DNA-based identification of *Quambalaria pitereka* causing severe leaf blight of *Corymbia citriodora* in China

XuDong Zhou[1,3*], Z.Wilhelm de Beer[2], YaoJian Xie[3], Geoff S. Pegg[4] and Michael J. Wingfield[1]

[1]Tree Protection Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI);
[2]Department of Microbiology and Plant Pathology; University of Pretoria, South Africa;
[3]China Eucalypt Research Centre, Chinese Academy of Forestry, P. R. China;
[4]Department of Primary Industries and Fisheries, Horticulture and Forestry Science, Indooroopilly, Brisbane, Australia.

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Quambalaria spp. include serious plant pathogens, causing leaf and shoot blight of *Corymbia* and *Eucalyptus* spp. In this study, a disease resembling *Quambalaria* leaf blight was observed on young *Corymbia citriodora* trees in a plantation in the Guangdong Province of China. Comparisons of rDNA sequence data showed that the causal agent of the disease is *Q. pitereka*. This study provides the first report of *Quambalaria* leaf blight from China, and it is also the first time that this pathogen has been found on trees outside the native range of Eucalypts.

Keywords: disease spread, plantation, sustainability

Introduction

The Ustilaginomycetes ('true smut fungi') are primarily known as parasites of vascular plants. The majority of the approximately 1500 smut species infect angiosperms, and most are parasites of monocots (Bauer *et al*., 1997). The *Exobasidiales* and *Microstromatales* differ from the other eight orders of the Ustilaginomycetes by their lack of teliospores and also their host preference. Most species in these two orders occur on woody bushes or trees, while by far the majority of other ustilaginomycete species parasitize non-woody herbs (Bauer *et al*., 1998). Consistent with this phylogeny, *Quambalaria*, established by Simpson (2000) for leaf pathogens of *Eucalyptus* and *Corymbia* trees (Eucalypts), was recently assigned to the *Microstromatales* (De Beer *et al*., 2006).

*Corresponding author: XuDong Zhou; e-mail: davidii_zhou@hotmail.com

Quambalaria comprises three valid species known to occur on eucalypts. These are *Q. cyanescens* (de Hoog & G.A. de Vries) Z.W. de Beer, Begerow & R. Bauer, *Q. pitereka* (J. Walker & Bertus) J.A. Simpson, and *Q. eucalypti* (M.J. Wingf., Crous & W.J. Swart) J.A. Simpson (De Beer *et al*., 2006). The taxonomic status of a fourth species, *Q. pusilla* (U. Braun & Crous) J.A. Simpson, known only by a single report from *Eucalyptus* leaves in Thailand (Braun, 1998), remains uncertain (De Beer *et al*., 2006).

Quambalaria cyanescens has been isolated from both *Eucalyptus pauciflora* and human skin (De Hoog and De Vries, 1973). While it has also been identified from tissue samples in immunocompromised patients, it is regarded as an opportunist rather than a primary pathogen (Sigler and Verweij, 2003). Apart from the single isolate from *E. pauciflora* in Australia, *Q. cyanescens* has not been reported from plant material. Its status as tree pathogen is thus unresolved.

Quambalaria pitereka and *Q. eucalypti* are well described tree pathogens, causing leaf and shoot blight on various *Corymbia* and *Eucalyptus* species (Simpson, 2000; Roux *et al*., 2006). In Australia, *Q. pitereka* causes significant damage to newly established *Corymbia* plantations in Queensland and New South Wales (Simpson, 2000; Pegg *et al*., 2005). This pathogen has not been reported from other hosts or from outside Australia. *Quambalaria eucalypti* leads to extensive shoot and leaf die-back, as well as stem cankers on young *E. grandis* and *E. nitens* trees in South Africa (Wingfield *et al*., 1993; Roux *et al*., 2006). In Brazil it causes stem girdling on seedlings and leaf and shoot blight on *Eucalyptus* hedge plants in clonal gardens (Alfenas *et al*., 2001), and in Uruguay it is associated with twig lesions of *E. globulus* (Bettucci *et al*., 1999). The host range of *Q. eucalypti* appears to be restricted to *Eucalyptus* spp. It is thus surprising that this species has been observed only on non-native *Eucalyptus* in plantations on other continents, and not on native *Eucalyptus* in Australia.

