genera can be distinguished from other members of the Cryphonectriaceae that produce orange conidiomata including *Amphilogia* (conical to pyriform conidiomata, paraphyses absent), *Cryptometrion*, *Cryphonectria* and *Endothia* (paraphyses absent), *Rostraureum* (clavate to rostrate conidiomata, conidiomatal necks present, paraphyses absent), *Ursicollum* (pyriform or rostrate conidiomata, conidiomatal necks present, paraphyses absent), *Aurifilum* (broadly convex conidiomata) and *Latruncellus* (conical with inflated neck conidiomata, conidiomatal necks present).

The unknown fungus from *Rapanea* could be distinguished from *Microthia* and *Holocryphia,* especially based on the size and position of conidiomata. The conidiomata of the *Rapanea* fungus (up to 1500 μm) were larger than those of *Microthia* and *Holocryphia* (smaller than 900 μm). The conidiomata of the unknown fungus were always immersed or semi-immersed in the bark of the host, while those of *Microthia* are usually superficial, or semi-immersed, and those of *Holocryphia* semi-immersed. These differences have previously been used to distinguish genera in the Cryphonectriaceae (Gryzenhout *et al.*, 2009; Vermeulen *et al.*, 2011). Conidia of the *Rapanea* fungus were also larger than those found in *Microthia* and *Holocryphia*.

