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RESEARCH ARTICLE

Quambalaria species associated with eucalypt diseases in southern China

Shuaifei CHEN[1,2], Qianli LIU[1], Guoqing LI[1], Michael J. WINGFIELD (✉)[2]

1 China Eucalypt Research Centre, Chinese Academy of Forestry, Zhanjiang 524022, China
2 Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria 0028, South Africa

Abstract The genus *Quambalaria* includes several important pathogens of species of *Eucalyptus* and *Corymbia*, mainly causing leaf and shoot blight. Recently, extensive shoot and leaf dieback and stem cankers suspected to be *Quambalaria* diseases have been found on young *Eucalyptus urophylla* × *E. grandis* trees in Guangdong and Hainan Provinces. The occurrence of *Quambalaria* species and their association with eucalypt hosts within China needs to be investigated for tree diseases management. The isolates from the diseased samples were identified based on their morphological structures and phylogenetic analyses with DNA sequence data for the internal transcribed spacer region and large ribosome subunit RNA of the nuclear rDNA. This work revealed that three species of *Quambalaria* were present: *Quambalaria pitereka* from *Corymbia citriodora*, *Q. eucalypti* from *E. urophylla* × *E. grandis*, both isolated from young eucalypt leaves and shoots in Guangdong Province, and *Quambalaria simpsonii*, which was isolated from stem cankers of *E. urophylla* × *E. grandis* at four different sites across Guangdong and Hainan Provinces. These results confirmed that *Quambalaria* agents were associated with the diseases occurring on eucalypt hosts in South China. This is the first report of *Q. eucalypti* in Asia and the first report of *Q. simpsonii* in China on *Eucalyptus* trees.

Keywords *Corymbia*, *Eucalyptus*, forest pathogens, plantations, Myrtaceae

1 Introduction

In China, *Eucalyptus* spp. (Myrtaceae) have been widely established in commercial plantations which cover about 4.5 million hectares in southern China[1]. They include mainly cloned hybrids of *Eucalyptus urophylla* and *E. grandis*, other *Eucalyptus* species include *E. camaldulensis*, *E. dunnii*, *E. globulus*, *E. pellita*, *E. smithii*, *E. urophylla*, as well as their hybrids and clones[2–4]. *Corymbia citriodora* (Myrtaceae), previously classified as a species of *Eucalyptus,* has also been widely planted in southern China[2,3] and the two genera are collectively referred to in this paper as eucalypts.

The extensive development of eucalypt plantations in China and the relatively limited numbers of clones planted in the past two decades has resulted in the appearance of numerous pests and pathogens that have caused increasing levels of damage[5]. Consequently, extensive surveys of eucalypt plantations have been undertaken in southern China, resulting in several important diseases being recorded. These include stem diseases caused by *Teratosphaeria zuluensis*[6,7], species of Botryosphaeriaceae[8], species of Cryphonectriaceae[9,10] and *Ceratocystis*[11]. Leaf diseases caused by *Calonectria* spp.[12–14], species of Mycosphaerellaceae and Teratosphaeriaceae[15,16] have also emerged as serious problems. The leaf and shoot pathogen, *Quambalaria pitereka* has been found on *Corymbia citriodora* in the Guangdong Province of southern China[17].

Six species of *Quambalaria* occur on eucalypts. They include *Q. coyrecup*, *Q. cyanescens*, *Q. eucalypti*, *Q. pitereka*, *Q. pusilla* and *Q. simpsonii* and all appear to be native to Australia where their host trees also occur naturally[18–27]. *Quambalaria eucalypti* has also been found on native *Myrceugenia glaucescens* (Myrtaceae) trees in Uruguay, although it seems likely to have been introduced into that country[28]. Of the six *Quambalaria* spp., *Q. eucalypti* and *Q. pitereka* cause leaf and shoot blight on eucalypts[20,21,24], *Q. coyrecup* causes cankers and shoot blight on *Corymbia* spp.[23], and *Q. cyanescens* is generally regarded as a saprophyte[22]. It remains unknown as to whether *Q. simpsonii* is pathogenic to eucalypts[26], and the taxonomic status of *Q. pusilla* remains unresolved[22,29].