Apart from the blight symptoms on leaves and stems, *Quambalaria*-infections are characterized by the occurrence of powdery white fungal spore masses on the lesions (Wingfield *et al*., 1993). In June 2006, a disease resembling *Quambalaria* leaf blight was observed on *C. citriodora* trees grown commercially in Guangdong Province of China, where more than 7 ha of plantations have been severely damaged. The aim of this study was to identify the causal agent of the disease using rDNA sequence data.

Materials and Methods

Field sampling and fungal isolations

Disease symptoms were observed in a young *C. citriodora* plantation near LeiZhou in Guangdong Province. Infected leaf samples with lesions covered with powdery white fungal spore masses were collected, placed in paper bags and transported to the laboratory in order to make isolations.

Isolations were made by scraping spore masses from the lesions on the leaf surfaces and transferring them to 2% MEA (20 g Biolab malt extract, 20 g Biolab agar, and 1000 mL deionised water). Plates were incubated at 25ºC and cultures purified. All cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, the plant pathology herbarium (BRIP) of the Queensland Department of Primary Industries and Fisheries, and the plant pathology herbarium (DAR) of the New South Wales Department of Agriculture, Australia.

DNA sequencing and phylogenetic analyses

Four single hyphal-tip cultures (CMW23610, CMW23611, CMW23612, and CMW23613) isolated from infected leaf material in China were selected for sequencing (Table 1). For comparative purposes, six *Q. pitereka* isolates from Australia, as well as the holotype of the species, were also included in the study (Table 1). DNA was extracted using PrepMan Ultra Sample reagent (Applied Biosystems) following the manufacturer's protocol. The ITS (internal transcribed spacer) region of the ribosomal RNA operon was amplified using primers ITS1-F (Gardes and Bruns, 1993) and ITS4 (White *et al*., 1990). PCR products were sequenced with the same primers. Conditions for PCR amplification and sequencing reactions were as described by Zhou *et al*. (2004). For phylogenetic analyses, ITS sequences of closely related taxa from previous studies (Table 2), were obtained from GenBank (http://www.ncbi.nlm.nih.gov/).

All sequences were aligned with the online version of MAFFT v. 5.667 (Katoh *et al*., 2002), using the iterative refinement method (FFT-NS-I settings). Phylogenetic analyses were conducted in MEGA3 (Kumar *et al*., 2004). Neighbor-joining analyses were done with the Kimura 2-parameter switched on. In addition, Maximum Parsimony analyses were done using 1000 replicates for bootstrapping. Trees were rooted against sequence data for an isolate of *Volvocisporium triumfetticola* (Table 2).

Table 1. *Quambalaria pitereka* isolates sequenced in this study.

Isolate/ Herbarium no.	GenBank no. (ITS)	Host	Origin	Collector
[a]BRIP48325	EF427366	*Corymbia citriodora* subsp. *variegata*	QLD, Australia	G Pegg
BRIP48361	EF427367	*C. citriodora* subsp. *variegata*	QLD, Australia	G Pegg
BRIP48370	EF427368	*C. torelliana* x *citriodora hybrid*	QLD, Australia	G Pegg
BRIP48384	EF427369	*C. citriodora* subsp. *variegata*	QLD, Australia	G Pegg
BRIP48386	EF427370	*C. citriodora* subsp. *variegata*	QLD, Australia	G Pegg
BRIP48531	EF427371	*C. citriodora* subsp. *variegata*	QLD, Australia	G Pegg
[b]CMW23610	EF427372	*C. citriodora*	Guangdong, China	YJ Xie
CMW23611	EF427373	*C. citriodora*	Guangdong, China	YJ Xie
CMW23612	EF427374	*C. citriodora*	Guangdong, China	YJ Xie
CMW23613	EF427375	*C. citriodora*	Guangdong, China	YJ Xie
[c]DAR19773 [T]	EF427376	*C. eximia*	NSW, Australia	AL Bertus, J Walker