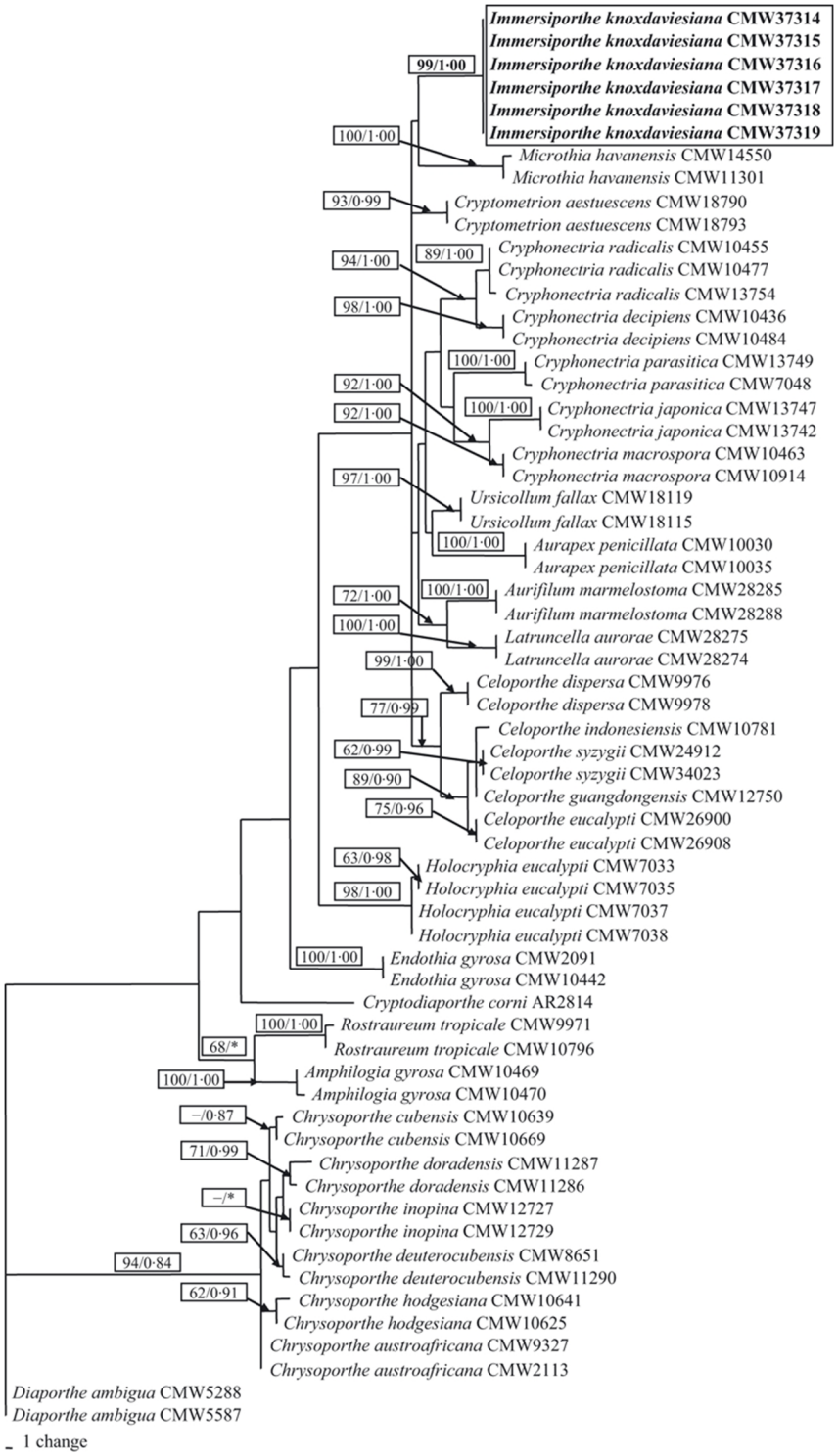

Figure 3. Cladogram based on maximum parsimony (MP) analysis of a combined DNA sequence data set of gene regions of the partial exon 4, exon 5, exon 6 and exon 7 of the BT genes, and the 5·8S rRNA gene region. Bootstrap values >50% for MP and posterior probabilities >0·70 obtained from Bayesian analysis are presented at branches. Bootstrap values lower than 50%, and posterior probabilities lower than 0·70 are marked with an *, and absent analysis values are marked with –. Isolates from *Rapanea melanophloeos* are in bold.

Based on the phylogenetic analyses for the LSU and 5·8S rRNA, exons in the BT gene regions, as well as the morphological characteristics, the fungus from *R. melanophloeos* clearly represents a previously undescribed genus and species in the Cryphonectriaceae. A new genus and a related species are described as follows:

- *Immersiporthe* S.F. Chen, M.J. Wingf., & Jol. Roux, gen. nov.

MycoBank No. MB564804

Etymology. Name is derived from the Latin word *immersus* describing the immersed conidiomata in the bark and *porthe* (destroyer), describing the pathogenic nature of the fungus.

Conidiomata pulvinate, immersed to semi-immersed, orange, uni- to multiloculate and convoluted, necks absent, stromatic tissue orange, pseudoparenchymatous at the edges, prosenchymatous in the centre. *Conidiophores* aseptate, occasionally with separating septa and branching, conidiogenous cells phialidic, cylindrical to flask-shaped with tapering apices. Long cylindrical cells, or *paraphyses*, occur between conidiophores. *Conidia* hyaline, cylindrical to fusoid, occasionally allantoid, aseptate, pushed through opening at stromatal surface as orange droplets or tendrils.

Notes. Only anamorphic structures were observed for *Immersiporthe*.

Typus: *Immersiporthe knoxdaviesiana* S.F. Chen, M.J. Wingf., & Jol. Roux, sp. nov.

- *Immersiporthe knoxdaviesiana* S.F. Chen, M.J. Wingf., & Jol. Roux, sp. nov.

MycoBank No. MB564866

Etymology. This species is named after Professor Peter Sidney Knox-Davies recognizing his great contributions to plant pathology, and the fact that he cherished the Harold Porter National Botanical Garden, where this fungus was first discovered, and where his remains have been scattered.

Conidiomata pulvinate (Fig. 4a), immersed (Fig. 4b) to semi-immersed (Fig. 4a), orange when young, umber to brown when mature, uni- to multiloculate and convoluted, necks absent, 120–320 μm high, 350–1500 μm diameter (Fig. 4a–c), locules 80–350 μm diameter (Fig. 4c), stromatic tissue pseudoparenchymatous at the edges, prosenchymatous in the centre (Fig. 4d). *Conidiophores* (5–)15–30(–61) μm long, occasionally with separating septa and branching, hyaline (Fig. 4f, g), conidiogenous cells 1·5–2 μm wide, cylindrical to flask-shaped with tapering apices, or not attenuated (Fig. 4g). Long cylindrical cells, seemingly sterile and *paraphyses* occurring between conidiophores, up to 150 μm long, branching occasionally into other sterile, cylindrical cells (Fig. 4e). *Conidia* (3·5–)5·0–5·5(–7·0) × 1–2 μm, hyaline, cylindrical, fusoid, occasionally allantoid, aseptate (Fig. 4h, i), exuded as orange droplets (Fig. 4b) or tendrils.

