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Quambalaria species associated with plantation and native eucalypts in Australia

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This study aimed to determine which species of *Quambalaria* are associated with shoot blight symptoms on *Corymbia* spp. An additional aim was to determine the presence and impact of quambalaria shoot blight on *Eucalyptus* species used in plantation development in subtropical and tropical regions of eastern Australia. Surveys identified three *Quambalaria* spp. – *Q. pitereka*, *Q. eucalypti* and *Q. cyanescens* – from native and plantation eucalypts, as well as amenity plantings, including the first confirmed report of *Q. eucalypti* from *Eucalyptus* plantations in Australia. Symptom descriptions and morphological studies were coupled with phylogenetic studies using ITS rDNA sequence data. *Quambalaria pitereka* was the causal agent of blight symptoms on species and hybrids in the *Corymbia* complex. *Quambalaria eucalypti* was identified from *Eucalyptus* species and a single *Corymbia* hybrid. *Quambalaria cyanescens* was detected from native and plantation *Corymbia* spp.

Keywords: *Corymbia* spp., *Eucalyptus* spp., plantation eucalypts and spotted gums, quambalaria shoot blight

Introduction

The first record of a species of *Quambalaria* causing damage to eucalypts was on nursery seedlings of *Corymbia maculata* (then *Eucalyptus maculata*) in New South Wales, Australia, in the 1950s (Walker & Bertus, 1971). The pathogen was described as *Ramularia pitereka*, but following re-examination of the four known species of *Ramularia* on eucalypts, it was transferred to the new genus *Quambalaria* (Simpson, 2000). A new family, Quambalariaceae, has since been described (de Beer *et al*., 2006) for species of *Quambalaria* that include a number of eucalypt (species of the genus *Eucalyptus* and *Corymbia*) pathogens.

There are five species of *Quambalaria* known to infect eucalypts in various parts of the world. These include *Q. pitereka*, *Q. eucalypti*, *Q. cyanescens* and *Q. coyrecup*. The fifth species, *Q. pusilla*, was described based on a single report from *Eucalyptus* spp. in Thailand (Braun, 1998). However, the taxonomic status of this fungus has been questioned (de Beer *et al*., 2006) and, as no type culture exists, it cannot be confirmed.

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Quambalaria pitereka has been isolated from foliage, stems and woody tissue of species of the genera *Corymbia*, *Blakella* and *Angophora* in Australia (Walker & Bertus, 1971; Bertus & Walker, 1974; Simpson, 2000). The disease that it causes is commonly known as quambalaria shoot blight (Pegg *et al*., 2005; Carnegie, 2007a). Old (1990) described this pathogen as being endemic to the coastal forests of eastern Australia, where seedlings and young trees of *Corymbia* species can be severely damaged. The pathogen was also reported causing shoot blight in *Corymbia maculata* plantings in Western Australia (Paap *et al*., 2008). *Quambalaria pitereka* was first found only in Australia, but a recent report of shoot blight on *Corymbia citriodora* in China was attributed to this pathogen (Zhou *et al*., 2007).

Quambalaria eucalypti was first reported (as *Sporothrix eucalypti*) in South Africa on *Eucalyptus grandis* (Wingfield *et al*., 1993). In that country, it was associated with leaf spots and serious shoot infections, but restricted to nurseries. It was subsequently reported from Brazil, where it caused stem girdling on seedlings of *Eucalyptus globulus* and leaf and shoot blight on mini-stumps of *E. saligna* × *E. maidenii* hybrids (Alfenas *et al*., 2001).

It was also identified from twig lesions on *E. globulus* in Uruguay (Bettucci *et al*., 1999). Roux *et al*. (2006) identified *Q. eucalypti* as the causal agent of extensive shoot and leaf dieback and stem cankers on 1-year-old

E. nitens in temperate regions of South Africa, the first report of the disease outside the nursery. Pathogenicity tests indicated that *Q. eucalypti* has a wider host range within *Eucalyptus*, including *E. dunnii* and *E. smithii* (Roux *et al*., 2006).

Quambalaria coyrecup is the causal agent of extensive perennial canker disease on *Corymbia calophylla* in Western Australia (Paap *et al*., 2008). This disease is causing serious decline of *C. calophylla* throughout its native range. Infection by *Q. coyrecup* gives rise to symptoms similar to the previously described canker fungus *Sporotrichum destructor*, which also caused a devastating disease of *Corymbia ficifolia* in Western Australia (Beard, 1963; Cass Smith, 1970). It has been hypothesized that *Q. coyrecup* represents the same fungus as *S. destructor*, but the latter fungus was never validly described and comparisons of the two fungi based on cultures have not been possible (Paap *et al*., 2008).

Quambalaria cyanescens was originally described from human skin (de Hoog & de Vries, 1973) and the first record on eucalypts was from a single location on *E. pauciflora* in New South Wales, Australia (de Beer *et al*., 2006). More recently it was identified from *C. ficifolia* and *C. calophylla* in Western Australia, where it sporulates on shoots and newly emerging leaves together with *Q. pitereka*. It also occurs on cankers caused by *Q. coyrecup* and on symptomless leaves and woody stems (Paap *et al*., 2008). More recently, *Q. cyanescens* was identified from bark beetles collected from a range of host tree species, including *Tilia*, *Quercus* and *Ficus*, in Hungary, Bulgaria and the Mediterranean (Kolarik *et al*., 2006).

Quambalaria shoot blight is a serious disease affecting the expanding eucalypt plantations in subtropical and tropical eastern Australia (Simpson, 2000; Self *et al*., 2002; Pegg *et al*., 2005; Carnegie, 2007b). The aim of this investigation was to determine which *Quambalaria* spp. are present in the area and to characterize the disease symptoms that they cause on various *Corymbia* and *Eucalyptus* hosts there.

Materials and methods

Surveys and symptoms

Commercial eucalypt plantations, as well as taxa and species trials in northern New South Wales and Queensland (Fig. 1), were surveyed between 2004 and 2006 for *Quambalaria* species. Surveys included the four *Corymbia* species commonly known as the 'spotted gums' [viz. *C. citriodora* subsp. *citriodora* (hereafter referred to as *C. citriodora*), *C. citriodora* subsp. *variegata* (hereafter referred to as *C. variegata*), *C. henryi* and *C. maculata*], *C. torelliana* and hybrids between this species and *C. citriodora*, *C. variegata* and *C. henryi*. Species of *Eucalyptus* included *E. argophloia*, *E. cloeziana*, *E. dunnii*, *E. grandis*, *E. grandis* × *E. camaldulensis*, *E. longirostrata*, *E. microcorys*, *E. nitens* and *E. pilularis*. Trees in native stands and residential plantings of eucalypts were also examined for the presence of *Quambalaria* infections.

For description of disease symptoms and collection of isolates for morphological and DNA-sequence-based comparisons, samples of shoots, juvenile and adult leaves (immature and mature) and green stem material exhibiting blight symptoms were used. These were collected, stored in paper bags and kept cool within a portable refrigerator unit prior to laboratory examination. Samples were examined under dissection and compound microscopes, with identification of *Quambalaria* spp. based on morphological characteristics previously described (Wingfield *et al*., 1993; Simpson, 2000), and verified using DNA sequence comparisons. Characters different from those in published records were described and recorded.