[a]BRIP the plant pathology herbarium for Queensland Department of Primary Industries and Fisheries, Australia.
[b]CMW the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), Pretoria, South Africa.
[c]DAR the plant pathology herbarium for the Department of Agriculture in NSW, Australia.
[T]Holotype for *Ramularia pitereka* J. Walker & Bertus [= *Q. pitereka*] (Walker and Bertus, 1971)

Results

Disease description and fungal isolates obtained

Quambalaria leaf blight on *C. citriodora* in LeiZhou was characterised by the formation of white lesions only on leaf surfaces (Figs 1A, B). The fungus sporulated on abaxial and adaxial leaf surface, and spores covered the entire area of the lesions. Infected plants were accompanied by damage caused by the leaf beetle (Coleoptera: Scarabaeidae) identified as *Anomala cupripes* Hope (Figs 1C, D), but lesions were not associated with insect wounds. In

Table 2. Isolates of selected species used for comparative purpose in this study.

Species	GenBank no. (ITS)	Isolation/ Herbarium no.	Host	Origin	Collector
Microstroma album	DQ317624	[a]RB2072	*Quercus robur*	Germany	R Bauer
M. juglandis	DQ317632	[b]F3381	*Juglans regia*	Germany	M Göker
	DQ317633	RB2054	*J. regia*	Germany	R Bauer
	DQ317634	RB2024	*J. regia*	Germany	R Bauer
Quambalaria cyanescens	DQ317622	[c]CBS357.73 [T]	*Skin of man*	Netherlands	TF Visser
	DQ317623	CBS876.73	*Eucalyptus pauciflora*	New South Wales, Australia	MJ Wingfield
Q. eucalypti	DQ317609	CBS118615	*E. nitens*	Rooihoogte, South Africa	ZL Mthalane, J Roux
	DQ317610	[d]CMW17253	*E. nitens*	Rooihoogte, South Africa	ZL Mthalane, J Roux
	DQ317611	CMW17254	*E. nitens*	Rooihoogte, South Africa	ZL Mthalane, J Roux
	DQ317612	CMW17255	*E. nitens*	Rooihoogte, South Africa	ZL Mthalane, J Roux
	DQ317613	CBS118616	*E. grandis* clone	Kwambonambi, South Africa	J Roux
	DQ317614	CMW14329	*E. grandis x E. camaldulensis*	Kwambonambi, South Africa	J Roux
	DQ317625	CBS118844 [T]	*Eucalyptus grandis*	Kwambonambi, South Africa	MJ Wingfield
	DQ317626	CBS119680	*E. grandis*	Kwambonambi, South Africa	L Lombard
Q. pitereka	DQ317627	CMW6707	*Corymbia maculata*	New South Wales, Australia	MJ Wingfield
	DQ317628	CBS118828	*Corymbia citriodora subsp. variegata*	Queensland, Australia	M Ivory
Rhodutorula bacarum	DQ317629	CBS6526 [T]	*Ribes nigrum*	UK	RWM Buhagiar
R. hinnulea	AB038130	CBS8079 [T]	*Banksia collina*	Australia	RG Shivas
R. phylloplana	DQ317630	CBS8073 [T]	*B. collina*	Australia	RG Shivas
Sympodiomycopsis paphiopedili	DQ317631	CBS7429 [T]	Nectar of *Paphiopedilum primurinum*	Japan	K Tokuoka
V. triumfetticola	DQ317637	RB2070[T]	*Triumfetta rhomboidea*	India	MS Patil

[a] RB Herbarium Robert Bauer, Tübingen, Germany.
[b] F Culture Collection, Tübingen, Germany.
[c] CBS the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.
[T] ex-holotype culture.
[d] CMW the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), Pretoria, South Africa.

total, 12 isolates morphologically resembling a species of *Quambalaria* were obtained.