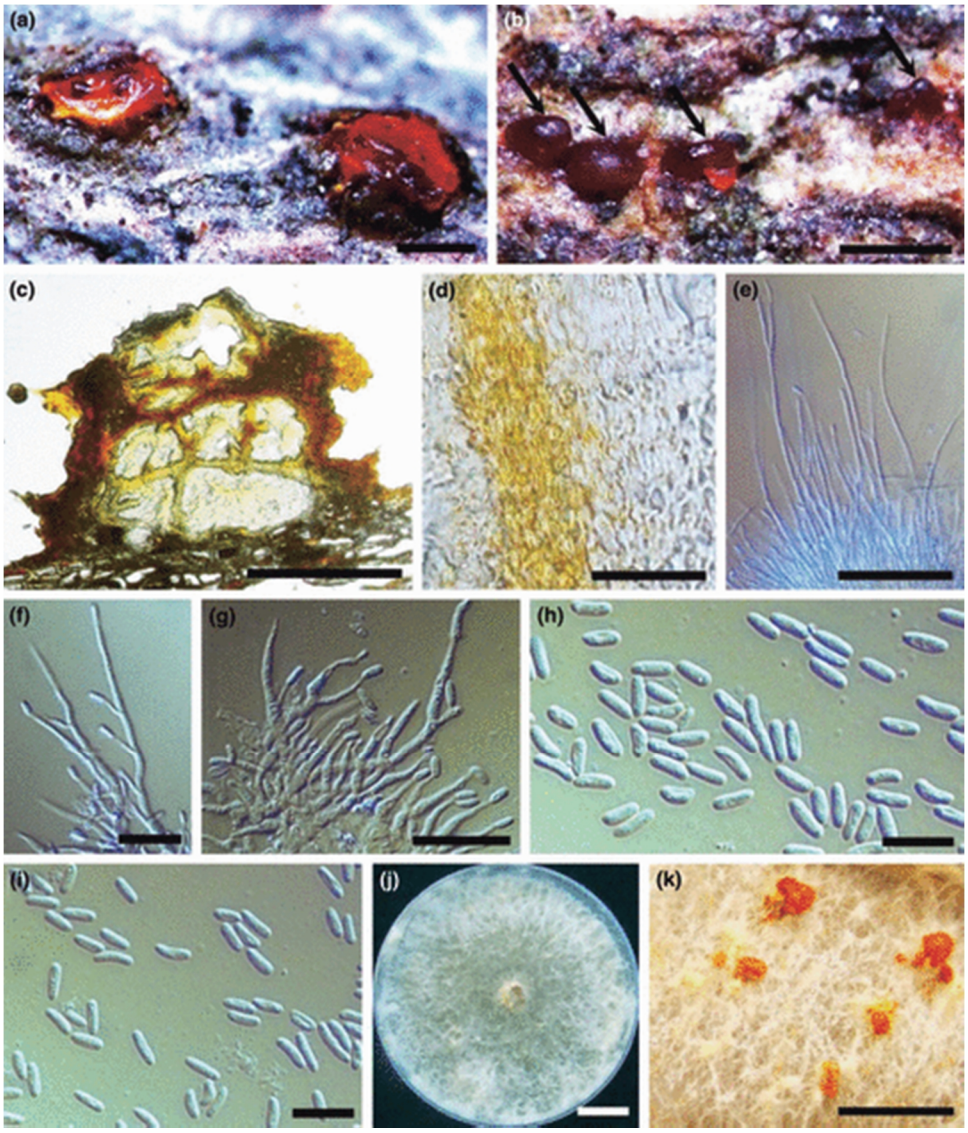

Figure 4. Fruiting structures of *Immersiporthe knoxdaviesiana* from *Rapanea melanophloeos* (specimen PREM60738). (a) Semi-immersed conidiomata on the bark; (b) immersed conidiomata in the bark (arrows indicate conidial spore mass); (c) longitudinal section through conidioma; (d) stromatic tissue of conidioma; (e) paraphyses; (f, g) conidiophores differing in length with conidigenous cells; (h, i) conidia differing in shape and size; (j) culture after 10 days of growth on MEA at 25°C; (k) asexual fruiting structures from primary isolations of the fungus. Bars: a = 100 μm; b, c = 200 μm; d = 20 μm; e = 50 μm; f, g = 20 μm; h, i = 10 μm; j, k = 10 mm.

Culture characteristics. On MEA *I. knoxdaviesiana* fluffy with an uneven margin, white when young, turning yellow white to sulphur yellow with yellow/sienna patches after 10 days (Fig. 4j). Colonies reverse white to yellow-white. Optimal growth temperature 25°C, covering the 90 mm plates after 7 days. No growth at 5°C and 35°C; colonies at 10°C reached 31·5 mm in 30 days (6 mm in 7 days). Asexual fruiting structures infrequently form in primary isolations of the fungus (Fig. 4k).