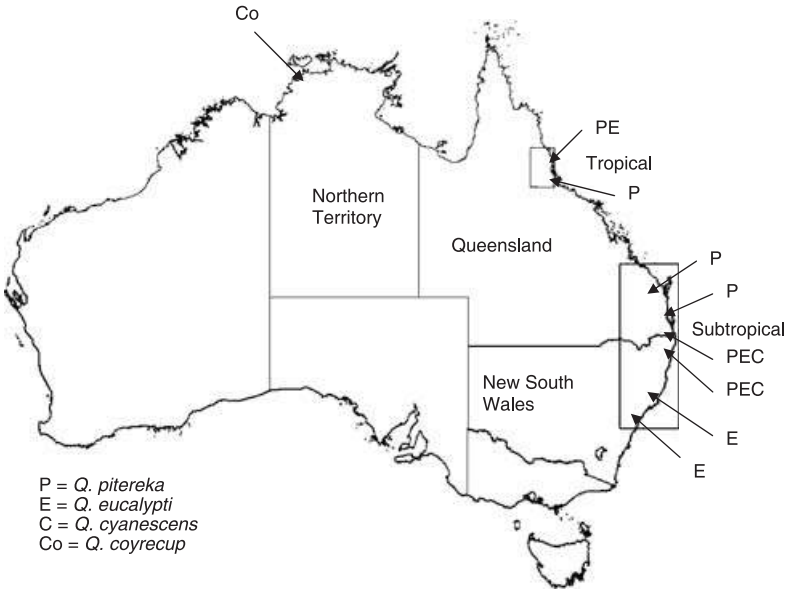

Figure 1 Map of Australia indicating where *Quambalaria pitereka* and *Q. eucalypti* haplotypes were identified from subtropical and tropical plantation areas surveyed in Queensland and New South Wales in this study.

Isolations from samples collected in Queensland and New South Wales were made from single lesions occurring on shoot, leaf and stem material exhibiting symptoms typical of *Quambalaria* spp. using potato-dextrose agar (PDA). Cultures, including the ex-holotype of *Q. pitereka* (DAR 19773), were incubated at 25°C in the dark for 2–3 days before being subcultured onto PDA and incubated for a further 2 weeks. All isolates and diseased samples collected in this study are stored in the Queensland Department of Primary Industries and Fisheries Plant Pathology Herbarium and culture collection (BRIP).

Fungal morphology

Comparisons of conidial morphology were made between isolates from different host materials. Spores were washed off leaves and stem lesions using sterile distilled water with a droplet placed onto a glass slide. Spore dimensions were recorded by measuring the lengths and widths of 30 randomly selected conidia at ×400 magnification (Olympus BH-2 compound microscope with graduated lens). Spore sizes for the different isolates were compared using ANOVA (STATVIEW).

Plant tissue samples representing a broad range of disease symptoms were also examined using scanning electron microscopy. Leaf samples were collected from infected trees in plantations and cut into 3-mm^2 pieces, focusing on areas with visible disease symptoms. Samples were prepared for examination according to van den Berg *et al*. (2003). However, drying was completed using hexamethylthyldisilazane (HMDS). All steps were completed using a Pelco Biowave®. Samples were coated with platinum for a period of 5 min to achieve a thickness of approximately 15 nm using an EIKO IB-5 Sputter Coater before being examined with a JOEL 6300 and JOEL 6400 scanning electron microscope at 8 kV.

DNA sequence comparisons

For DNA sequence analysis, mycelium from isolates of *Quambalaria* spp. (Table 1) was harvested by scraping it from the surface of PDA cultures using a sterilized scalpel. The mycelium was then frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle that had been sterilized with 70% ethanol. DNA was extracted using a Wizard® Genomic DNA Purification Kit – Isolation of Genomic DNA from Plant Tissue (Promega), according to the manufacturer's instructions. DNA was resuspended in TE buffer (10 mm Tris-HCl, 1 mM EDTA, pH 8·0) and quantified using a Biorad SmartSpec™ Spectrophometer prior to further analysis.

DNA of the ribosomal RNA gene region (ITS1-5·8S-ITS2) was amplified using the universal primers ITS4 and ITS5 (White *et al*., 1990). Each 25-μL PCR reaction solution in each tube contained 12·5 ng template DNA μL^{-1}, 100 μM dNTPs, 1·5 mM $MgCl_2$, 1 U *Taq* DNA polymerase (Biotech) and 0·6 μM primers. DNA amplifications were performed in a PTC-100 Programmable Thermal Controller (MJ Research). An initial denaturation step at 95°C for 5 min was followed by 40 cycles of 95°C for 30 s, annealing for 30 s at 60°C and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min.

The PCR amplicons were size-fractionated in a 1·0% agarose gel and visualized using ethidium bromide under UV light. The ITS amplicons were purified from the agarose gel using a High Pure PCR Product Purification Kit (Roche Diagnostics). The purified amplicons were sequenced with the same primers that were used to amplify the respective DNA regions using an ABI Prism BigDye Terminator Sequencing Reaction Kit (v3·1) on an ABI 3700 DNA Analyser (Applied Biosystems).

To compare the *Quambalaria* species used in this study with other *Quambalaria* species, ITS sequences of closely related species, including the newly described *Q. coyrecup* (Paap *et al*., 2008), were obtained from GenBank and used in phylogenetic analyses (Table 1). DNA sequences were automatically aligned using CLUSTALX (Jeanmougin *et al*., 1998) and the alignments adjusted manually in BIOEDIT v5·0 (Hall, 1999). All sequences obtained in this study were deposited in GenBank and accession numbers are shown in Table 1. Haplotypes of *Quambalaria* species were defined for the purpose of this study as isolates having identical DNA sequences in the ITS1-5·8S-ITS2 rRNA region.

Analyses of sequence data were performed on the aligned ITS-5·8S-ITS2 sequences in PAUP (phylogenetic analysis using parsimony) v4·0b10 (Swofford, 2002). The most parsimonious trees were obtained by using heuristic searches with random stepwise addition in 100 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option off. Maxtrees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) were determined (Hillis & Huelsenbeck, 1992). Characters were unweighted and unordered, branch and branch node supports were determined using 1000 bootstrap replicates (Felsenstein, 1985), and characters were sampled with equal probability. Gaps were treated as a fifth character in the parsimony analysis. Trees were rooted to *Tilletiopsis washingtonensis*, *Entyloma calendulae* and *Microstroma juglandis*, which were treated as the outgroup taxa.