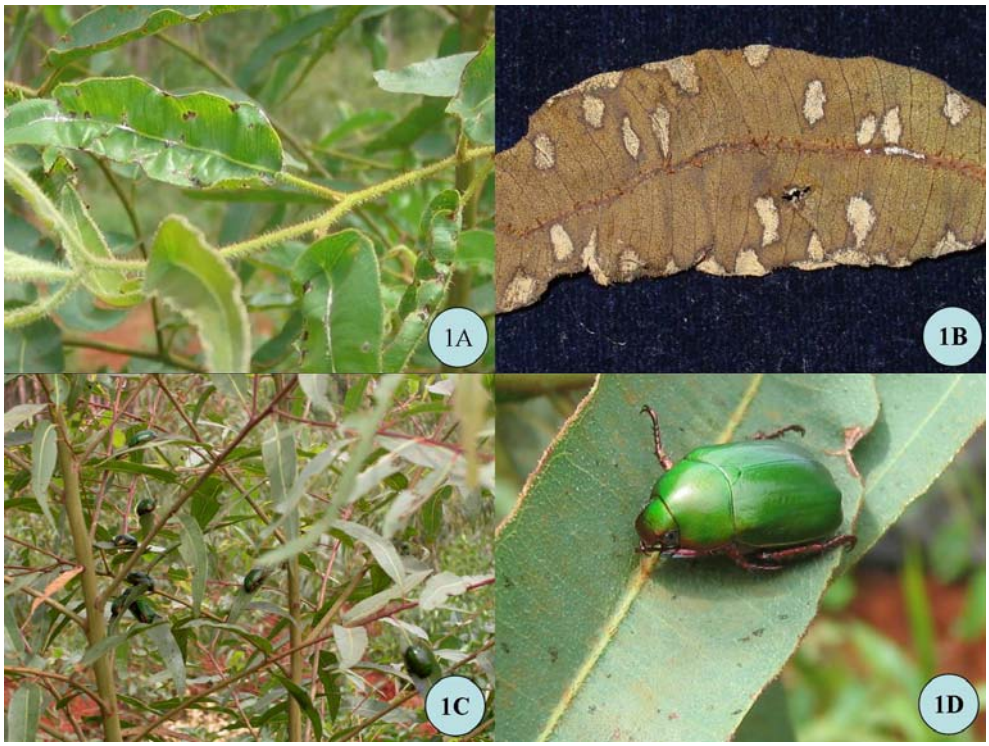

Fig. 1. Symptoms of *Q. pitereka* infection on *Corymbia citriodora*. **(A, B)** leaf spots with white fungal spore masses, **(C, D)** *Quambalaria*-infected plants with the presence of *Anomala cupripes*.

DNA Sequence analyses

PCR of the ITS regions for the four isolates from China, six from Australia and the holotype of *Q. pitereka* produced fragments of 607 bp in size. Phylogenetic analyses (Fig. 2) showed that the DNA sequences of the four isolates from China were identical to each other and to those of two isolates from *C. citriodora* subsp. *variegata* in Queensland (BRIP48384 and BRIP48531). These two Australian isolates as well as the China isolates formed a larger group that also included the type specimen of *Q. pitereka* from *C. eximia* in New South Wales, and other *Q. pitereka* isolates from *C. citriodora, C. maculata,* and a *C. torelliana x C. citriodora* hybrid, all from Queensland (Fig. 2). Sequences of *Q. eucalypti* and *Q. cyanescens* formed two well-supported groups, clearly distinct from *Q. pitereka*.