Teleomorph. Not observed.

Habitat. Causing cankers on stems and branches of *Rapanea melanophloeos*.

Hosts and distribution. Rapanea melanophloeos in the Harold Porter National Botanical Garden, Betty's Bay, Western Cape Province, South Africa.

Specimens examined. SOUTH AFRICA. Western Cape province, the Harold Porter National Botanical Garden (18°55′56″E and 34°20′99″S; 40–45 m a.s.l.), from bark of *Rapanea melanophloeos*. Feb., 2011, Michael J. Wingfield & Jolanda Roux, HOLOTYPE PREM60738, ex-type culture CMW37314 = CBS132862; Feb., 2011, Michael. J. Wingfield & Jolanda Roux, PARATYPE PREM60739, living culture CMW37315 = CBS132863; Mar., 2011, Jolanda Roux, ShuaiFei Chen & Francois Roets, PREM60740, living culture CMW37318 = CBS132864.

Notes. Immersiporthe knoxdaviesiana is morphologically most similar to *Microthia havanensis* and *Holocryphia eucalypti*, but can be distinguished from these fungi by the size of their conidia. The conidia of *I. knoxdaviesiana* (conidia medium, av. >5 μm, up to 7·0 μm) are longer than those of *M. havanensis* (conidia minute, av. <5 μm, up to 5·0 μm) and *Holocryphia* (conidia minute, av. <5 μm, up to 5·0 μm).

Pathogenicity tests

Inoculations on *R. melanophloeos* trees showed that *I. knoxdaviesiana* is an aggressive pathogen of these trees (Figs 5a,b & 6) and has the ability to kill inoculated stems within 6 weeks (Fig. 5a). Fruiting structures of *I. knoxdaviesiana* were produced on the bark of inoculated branches within 6 weeks (Fig. 5b). All four isolates of *I. knoxdaviesiana* produced well-developed lesions within 6 weeks (Figs 5a & 6), while the control inoculations using sterile agar were covered by callus after the same period (Fig. 5a). The mean comparison tests showed that the lesions produced by the *I. knoxdaviesiana* isolates were all significantly longer ($P < 0{\cdot}001$) than those of the controls (Fig. 6). The lesions produced by the four isolates were not significantly different from each other ($P = 0{\cdot}1354$; Fig. 6). All the inoculated fungi were reisolated from the lesions, but not from the control inoculations.

Culture characteristics. On MEA *I. knoxdaviesiana* fluffy with an uneven margin, white when young, turning yellow white to sulphur yellow with yellow/sienna patches after 10 days (Fig. 4j). Colonies reverse white to yellow-white. Optimal growth temperature 25°C, covering the 90 mm plates after 7 days. No growth at 5°C and 35°C; colonies at 10°C reached 31·5 mm in 30 days (6 mm in 7 days). Asexual fruiting structures infrequently form in primary isolations of the fungus (Fig. 4k).

Teleomorph. Not observed.

Habitat. Causing cankers on stems and branches of *Rapanea melanophloeos*.

Hosts and distribution. Rapanea melanophloeos in the Harold Porter National Botanical Garden, Betty's Bay, Western Cape Province, South Africa.

Figure 5. Symptoms after inoculations on *Rapanea melanophloeos* stems/branches with *Immersiporthe knoxdaviesiana*. (a) Negative control inoculation (thin grey arrow) showing wound and the absence of lesion development, and canker and lesions in the cambium (thin black arrow), and branch death (bold black arrow) caused by isolates of *I. knoxdaviesiana*; (b) canker with fruiting structures on the bark directly adjacent to the point of inoculation on branch. Bars: a, b = 10 mm.

Specimens examined. SOUTH AFRICA. Western Cape province, the Harold Porter National Botanical Garden (18°55′56″E and 34°20′99″S; 40–45 m a.s.l.), from bark of *Rapanea melanophloeos*. Feb., 2011, Michael J. Wingfield & Jolanda Roux, HOLOTYPE PREM60738, ex-type culture CMW37314 = CBS132862; Feb., 2011, Michael. J. Wingfield & Jolanda Roux, PARATYPE PREM60739, living culture CMW37315 = CBS132863; Mar., 2011, Jolanda Roux, ShuaiFei Chen & Francois Roets, PREM60740, living culture CMW37318 = CBS132864.