Results

Isolates

Three species of *Quambalaria* were identified from surveys in northern New South Wales and Queensland in this study. These were *Q. pitereka*, *Q. eucalypti* and *Q. cyanescens* (Fig. 2). Forty of the isolates collected in this study (Tables 1 and 2) were from *Corymbia* species (*C. variegata*, *C. citriodora*, *C. henryi*, *C. polycarpa*, *C. torelliana* and *Corymbia* hybrids). Twelve of these isolates were collected from New South Wales, 15 from southern

Table 1 *Quambalaria* isolates, from *Corymbia* and *Eucalyptus* species, used for DNA sequence comparisons

Culture no.	*Quambalaria* sp.	Host	Location[a]	Collector	Haplotype[b]	GenBank accession no.
DAR19773	*Q. pitereka*	*C. exima*	NSW, Australia	Walker	QP1	DQ823423
BRIP48346	*Q. pitereka*	*C. citriodora* subsp. *citriodora*	Davies Creek, QLD, Australia	G. S. Pegg	QP1	EF444845
BRIP48387	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Beaudesert, QLD, Australia	G. S. Pegg	QP1	EF444846
BRIP48425	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Grafton, NSW, Australia	G. S. Pegg	QP1	EF444847
BRIP48429	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Grafton, NSW, Australia	G. S. Pegg	QP1	EF444848
BRIP48433	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Grafton, NSW, Australia	G. S. Pegg	QP1	EF444849
BRIP48492	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Tipperary, NSW, Australia	A. J. Carnegie	QP1	EF444850
BRIP48505	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Moggill, QLD, Australia	G. S. Pegg	QP1	EF444851
BRIP48506	*Q. pitereka*	Native *C. citriodora*	Moggill, QLD, Australia	G. S. Pegg	QP1	EF444852
BRIP48515	*Q. pitereka*	Native *C. henryi*	Grafton, NSW, Australia	G. S. Pegg	QP1	EF444853
CMW5318	*Q. pitereka*	*C. citriodora* subsp. *variegata*	QLD, Australia	M. Ivory	QP2	DQ317621
BRIP48317	*Q. pitereka*	*C. henryi*	Coolabunia, QLD, Australia	G. S. Pegg	QP2	EF444854
BRIP48321	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Beaudesert, QLD, Australia	G. S. Pegg	QP2	EF444855
BRIP48512	*Q. pitereka*	Native *C. henryi*	Grafton, NSW, Australia	G. S. Pegg	QP2	EF444856
BRIP48381	*Q. pitereka*	*C. citriodora* subsp. *citriodora*	Silkwood, QLD, Australia	G. S. Pegg	QP3	EF444858
BRIP48361	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Walkamin, QLD, Australia	G. S. Pegg	QP3	EF427367
BRIP48343	*Q. pitereka*	*C. citriodora* subsp. *citriodora*	Walkamin, QLD, Australia	G. S. Pegg	QP3	EF444857
BRIP48383	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Beaudesert, QLD, Australia	G. S. Pegg	QP4	EF444859
WAC12956	*Q. pitereka*	*C. ficifolia*	Western Australia	T. Paap	QP5	DQ823428
WAC12957	*Q. pitereka*	*C. ficifolia*	Western Australia	T. Paap	QP5	DQ823426
WAC12958	*Q. pitereka*	*C. calophylla*	Western Australia	T. Paap	QP5	DQ823427
BRIP48349	*Q. pitereka*	*C. torelliana* × *C. citriodora* subsp. v*ariegata*	Mareeba, QLD, Australia	G. S. Pegg	QP6	EF444860
BRIP48350	*Q. pitereka*	*C. torelliana* x *C. citriodora* subsp. *variegata*	Mareeba, QLD, Australia	G. S. Pegg	QP6	EF444861
BRIP48360	*Q. pitereka*	*C. torelliana* × *C. citriodora* subsp. *variegata*	Walkamin, QLD, Australia	G. S. Pegg	QP6	EF444862
BRIP48368	*Q. pitereka*	*C. torelliana* × *C. citriodora* subsp. *citriodora*	Mareeba, QLD, Australia	G. S. Pegg	QP6	EF444863
BRIP48370	*Q. pitereka*	*C. torelliana* × *C. citriodora* subsp. *citriodora*	Mareeba, QLD, Australia	G. S. Pegg	QP6	EF427368
BRIP48388	*Q. pitereka*	*C. torelliana*	Atherton, QLD, Australia	G. S. Pegg	QP6	EF444864
BRIP48325	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Binjour, QLD, Australia	G. S. Pegg	QP7	EF427366
BRIP48335	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Imbil, QLD, Australia	G. S. Pegg	QP7	EF444865
BRIP48340	*Q. pitereka*	*C. torelliana* × *C. henryi*	Childers, QLD, Australia	G. S. Pegg	QP7	EF444866
BRIP48386	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Beaudesert, QLD, Australia	G. S. Pegg	QP7	EF427370
BRIP48413	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Casino, NSW, Australia	G. S. Pegg	QP7	EF444867
BRIP48424	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Grafton, NSW, Australia	G. S. Pegg	QP7	EF444868
BRIP48508	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Moggill, QLD, Australia	G. S. Pegg	QP7	EF444869
BRIP48513	*Q. pitereka*	Native *C. henryi*	Grafton, NSW, Australia	G. S. Pegg	QP7	EF444870
BRIP48516	*Q. pitereka*	Native *C. henryi*	Grafton, NSW, Australia	G. S. Pegg	QP7	EF444871
BRIP48328	*Q. pitereka*	Native *C. citriodora* subsp. *variegata*	Dilkoon, NSW, Australia	G. S. Pegg	QP8	EF444872
BRIP48384	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Beaudesert, QLD, Australia	G. S. Pegg	QP8	EF427369
BRIP48432	*Q. pitereka*	*C. citriodora* subsp. *variegata*	Grafton, NSW, Australia	G. S. Pegg	QP9	EF444873
CMW23610	*Q. pitereka*	*C. citriodora*	China	Y. J. Xie	QP10	EF427373
CMW23611	*Q. pitereka*	*C. citriodora*	China	Y. J. Xie	QP10	EF427374
CMW23612	*Q. pitereka*	*C. citriodora*	China	Y. J. Xie	QP10	EF427375
CMW23613	*Q. pitereka*	*C. citriodora*	China	Y. J. Xie	QP10	EF427376
CBS118844	*Q. eucalypti*	*E. grandis*	South Africa	M. J. Wingfield	QE1	DQ317626
CBS119680	*Q. eucalypti*	*E. grandis*	South Africa	L. Lombard	QE1	DQ317625