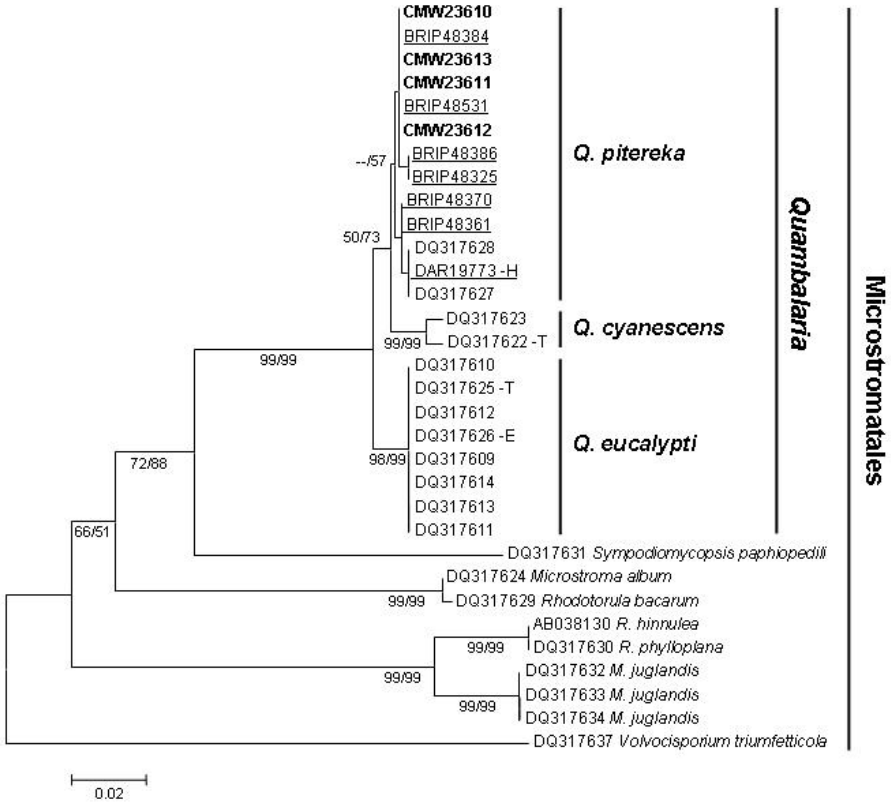

Fig. 2. NJ tree obtained from ITS sequence data of *Q. pitereka* isolates from Australia (underlined) and China (bold type). Bootstrap values at nodes are for 1000 replicates (Maximum Parsimony/ Neigbor-Joining). Sequences obtained in this study are referred to as isolate numbers (Table 1), and published sequences are referred to by GenBank accession numbers (Table 2). H = holotype, T = ex-type culture, E = ex-epitype culture.

Discussion

Eucalypts (including *Eucalyptus* and *Corymbia*) have been successfully established in plantations in China during the course of the past twenty years and these are rapidly expanding (Xie, 2003). However, very little is known about diseases of these trees in China (Ran, 2002; Cortinas *et al*., 2006; Burgess *et al*., 2006, 2007). This study provides the first report of *Quambalaria* leaf blight on *C. citriodora* in China and we have confirmed that

the causal agent of the disease is *Q. pitereka*. This is, furthermore, the first record of *Q. pitereka* outside the continent of Australia and it suggests that the pathogen is likely to spread to other areas of the world where Eucalypts, particularly *Corymbia* spp. are being grown.

Quambalaria pitereka was first reported in 1971 in Australia (Walker and Bertus, 1971). It has subsequently been recognised as an economically important pathogen in young *Corymbia* plantations in Queensland and New South Wales (Self *et al*., 2003; Pegg *et al*., 2005). Results of this study show that there is a relatively high degree of variability in ITS sequences between *Q. pitereka* isolates from different locations and from different *Corymbia* species in Australia (Fig. 2). Pegg *et al*. (2005) also mentioned variability in morphology and virulence between isolates of this species. This level of variability in *Q. pitereka,* in contrast to the apparent clonality of *Q. eucalypti* isolates from South Africa (Fig. 2), supports earlier suggestions that Australia is the centre of origin of *Quambalaria* (De Beer *et al*., 2006; Roux *et al*., 2006). These suggestions were based merely on the fact that all *Quambalaria* species have been reported only from trees native to Australia that have been introduced into other countries (Wingfield *et al*., 1993; Braun, 1998; Bettucci *et al*., 1999; Alfenas *et al*., 2001; De Beer *et al*., 2006; Roux *et al*., 2006).