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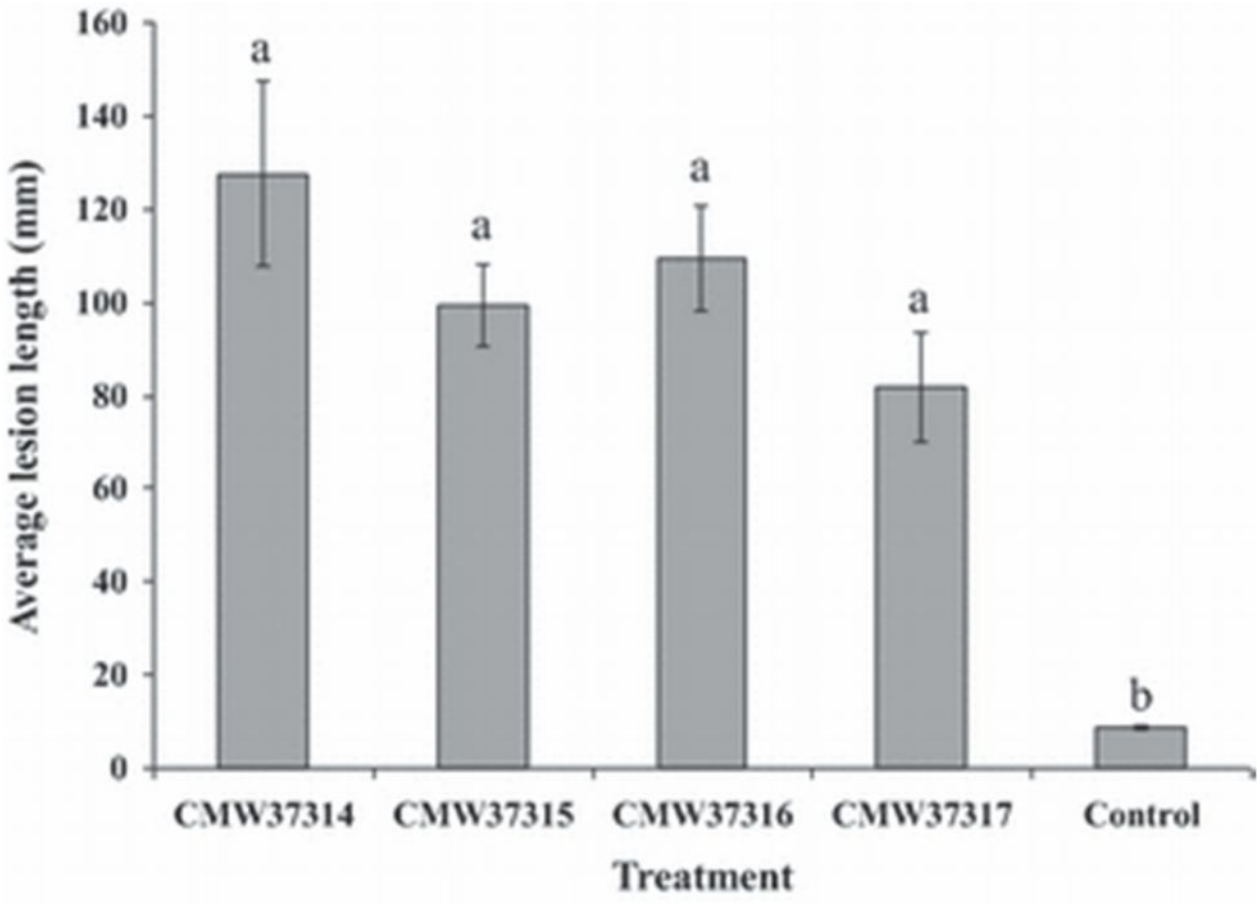

Figure 6. Bar graph showing the average lesion length (mm) resulting from inoculation trials with *Immersiporthe knoxdaviesiana* onto stems/branches of *Rapanea melanophloeos*. Vertical bars represent standard error of means. Different letters above the bars indicate treatments that were significantly different ($P = 0{\cdot}05$).

Fruiting structures of *I. knoxdaviesiana* were produced on the bark of inoculated branches within 6 weeks (Fig. 5b). All four isolates of *I. knoxdaviesiana* produced well-developed lesions within 6 weeks (Figs 5a & 6), while the control inoculations using sterile agar were covered by callus after the same period (Fig. 5a). The mean comparison tests showed that the lesions produced by the *I. knoxdaviesiana* isolates were all significantly longer ($P < 0{\cdot}001$) than those of the controls (Fig. 6). The lesions produced by the four isolates were not significantly different from each other ($P = 0{\cdot}1354$; Fig. 6). All the inoculated fungi were reisolated from the lesions, but not from the control inoculations.