Table 1 *Continued*

Culture no.	*Quambalaria* sp.	Host	Location[a]	Collector	Haplotype[b]	GenBank accession no.
CMW14329	*Q. eucalypti*	*E. grandis* × *E. camaldulensis*	South Africa	J. Roux	QE1	DQ317614
CBS118616	*Q. eucalypti*	*E. grandis*	South Africa	J. Roux	QE1	DQ317613
CMW17254	*Q. eucalypti*	*E. nitens*	South Africa	J. Roux	QE1	DQ317611
CBS118615	*Q. eucalypti*	*E. nitens*	South Africa	J. Roux	QE1	DQ317609
BRIP48367	*Q. eucalypti*	*C. torelliana* × *C. citriodora* subsp. *variegata*	Walkamin, QLD, Australia	G. S. Pegg	QE1	EF444823
BRIP48416	*Q. eucalypti*	*E. dunnii*	Casino, NSW, Australia	G. S. Pegg	QE1	EF444824
BRIP48420	*Q. eucalypti*	*E. dunnii*	Grafton, NSW, Australia	A. J. Carnegie	QE1	EF444825
BRIP48494	*Q. eucalypti*	*E. dunnii*	Dyraaba, NSW, Australia	A. J. Carnegie	QE1	EF444827
BRIP48496	*Q. eucalypti*	*E. dunnii*	Tabulam, NSW, Australia	A. J. Carnegie	QE1	EF444828
BRIP48504	*Q. eucalypti*	*E. dunnii*	Casino, NSW, Australia	A. J. Carnegie	QE1	EF444829
BRIP48511	*Q. eucalypti*	*E. longirostrata*	Grafton, NSW, Australia	A. J. Carnegie	QE1	EF444830
BRIP48519	*Q. eucalypti*	*E. longirostrata*	Moggill, QLD, Australia	G. S. Pegg	QE1	EF444831
BRIP48422	*Q. eucalypti*	*E. dunnii*	Bonalbo, NSW, Australia	A. J. Carnegie	QE2	EF444832
BRIP48489	*Q. eucalypti*	*E. grandis*	Lismore, NSW, Australia	G. S. Pegg	QE2	EF444833
BRIP48490	*Q. eucalypti*	*E. grandis*	Lismore, NSW, Australia	G. S. Pegg	QE2	EF444834
BRIP48493	*Q. eucalypti*	*E. grandis*	Taree, NSW, Australia	A. J. Carnegie	QE2	EF444826
BRIP48499	*Q. eucalypti*	*E. grandis*	Mandalong, NSW, Australia	A. J. Carnegie	QE2	EF444835
BRIP48500	*Q. eucalypti*	*E. dunnii*	Taree, NSW, Australia	A. J. Carnegie	QE2	EF444836
BRIP48501	*Q. eucalypti*	*E. dunnii*	Casino, NSW, Australia	A. J. Carnegie	QE2	EF444837
BRIP48502	*Q. eucalypti*	*E. dunnii*	Kyogle, NSW, Australia	A. J. Carnegie	QE2	EF444838
BRIP48509	*Q. eucalypti*	*E. grandis*	Lismore, NSW, Australia	A. J. Carnegie	QE2	EF444839
BRIP48510	*Q. eucalypti*	*E. grandis*	Lismore, NSW, Australia	A. J. Carnegie	QE2	EF444840
BRIP48518	*Q. eucalypti*	*E. longirostrata*	Moggill, QLD, Australia	G. S. Pegg	QE2	EF444841
BRIP48520	*Q. eucalypti*	*E. grandis*	Moggill, QLD, Australia	G. S. Pegg	QE2	EF444842
BRIP48497	*Q. eucalypti*	*E. grandis*	Nana Glen, NSW, Australia	A. J. Carnegie	QE3	EF444843
BRIP48498	*Q. eucalypti*	*E. grandis*	Kew, NSW, Australia	A. J. Carnegie	QE3	EF444844
BRIP48507	*Q. eucalypti*	*E. grandis*	Moggill, QLD, Australia	G. S. Pegg	QE4	EF444822
BRIP48414	*Q. eucalypti*	*E. grandis*	Moggill, QLD, Australia	G. S. Pegg	QE4	EF444821
BRIP48396	*Q. cyanescens*	Native *C. citriodora*	Beaudesert, QLD, Australia	G. S. Pegg		EF444874
BRIP48398	*Q. cyanescens*	Native *C. citriodora*	Beaudesert, QLD, Australia	G. S. Pegg		EF444875
BRIP48403	*Q. cyanescens*	Native *C. citriodora*	Beaudesert, QLD, Australia	G. S. Pegg		EF444876
WAC12952	*Q. cyanescens*	*C. calophylla*	Western Australia	T. Paap		DQ823419
WAC12955	*Q. cyanescens*	*C. calophylla*	Western Australia	T. Paap		DQ823421
CBS876·73	*Q. cyanescens*	*E. pauciflora*	New South Wales	V. F. Brown		DQ317623
WAC12948	*Q. coyrecup*	*C. calophylla*	Western Australia	T. Paap		DQ823433
WAC12949	*Q. coyrecup*	*C. calophylla*	Western Australia	T. Paap		DQ823432
WAC12950	*Q. coyrecup*	*C. ficifolia*	Western Australia	T. Paap		DQ823429
BRIP48338	*Q. coyrecup*	*C. polycarpa*	Darwin, NT, Australia	R. Pitkethley		EF444877
BRIP48339	*Q. coyrecup*	*C. polycarpa*	Darwin, NT, Australia	R. Pitkethley		EF444878

[a]NSW = New South Wales, NT = Northern territory, QLD = Queensland.

[b]QP = *Q. pitereka*, QE = *Q. eucalypti*.