The fact that *Q. eucalypti* has not been reported from Australia, has been ascribed to ecological homeostasis precluding the proliferation of the fungus in its natural environment (Wingfield *et al*., 1993). It is known that both South Africa and Brazil have commonly imported *Eucalyptus* seed from Australia, and also that they have exchanged seed between themselves as well (Roux *et al*., 2006). It therefore, seems highly probable that the movement of *Q. eucalypti* has been facilitated by the exchange of seed. Results of this study, reporting the first appearance of *Q. pitereka* on non-native trees outside Australia, suggest that it was introduced into China through the exchange of seed. It is known that the forestry industry in China regularly imports germplasm, which would have provided an opportunity for introduction.

The leaf blight disease reported in this study now threatens the sustainability of the plantation industry in China, which to date has been relatively free of pest and disease problems. In Australia, the disease originally prevented the use of *C. maculata* as a plantation species. However, extensive selection and tree improvement programs in Australia have been relatively successfully in reducing the impact of *Quambalaria* shoot blight. Several seed provenances showing elevated tolerance to the disease have already been selected (Dickinson *et al*., 2004). The industry in China will clearly benefit greatly by considering resistance towards *Quambalaria* species in their future breeding programs.

The life cycles and infection strategies of *Quambalaria* species have not been studied. The fact that these fungi are related to the smut fungi, suggests that their life cycle might not be as simple as those of Ascomycetous leaf pathogens. The variability among isolates of *Q. pitereka* possibly indicates that the fungus is reproducing sexually in its native environment. However, a sexual state has not been observed for any of the *Quambalaria* species. The lack of understanding the biology of these fungi clearly hampers progress towards effective control of the various manifestations of disease associated with them. Biological and ecological studies on *Quambalaria* spp. are thus urgently needed to provide the background knowledge of these pathogens that are undoubtedly increasing in their global importance.

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References

Alfenas, A.C., Zauza, E.A.V., Rosa, O.P.P. and Assi, T.F. (2001). *Sporothrix eucalypti*, a new pathogen of *Eucalyptus* in Brazil. Fitopatologia Brasiliera 26: 221.

Bauer, R., Begerow, D. and Oberwinkler, F. (1998). Fortschritte in der Systematik der Brandpilze. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 105: 224-238.

Bauer, R., Oberwinkler, F. and Vánky, K. (1997). Ultrastructural markers and systematics in smut fungi and allied taxa. Canadian Journal of Botany 75: 1273-1314.

Bettucci, L., Alonso, R. and Tiscornia, S. (1999). Endophytic mycobiota of healthy twigs and the assemblage of species associated with twig lesions of *Eucalyptus globulus* and *E. grandis* in Uruguay. Mycological Research 103: 468-472.

Braun, U. (1998). *A monograph of Ramularia, Cercosporella and allied genera (phytopathogenic hyphomycetes)*. IHW-Verlag, Eching.

Burgess, T.I., Andjic, V., Hardy, G.E.S., Dell, B. and Xu, D. (2006). First report of *Phaeophleospora destructans* in China. Journal of Tropical Forest Science 18: 144-146.

Burgess, T.I., Barber, P.A., Sufaati, S., Xu, D., Hardy, G.E. StJ. and Dell, B. (2007) *Mycosphaerella* spp. on *Eucalyptus* in Asia; new species, new hosts and new records. Fungal Diversity 24: 135-157.

Cortinas, M.N., Burgess, T.I., Dell, B., Xu, D.P., Crous, P.W., Wingfield, B.D. and Wingfield, M.J. (2006). First record of *Colletogloeopsis zuluense* comb. nov., causing a stem canker of *Eucalyptus* in China. Mycological Research 110: 229-236.