Discussion

In this study, a new and serious stem canker disease of native *R. melanophloeos* trees was discovered in the Harold Porter National Botanical Garden in the Western Cape province of South Africa. This disease was shown to be caused by a previously unknown fungus in the Cryphonectriaceae and is described in the new genus *Immersiporthe* as *I. knoxdaviesiana*. The identification of this fungus was supported by phylogenetic analyses as well as morphological characteristics. Inoculation tests showed that *I. knoxdaviesiana* is a virulent pathogen on *R. melanophloeos* and is able to kill inoculated stems and branches in a relatively short period of time.

The canker disease on *R. melanophloeos* is aggressive and severely damaging. It had not been observed in regular visits to the Harold Porter National Botanical Garden during the course of the past two decades (M. Wingfield, FABI, University of Pretoria, personal communication) and it appears to have emerged relatively recently. The origin of the pathogen is unknown and the disease has not been found in other areas where *R. melanophloeos* grows. All indications are that this is a new and serious disease problem, potentially caused by an introduced pathogen that might threaten an important native tree in southern Africa. In this regard, its recent appearance might be analogous to the first observation of the chestnut blight pathogen in the New York Botanical Garden in 1906 (Merkel, 1906).

Only asexual anamorphic structures were found for *I. knoxdaviesiana*. However, these, together with phylogenetic data, were sufficient to clearly show the unique nature of the fungus on *R. melanophloeos*. The characteristics of anamorphic structures have previously been used to distinguish different genera in the Cryphonectriaceae (Gryzenhout *et al.*, 2006b,c, 2009; Vermeulen *et al.*, 2011). These, for example, include *Aurapex* and *Ursicollum*, the two other genera of Cryphonectriaceae for which a teleomorph is not known. In this study, *Immersiporthe* could be distinguished relatively easily from other morphologically similar genera of Cryphonectriaceae by the colour, size and position of conidiomata.

Immersiporthe knoxdaviesiana is the fifth species in the Cryphonectriaceae that has been found in southern Africa and the fourth in South Africa. Other species include *Celoporthe dispersa*, *Chrysoporthe austroafricana, Holocryphia eucalypti* and *Latruncellus aurorae*. All of these are associated with canker diseases on trees from which they were identified (Van der Westhuizen *et al.*, 1993; Gryzenhout *et al.*, 2003, 2009; Nakabonge *et al.*, 2006; Vermeulen *et al.*, 2011), and

Chr. austroafricana (Van der Westhuizen *et al.*, 1993; Gryzenhout *et al.*, 2003, 2009; Nakabonge *et al.*, 2006) is amongst the most serious tree pathogens known in Africa (Wingfield *et al.*, 1989; Wingfield, 2003; Gryzenhout *et al.*, 2009). In this regard, *I. knoxdaviesiana* described in this study has a similar biology and level of importance.

Immersiporthe knoxdaviesiana and *Chr. austroafricana* both cause serious canker diseases on their hosts, but they also differ in various respects. *Chrysoporthe austroafricana* is found on the branches and stems of native *Syzygium cordatum* in South Africa (Heath *et al.*, 2006), but apart from where trees are severely stressed, there is no evidence that it causes serious damage to these trees. In contrast, there is good evidence to show that it has undergone a host shift to non-native *Eucalyptus* spp. and *Tibouchina* spp. (Slippers *et al.*, 2005; Gryzenhout *et al.*, 2009) where it causes serious damage and can kill trees (Wingfield *et al.*, 1989; Conradie *et al.*, 1990; Myburg *et al.*, 2002; Gryzenhout *et al.*, 2009). *Immersiporthe knoxdaviesiana* occurs on the stems and small branches of *R. melanophloeos*, a tree native to South Africa, and the damage that it causes is dramatic and suggestive of an introduced pathogen. Population biology studies on the pathogen will clearly be needed to clarify this.

The first appearance of a serious new pathogen in a botanical garden is not unique. These are areas where plants are commonly introduced from other areas, thereby increasing opportunities for new disease introductions and host shifts (Slippers *et al.*, 2005). Following this reasoning, botanical gardens are increasingly being seen as important sites for the early detection of new tree pathogens. In this regard, the discovery of *I. knoxdaviesiana* could represent an example of an important new disease that has emerged for the first time in a botanical garden, and surveys of this tree and its relatives in other areas should be intensified to better understand its relative importance.

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