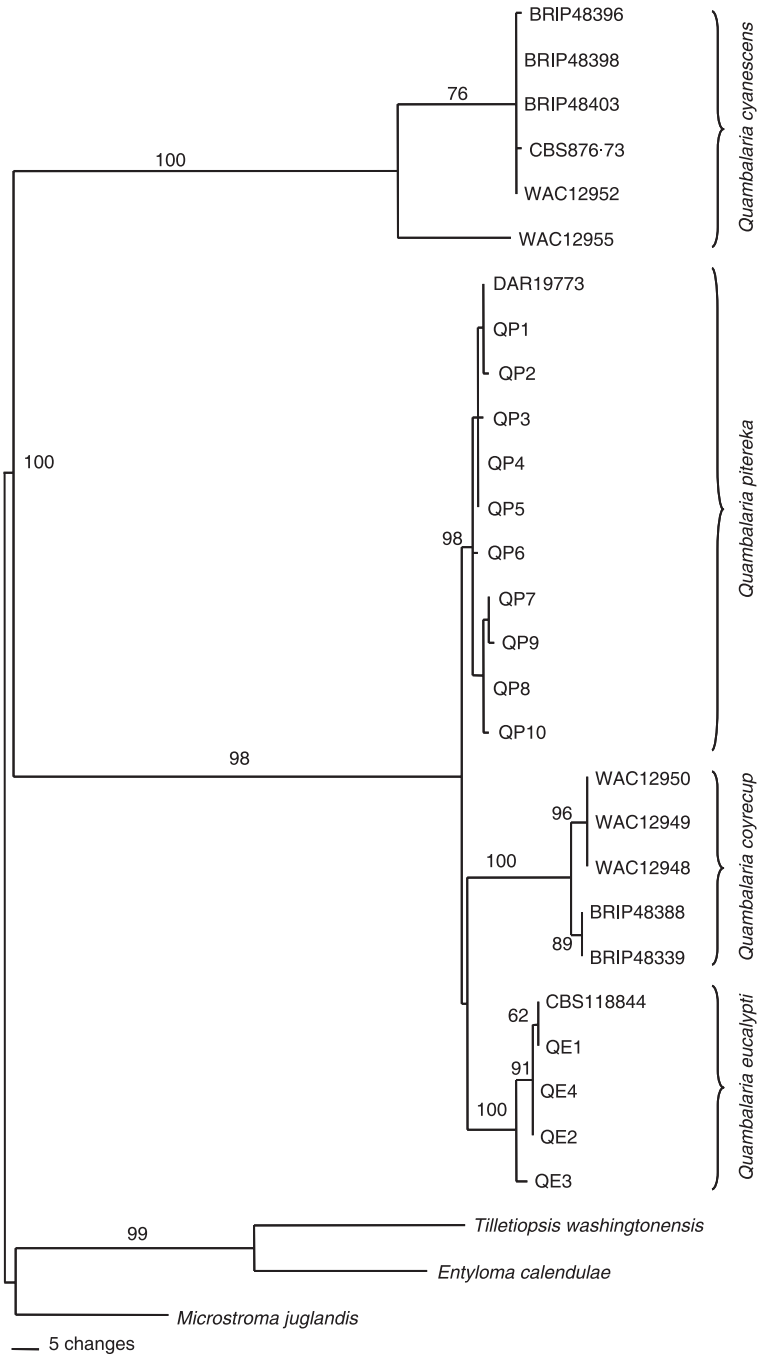

Figure 2 A phylogram of one of the 50 most-parsimonious trees obtained from the ITS sequence data of the *Quambalaria* isolates. Bootstrap values are given above the branch. The tree is rooted to *Tilletiopsis washingtonensis*, *Entyloma calendulae* and *Microstroma juglandis*. Isolates of *Q. pitereka* and *Q. eucalypti* are represented by haplotypes, QP and QE, respectively. GenBank accession numbers for these haplotypes are listed in Table 1.

Queensland, 11 from far-north Queensland and two from the Northern Territory. Two additional isolates were collected from *C. maculata* in northern New South Wales. Twenty-three isolates were collected from *Eucalyptus* spp. (*E. grandis*, *E. longirostrata*, *E. dunnii*), with the majority from New South Wales. Additional isolates not used in DNA sequence analysis were collected from *E. microcorys* in southern Queensland and from *E. grandis* and an *E. grandis* × *E. camaldulensis* hybrid in far-north Queensland.

Surveys and symptoms

Quambalaria pitereka was found in all *Corymbia* plantations, species and taxa trials surveyed within Queensland and northern New South Wales. The pathogen was also found in native forests and residential gardens in these regions. *Quambalaria pitereka* was identified from all spotted gum species examined including *C. variegata*, *C. citriodora*, *C. henryi* and *C. maculata*, as well as *C. torelliana* and its hybrids with other *Corymbia*

Table 2 Geographic origins, hosts and DNA haplotypes of *Quambalaria* species collected from subtropical and tropical regions of eastern Australia on *Eucalyptus* and *Corymbia* species

Location and host species	*Quambalaria* species	Haplotype
Northern New South Wales		
C. variegata	*Quambalaria pitereka*	QP1, QP7, QP9
	Quambalaria cyanescens	
C. henryi	*Quambalaria pitereka*	QP1, QP2, QP7
C. maculata	" "	
E. dunnii	*Quambalaria eucalypti*	QE1, QE2
E. grandis	" "	QE2, QE3
E. longirostrata	" "	QE1
Southern Queensland		
C. variegata	*Quambalaria pitereka*	QP1, QP2, QP4, QP7, QP8
	Quambalaria cyanescens	
C. henryi	*Quambalaria pitereka*	QP2
C. torelliana × *C. henryi*	" "	QP7
E. dunnii	*Quambalaria eucalypti*	
E. grandis	" "	QE2, QE4
E. longirostrata	" "	QE1, QE2
E. microcorys	" "	
North Queensland		
C. variegata	*Quambalaria pitereka*	QP3
C. citriodora	" "	QP1, QP3
C. torelliana	" "	QP6
C. torelliana × *C. variegata*	" "	QP6
	Quambalaria eucalypti	QE1
C. torelliana × *C. citriodora*	*Quambalaria pitereka*	QP6
E. grandis × *E. camaldulensis*	*Quambalaria eucalypti*	
E. grandis	" "	

species. Quambalaria shoot blight on *C. torelliana* was found only in plantation and amenity plantings in tropical areas of far-north Queensland. The fungus was, however, not identified from any *Eucalyptus* spp. sampled.

Symptoms caused by *Q. pitereka* on *C. variegata*, *C. citriodora*, *C. maculata*, *C. henryi*, *C. torelliana* and *Corymbia* hybrids were similar to those described previously (Walker & Bertus, 1971; Simpson, 2000). Disease symptoms on new shoots and expanding juvenile foliage consisted of distorted leaves and amphigenous necrotic lesions of irregular shape and size (Fig. 3). White-coloured pustules were present on the adaxial and abaxial leaf surfaces covering the lesions. Lesions covered with white pustules were also present on the leaf midribs. Restricted necrotic lesions occurred on mature juvenile foliage with scattered white pustules occurring on parts of the lesions. Similar shoot blight symptoms caused by *Q. pitereka* were detected on immature adult foliage on native and amenity spotted gum trees. No disease symptoms were detected on mature adult foliage.

An unusual foliage symptom was detected on *Corymbia* hybrids grown in taxa and species trials in tropical regions of northeastern Australia. Leaf blight symptoms caused by *Q. pitereka* occurred on expanding and fully expanded foliage of *Corymbia* hybrids in species trials (Fig. 3d). These symptoms consisted of amphigenous, irregular shaped necrotic lesions surrounded by water-soaked margins. The size of lesions was variable (2–15 mm) and appeared to increase as leaves expanded. Lesions often coalesced to form large necrotic areas on the leaves. White masses of erumpent conidiophores occurred primarily in the centres of the lesions on the abaxial leaf surfaces. However, on immature expanding foliage, pustules were observed covering the entire lesion surface. Lesions also occurred on the leaf midrib, resulting in distorted growth. Restricted, sunken, necrotic lesions were present on young green stems, with pustules occurring over the entire necrotic area.

On green apical stems and side branches of spotted gum species, sunken necrotic lesions caused by *Q. pitereka* were observed (Fig. 4a). White pustules were often present on the entire area of the lesion. Stem lesions were associated with distorted growth or death of the stem when girdling occurred. Repeated loss of new shoots and death of stems resulted in the loss of apical dominance and a bush-like growth habit. A reduction in foliage occurred as a result of premature senescence of infected leaves and green stems. Distortion and death of the leading shoots and stems of *Corymbia* hybrids was detected less frequently than that on spotted gum in subtropical regions, but was observed in tropical regions of eastern Australia (Fig. 4b). Lesions were also detected on stems of *C. torelliana* in this region. *Quambalaria pitereka* was also observed in association with wounds caused by hailstones (Fig. 4c) and cankers on woody branches of infected *C. citriodora* amenity trees (Fig. 4d).