De Beer, Z.W., Begerow, D., Bauer, R., Pegg, G.S., Crous, P.W. and Wingfield, M.J. (2006). Phylogeny of *Quambalariaceae* fam. nov., including important *Eucalyptus* pathogens from South Africa and Australia. Studies in Mycology 55: 289-298.

De Hoog, G.S. and De Vries, G.A. (1973). Two new species of *Sporothrix* and their relation to *Blastobotrys nivea*. Antonie van Leeuwenhoek 39: 515-520.

Dickinson, G.R., Lee, D.J. and Huth, J.R. (2004). Early growth and tolerance to *Ramularia* shoot blight of provenances of three spotted gum taxa on a range of sites in Queensland. Australian Forestry 67: 122-130.

Gardes, M. and Bruns, T.D. (1993). ITS primers with enhanced specificity for *Basidiomycetes*–application to the identification of mycorrhiza and rusts. Molecular Ecology 2: 113–118.

Katoh, K., Misawa, K., Kuma, K. and Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research 30: 3059–3066.

Kumar S., Tamura, K. and Nei, M. (2004). MEGA3: Integrated Software for Molecular Evolutionary Genetics Analysis and Sequence Alignment. Briefings in Bioinformatics 5: 150-163.

Pegg, G.S., Drenth, A. and Wingfield, M.J. (2005). *Quambalaria pitereka* on spotted gum plantations in Queensland and northern New South Wales, Australia. Proceedings of the XXII IUFRO World Congress, 8-13 August 2005, Brisbane, Australia. International Forestry Review 7: 337.

Ran, L.X. (2002). Suppression of bacterial wilt in Eucalyptus and bacterial speck in *Arabidopsis* by fluorescent *Pseudomonas* spp. strains: conditions and mechanisms. Ph.D Thesis, Universities of Utrecht, Netherlands.

Roux, J., Mthalane, Z.L., De Beer, Z.W., Eisenberg, B. and Wingfield, M.J. (2006). *Quambalaria* leaf and shoot blight on *Eucalyptus nitens* in South Africa. Australasian Plant Pathology 35: 427-433.

Self, N.M., Aitken, E.A.B. and Dale, M.D. (2003). Susceptibility of provenances of spotted gums to *Ramularia* shoot blight. New Zealand Plant Protection 55: 68-72.

Sigler, L. and Verweij, P.E. (2003). *Aspergillus*, *Fusarium*, and other opportunistic moniliaceous fungi. In: *Manual of Clinical Microbiology* (Ed, P.R. Murray). ASM Press, Washington, D. C., USA: 1726-1760.

Simpson, J.A. (2000). *Quambalaria*, a new genus of eucalypt pathogens. Australian Mycologist 19: 57-62.

Walker, J. and Bertus, A.L. (1971). Shoot blight of *Eucalyptus* spp. caused by an undescribed species of *Ramularia*. Proceedings of the Linnean Society of New South Wales, ser. 2. 96: 108-115.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols*: *a guide to methods and applications* (eds. M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White). Academic Press, San Diego, USA: 315-322.

Wingfield, M.J., Crous, P.W. and Swart, W.J. (1993). *Sporothrix eucalypti* (sp.nov.), a shoot and leaf pathogen of *Eucalyptus* in South Africa. Mycopathologia 123: 159-164.

Xie, Y.J. (2003). Developing a strategy for sustainable management of *Eucalyptus* plantations in China. In: *Eucalypts in Asia*. (ed J.W. Turnbull). Proceedings of an international conference held in Zhanjiang, Guangdong, People's Republic of China, 7-11 April 2003. Canberra, ACIAR Proceedings No. 111, Australia: 32-38.

Zhou, X.D., De Beer, Z.W., Harrington, T.C., McNew, D., Kirisits, T., Wingfield, B.D. and Wingfield, M.J. (2004). Epityfication of *Ophiostoma galeiforme* and phylogeny of species in the *O. galeiforme* complex. Mycologia 96: 1306–1315.

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