Quambalaria eucalypti was identified from leaf spots on *E. grandis*, *E. dunnii*, *E. longirostrata*, *E. grandis* and *E. grandis* × *E. camaldulensis* in commercial plantations, species trials and residential gardens in subtropical and tropical regions of eastern Australia. *Quambalaria eucalypti* was not detected on *E. cloeziana*, *E. pilularis* or *E.*

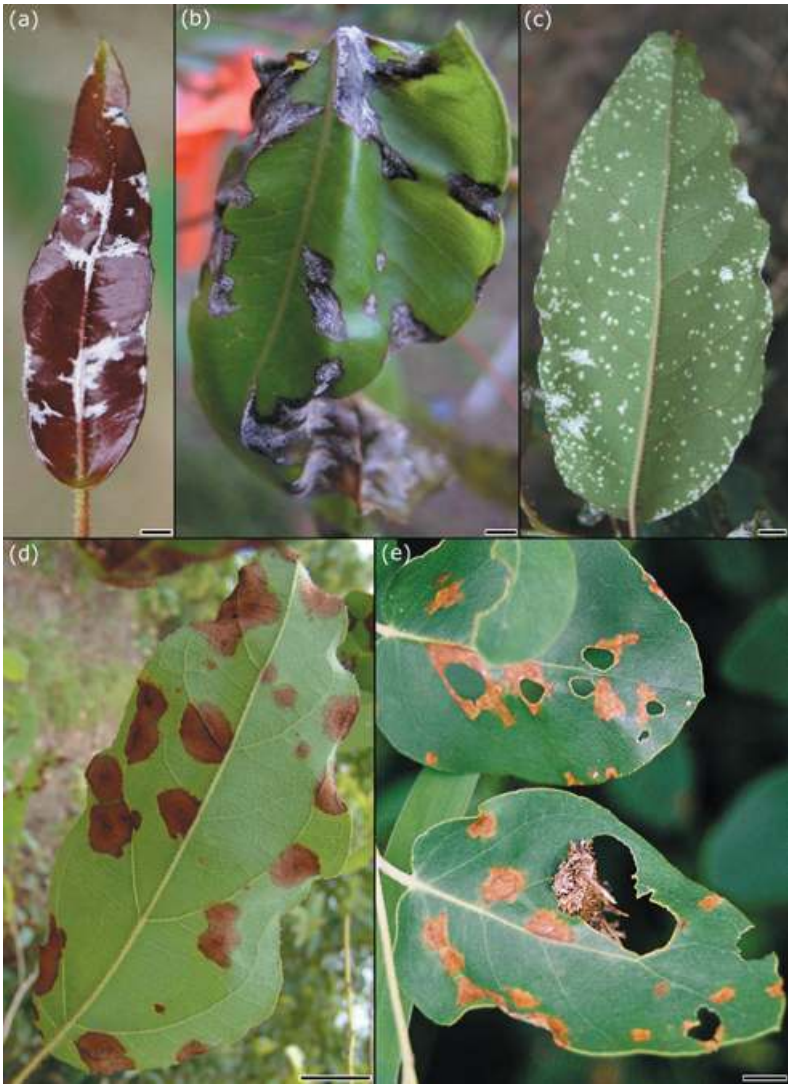

Figure 3 Symptoms of quambalaria shoot blight on eucalypt foliage. Immature (a) and mature (b) juvenile foliage of *Corymbia variegata* infected by *Quambalaria pitereka* resulting in distorted leaves and irregularly shaped and sized amphigenous necrotic lesions covered in white-coloured pustules. Symptoms of *Q. pitereka* infection on immature (c) and mature juvenile (d) foliage of *Corymbia torelliana* × *C. variegata* hybrid. (e) *Quambalaria eucalypti* symptoms on *Eucalyptus dunnii*. Scale bar = 5 mm.

argophloia in the survey. Infection was characterized by restricted, amphigenous lesions of irregular shape and size with white pustules predominantly present on the abaxial leaf surfaces (Fig. 3e). On expanding *E. grandis* juvenile leaves, pustules were observed on the adaxial and abaxial leaf surfaces and associated with leaf distortion or buckling. On all hosts, lesions were detected only on juvenile foliage and were often associated with wounds. In this case, wounds were caused by weevil (*Oxyops* spp. and *Gonipterus* spp.) and flea beetle (*Chaetocnema* sp.) feeding damage, but it is likely that *Q. eucalypti* would also be associated with other insect activity. On several disease samples of *E. dunnii* collected in New South Wales, small elongated lesions were observed on small green stems, which often resulted in splits in the stems. White pustules were evident on these lesions (Fig. 4e).

Quambalaria cyanescens was isolated from small stem lesions on native *C. variegata* and *C. citriodora* in Queensland and northern New South Wales, as well as in a limited number of *C. variegata* plantations in New South Wales. Extensive surveys to determine the host range and impact of this *Quambalaria* species have not been conducted. Discrete lesions were detected on woody stems and branches. Lesions were more or less elliptical in shape and covered in white/pink-coloured spore masses (Fig. 4f). Discoloration was present immediately under the lesion and appeared to be restricted to the cambium.

Quambalaria coyrecup was identified from a stem sample sent from the Northern Territory, Australia, with symptoms of cankering on small-diameter branches of *C. polycarpa*. Symptoms included stem swelling and bark splitting, with masses of erupting, white-coloured conidiophores and conidia present. Branch death occurred where cankers girdled the stems. This fungus was not detected in Queensland or New South Wales during the study.

Figure 4 *Quambalaria* species associated with stem lesions on eucalypts in subtropical and tropical region of eastern Australia. (a) Distorted apical shoot of *Corymbia maculata* infected by *Q. pitereka* covered in white masses of conidia and conidiophores; (b) lesions on the stem of a juvenile branch of *C. torelliana* × *C. variegata* caused by *Q. pitereka*; (c) stem lesions on *C. variegata* caused by hail damage and subsequently infected with *Q. pitereka*; (d) *Q. pitereka* sporulating (indicated by arrow) on a woody canker on *C. citriodora*; (e) *Q. eucalypti* associated with a lesion on juvenile *Eucalyptus dunnii* twig; (f) *Q. cyanescens* sporulating on a canker on a woody branch of *C. variegata* (indicated by arrow). Scale bar in all figures = 10 mm.

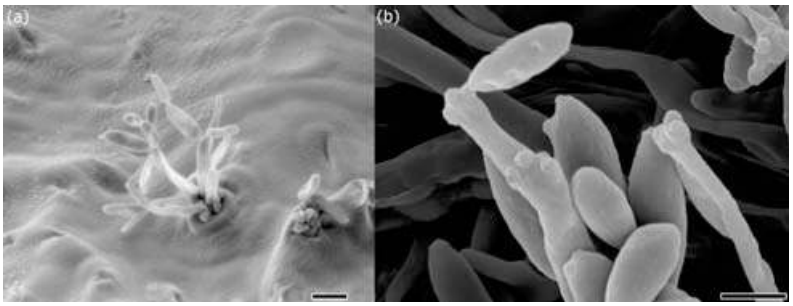

Figure 5 Conidia and conidiophores of (a) *Quambalaria pitereka* and (b) *Quambalaria eucalypti* borne on simple or aggregated conidiophores. Conidiophores of *Q. eucalypti* were uniform in width with a swollen cluster of conidium bearing denticles. Scale bar = 1 μm.

Spore morphology

Spore dimensions for *Q. pitereka* collected from spotted gum species *C. variegata*, *C. citriodora*, *C. maculata* and *C. henryi* were similar to those of the holotype of *Q. pitereka* (DAR 19773). Primary conidia were variable in shape and ranged from clavate to elongate-clavate, cylindrical, fusiform, narrowly pyriform or obovoid. Primary conidia, perhaps better described as ramoconidia (Schubert *et al*., 2007), were of variable length (6–13 × 2–4 μm) and were not significantly different from secondary conidia. Conidia were borne on simple single or aggregated conidiophores (Fig. 5). Spore size was also variable for isolates collected from *C. torelliana* and *Corymbia* hybrids in north Queensland. Spores collected from *C. torelliana* (4–8 × 2–5 μm) were significantly ($P \leq 0{\cdot}01$) smaller than those collected from *Corymbia* hybrids (4–10 × 2–6 μm), *C. citriodora* and *C. variegata*.

Spore dimensions for *Q. eucalypti* from isolates collected from *Eucalyptus* species were smaller (4–9 × 2–3 μm) than those of *Q. pitereka*. The presence of secondary conidia half the size of the primary conidia was also noted; this is a key taxonomic feature detailed by Simpson (2000) and Wingfield *et al*. (1993) for *Q. eucalypti*. Conidiophores were uniform in width with a swollen cluster of conidium-bearing denticles. Denticles were observed on both terminal and short side branches (Fig. 5).

DNA sequence comparisons

DNA sequences from the ex-type cultures and representatives of each haplotype of *Q. pitereka* and *Q. eucalypti* were included in a dataset containing other *Quambalaria* spp., *Q. cyanescens* and *Q. coyrecup*, and outgroup taxa (TreeBASE SN3314-14388; Table 1). The aligned dataset consisted of 676 bp, of which 176 characters were parsimony-informative. These data contained significant phylogenetic signal (g1 = –1·44). Heuristic searches in PAUP resulted in 50 most parsimonious trees of 303 steps (CI = 0·82, RI = 0·89), one of which is presented in Fig. 2.

Forty-three isolates of *Q. pitereka* represented 10 haplotypes based on ITS profile (TreeBASE SN3314-14386; Tables 1 and 3, Fig. 2). All the haplotypes of this species were clustered in a single, strongly supported clade (Fig. 2). Twelve isolates tested had identical ITS sequences to the ex-holotype culture of *Q. pitereka* (DAR 19773). Haplotype 6 was present in isolates collected from *C. torelliana* and *C. torelliana* × *C. variegata* and *C. torelliana* × *C. citriodora* hybrids in north Queensland (Fig. 2, Tables 1 and 2). These isolates formed a discrete group separate from isolates collected from *C. variegata* and *C. citriodora* trees in the same region and an isolate collected from a *C. torelliana* × *C. henryi* hybrid in central Queensland. Other groupings were not host- or region-specific. Five haplotypes: 1, 2, 4, 7 and 8 (Fig. 2, Tables 1 and 2), were found to be present in a single plantation in southeast Queensland.

Twenty-six isolates of *Q. eucalypti* could be divided into four haplotypes (TreeBASE SN3314-14387; Tables 1 and 4, Fig. 2). All the *Q. eucalypti* haplotypes grouped together in a single, strongly supported clade (Fig. 2). Eleven isolates had identical ITS sequences to the ex-type culture (CBS 118844) collected in South Africa and used by Wingfield *et al.* (1993) to describe *Q. eucalypti*. Two isolates with a single 5-bp indel were identified from leaf lesions on *E. grandis* in southeast Queensland (Table 4).

Table 3 Haplotypes of *Quambalaria pitereka* as determined from the polymorphic nucleotides within the aligned sequence data of ITS region of the rDNA for isolates collected from spotted gum species and *Corymbia* hybrids in Queensland and New South Wales

Haplotype	14[a]	40	86	91	185	190	421	567	575
1	T[b]	A	G	G	T	G	C	C	A
2	T	A	G	A	T	G	C	C	A
3	T	A	G	A	T	A	C	C	A
4	T	A	G	G	T	A	C	C	A
5	T	A	G	G	T	A	A	C	A
6	T	A	G	G	T	A	C	T	G
7	A	G	G	G	G	A	C	C	G
8	A	G	G	G	T	A	C	C	G
9	T	G	G	G	G	A	C	C	G
10	A	G	A	G	T	A	C	C	G

[a]Base pair (bp) positions in aligned data, TreeBase SN3314-14388.
[b]Polymorphisms shared with the first haplotype are highlighted.

Table 4 Haplotypes of *Quambalaria eucalypti* as determined from the polymorphic nucleotides within the aligned sequence data of ITS region of the rDNA for isolates collected from species of *Eucalyptus* in Queensland and New South Wales

Haplotype	100[a]	141–145	524
1	T[b]	–	T
2	T	–	C
3	C	–	C
4	T	+	C

[a]Base pair (bp) positions in aligned data, TreeBase SN3314-14388.
[b]Polymorphisms shared with the first haplotype are highlighted.

Discussion

Three species of *Quambalaria*, namely *Q. pitereka*, *Q. eucalypti* and *Q. cyanescens*, were found associated with plantation eucalypts in subtropical and tropical regions of eastern Australia. This is the first confirmed record of *Q. eucalypti* in Australia and of *Q. cyanescens* in plantations as well as native *C. variegata*. In addition, *Q. coyrecup* was detected for the first time outside Western Australia, where it caused branch cankers on *C. polycarpa* in the Northern Territory, although it was not found in Queensland or New South Wales.

Quambalaria pitereka affects all spotted gum plantation areas in the subtropical and tropical regions of Australia. Infection of the growing points has the most significant impact on forest production, with the loss of apical dominance and multi-branching often resulting in a shrub-like growth habit, a form not viable for commercial forest production. *Quambalaria pitereka* has also been reported causing shoot blight on plantations of *C. maculata* in Western Australia (Paap *et al.*, 2008) and *C. citriodora* plantations in China (Zhou *et al.*, 2007). It was not found on *Eucalyptus* species in this study and appears to be restricted to hosts in the genus *Corymbia*.

In Queensland and northern New South Wales, plantations of *Corymbia* spp. are planted in close proximity to native stands of spotted gum, with mature habitat trees often retained within a plantation. These native spotted gums are the likely source of infection for *Q. pitereka*, which can be found sporulating on juvenile foliage of seedlings and saplings and immature adult foliage of mature native spotted gum trees. Simpson (2000) also reported the occurrence of *Q. pitereka* from large *C. maculata* trees and its common occurrence on young spotted gum natural regeneration.

Results of the survey provide the first report of *Q. pitereka* associated with cankers on woody stems of *C. citriodora* and an association with stem wounding caused by hail damage on *C. variegata*, *C. citriodora*, *C. henryi* and *C. maculata*. However, Simpson (2000) reported the presence of *Q. pitereka* in apparently healthy woody tissue of *C. henryi*, *C. maculata* and *C. variegata*. Little is known regarding the life cycle of *Q. pitereka* and further work is required to determine the role of spotted gum in native forests in disease development in plantations.

Likewise, it will be intriguing to discover whether the existence of the pathogen in woody stems serves as a long-term survival mechanism.

Quambalaria pitereka was also isolated from *C. torelliana* in taxa trials and amenity plantings in tropical regions of north Queensland. This is the first record of *Q. pitereka* causing disease on *C. torelliana* within a plantation or native forest ecosystem. This is a significant finding with respect to the current breeding programme that has been established using hybrids between *C. torelliana* and spotted gum species (Lee, 2007). One of the justifications for selecting *C. torelliana* is the belief that it is resistant to infection by *Q. pitereka*. Indeed, the pathogen has not been detected in trial plantings or on amenity trees of *C. torelliana* in the subtropical regions. Likewise, the influence of *Q. pitereka* on the hybrid material is more apparent in the tropical regions of north Queensland than in subtropical plantings.

Corymbia hybrids have been shown to significantly out-perform parent material from a plantation point of view and initially in small-scale plantations did not appear to be affected by *Q. pitereka* (Lee, 2007). Foliage symptoms seen in subtropical regions in this study were similar to those detected on spotted gum. The disease was, however, limited to new shoots and expanding foliage and infection rarely resulted in stem death or loss of apical dominance. Yet, in species trials in north Queensland, *Q. pitereka* caused necrotic lesions on mature foliage of *C. torelliana* hybrids, with high levels of infection resulting in premature leaf senescence. Unlike symptoms on spotted gum, sporulation on the hybrids was restricted to the abaxial leaf surfaces and often only occurred in the centres of the lesions. The lesions were surrounded by a water-soaked margin, which was also a symptom not observed on spotted gum. It is unclear whether this unusual symptom is the result of host reaction or climatic conditions in tropical north Queensland. In light of this finding, the development of disease screening considering both parent species should be an essential part of future breeding programmes.

DNA sequence comparisons showed that the isolates collected from *C. torelliana* and the hybrids in tropical north Queensland were genetically unique and not found in any other region or on spotted gum species within the same plantations or districts. Isolates of *Q. pitereka* taken from other hybrid trees in southeast Queensland were also different from those in north Queensland, suggesting geographic barriers to gene flow. Interestingly, *C. torelliana* has a restricted native range in northern Queensland west of Ingham to south of Cooktown in the coastal foothills, in addition to occasional stands in the rainforest (Brooker & Kleinig, 1996). It is possible that the *Q. pitereka* haplotype found on this species has evolved in this region in association with the natural population of *C. torelliana*. This could be significant in relation to the selection of geographic locations for screening of *Corymbia* hybrids for use in future plantation development.

Quambalaria eucalypti was detected from shoot and leaf blight, leaf spots and stem lesions on *Eucalyptus* species over a wide geographic range and it was often associated with insect damage. Carnegie (2007a) provided a tentative report of this fungus from New South Wales, but this study, including DNA sequence comparisons, confirms its presence in Australia. This is also the first record of *Q. eucalypti* occurring on a *Corymbia* species, albeit a hybrid (*C. torelliana* × *C. variegata*). Lesions caused by *Q. eucalypti* were restricted and often limited to sites where insect browsing had occurred, particularly that of weevils. While the mode of infection of *Quambalaria* spp. is not yet known, it is possible that the wounds created by insects provided entry points for the fungus. The role of insects as vectors of the fungus should be considered, especially given the fact that *Q. cyanescens* was found associated with bark beetles in Hungary, Bulgaria and the Mediterranean (Kolarik *et al*., 2006).

Quambalaria eucalypti does not appear to be a significant threat to plantation health in Australia. It was detected only in one instance (in 2004) in forest health surveys in New South Wales from 1996 to 2005 (Carnegie, 2007a), but was relatively common in the 2006 surveys that formed part of this study. Similarly, *Q. eucalypti* was not found in Queensland in surveys that began in 1996, and its appearance in this survey in 2006 is intriguing. This may indicate that levels of *Q. eucalypti* are increasing. Why this might be occurring is uncertain, but it may be caused by the expansion of high density planting of *Eucalyptus* species in subtropical regions of eastern Australia. However, this does not explain the occurrence of the disease in residential gardens, geographically isolated from any form of plantation development. *Quambalaria eucalypti* is presently seen as one of the most serious pathogens of *Eucalyptus* spp. in nurseries in Brazil (Alfenas *et al*., 2001) and it has more recently been detected causing severe shoot and leaf blight and stem cankers in young trees in provenance trials in South Africa (Roux *et al*., 2006). In South Africa it was not initially believed to be a serious threat (Wingfield *et al*., 1993) as it was restricted to nurseries. As more plantations of *Eucalyptus* spp. are developed in Australia, it seems likely that this fungus will gain importance.

Quambalaria cyanescens was detected on woody stems and branches on native and plantation spotted gum, but its role as a pathogen on spotted gum appears to be limited. This study provides the first record of *Q. cyanescens* associated with plantation and native spotted gum in Australia. However, this is not the first report of its association with eucalypts, as a fungus was isolated from *E. pauciflora* in New South Wales under the name *S. cyanescens* (de Hoog & de Vries, 1973). More recently, *Q. cyanescens* was found to be associated with cankers on *C. calophylla* and *C. ficifolia* in combination with *Q. pitereka* in Western Australia (Paap *et al*., 2008); in that study pathogenicity trials confirmed that *Q. cyanescens* was non-pathogenic to *Corymbia* spp. This fungus has also been identified from human skin and tissue samples (de Hoog & de Vries, 1973), as well as being associated with processed oats (Da Silva *et al*., 1999). The presence

of *Q. cyanescens* in both plantations and native spotted gum trees is of interest, but there currently appears to be no damage to tree health or wood quality. Lesions were not found to extend into the cambium or result in the development of resin pockets within the timber of juvenile or mature timber. However, additional research will be required to eliminate this fungus as a potential cause of stem degradation in commercial plantations in subtropical Australia.

Previous studies on *Quambalaria* spp. suggested that *Q. pitereka* and *Q. eucalypti* are endemic to Australia and have been introduced into new areas with seed used for plantation development (Wingfield *et al*., 1993; Roux *et al*., 2006; Zhou *et al*., 2007). DNA sequence comparisons for isolates of *Q. eucalypti* from South Africa (Roux *et al*., 2006) and *Q. pitereka* from China (Zhou *et al*., 2007) found no variation amongst isolates of these two species. Conversely, isolates of *Q. eucalypti* and *Q. pitereka* collected in this study were variable, with four and nine ITS haplotypes, respectively. The genetic diversity observed in Australia compared to that elsewhere in the world, combined with the native distribution and abundance of *Eucalyptus* and *Corymbia* species, supports the view that Australia is the likely centre of origin of *Q. eucalypti* and *Q. pitereka*. The apparently clonal population of *Q. pitereka* collected from *C. maculata* in Western Australia (Paap *et al*., 2008) also suggests that the fungus has been introduced into that part of the continent, where *C. maculata* does not occur naturally. Interestingly, there was no commonality between the Western Australian isolates and those collected from eastern subtropical and tropical regions of Australia. Further collections of *Q. pitereka* isolates for population genetic studies may help to develop an improved understanding of the origin and population structure of this pathogen in Australia.

Quambalaria species are becoming globally more significant as pathogens in eucalypt plantations. This is the first comprehensive survey to be undertaken and both *Q. pitereka* and *Q. eucalypti* were found to be widely distributed on a range of hosts throughout the subtropical and tropical regions of eastern Australia. The variability identified from DNA sequence comparisons for both species provides an insight into their population biology. Further understanding of this variation and how it might relate to pathogenicity to particular host species will be significant in the development of future tree breeding and risk management programmes. Understanding the factors influencing disease development, the impact of infection on growth rates and wood quality and host pathogen interactions will also be vital in the development of disease management strategies.

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