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Correspondence: mike.wingfield@fabi.up.ac.za

Recently, leaf and shoot blight with symptoms typical of those caused by species of *Quambalaria* was observed on young *C. citriodora* and *E. urophylla* × *E. grandis* trees in southern China. In addition, a fungus with morphological characteristics typical of *Quambalaria* was isolated from cankers on the stems of *E. urophylla* × *E. grandis* trees. The aims of this study were to identify these *Quambalaria* spp. based on comparisons of DNA sequence data and morphological characteristics.

2 Materials and methods

2.1 Collections of fungal isolates

Leaf and shoot blight was observed on *C. citriodora* trees of different provenances in two experimental plantations and an *E. urophylla* × *E. grandis* plantation in Guangdong Province in southern China (Figs. 1a, 1c, 1d and 1e). White masses of conidia and conidiophores characteristic of the

Fig. 1 Symptoms of infection by *Quambalaria* spp. on eucalypt trees. Shoot (a) and juvenile leave (b) of *Corymbia citriodora* infected by *Quambalaria pitereka* covered in white masses of conidia and conidiophores. New shoot (c) produced from the infected *C. citriodora*, and reinfected by *Q. pitereka*. Death of apical shoot (d) of *Eucalyptus urophylla* × *E. grandis* clone infected by *Quambalaria simpsonii*. Mature leaf (e) and young apical shoot (f) of *E. urophylla* × *E. grandis* clone infected by *Q. simpsonii*. Arrows indicate infected sites.

Quambalaria[21,22] were common on the surface of the infected leaves and shoots (Figs. 1b and 1f). Isolations were made by scraping conidial masses from the leaf and shoot surfaces and transferring these to 2% malt extract agar (MEA) medium (20 g malt extract and 20 g agar per liter water) and incubated at 25°C. During the process of isolating the stem canker pathogen *Teratosphaeria zuluensis* (unpublished data) from cankered *E. urophylla* × *E. grandis* hybrid trees, a fungus with the morphological characteristics of *Quambalaria* species was isolated and these cultures were included in the present study. All *Quambalaria* isolates were collected during August 2015 and June 2016.

After the fungi had been cultured for 10 d on 2% MEA, single germ tubes emerging from colonies were subculture on 2% MEA media to obtain pure cultures. Cultures were deposited in the culture collection of the China Eucalypt Research Centre, Chinese Academy of Forestry, Zhanjiang, China. Representative isolates were also deposited at the China Forestry Culture Collection Centre, Beijing, China (Table 1).

2.2 DNA extraction, PCR and sequence reactions

Isolates collected from eucalypt trees in this study were identified based on DNA sequence comparisons (Table 1). For DNA extraction, isolates were grown on 2% MEA at 25°C for 10 d after which actively growing mycelium for each isolate was scraped from the surface of the medium using sterile scalpel blades and transferred to 1.5-mL Eppendorf tubes. DNA was extracted using "method 5" described by Van Burik et al.[31]. The concentration of resulting DNA was checked using a Nano-Drop 2000 Spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Two gene regions, the internal transcribed spacer (ITS) regions including the 5.8S gene of the rDNA operon and the conserved nuclear large subunit (LSU) rDNA were amplified as described by Chen et al.[32]. Nucleotide sequences were edited using MEGA version 4 software[33]. All sequences obtained in this study were deposited in GenBank (Table 1).

2.3 Phylogenetic analyses

To identify the isolates, sequences of ITS and LSU gene regions were compared to sequences of all described *Quambalaria* species, including the ex-type cultures of all the identified species from GenBank (Table 1). Also, in order to examine the diversity of the *Quambalaria* species, the haplotypes were determined from the polymorphic nucleotides within the aligned sequence data of ITS and LSU regions for isolates collected in this and previous studies.

To characterize the haplotypes from ITS sequences, all haplotypes designated by Pegg et al.[24] were determined for all isolates of *Quambalaria* spp. from this and previous studies (Table 1). For phylogenetic analyses, two isolates representing each haplotype were used. Where only one isolate was available for a particular haplotype, this isolate was duplicated in the phylogenetic analyses to determine whether it would reside in an independent clade. *Microstroma juglandis* was used as the outgroup taxon (Table 1).

For haplotype determination using LSU sequences, representative Chinese isolates which included all the haplotypes determined based on the ITS sequences, and all isolates for which the LSU had been sequenced in previous studies were included (Table 1). All isolates used for haplotype determination by LSU sequences were used in the phylogenetic analyses. Where only one isolate was available for a particular haplotype, the isolates were duplicated in the phylogenetic analyses. *M. juglandis* was also used as the outgroup taxon for the LSU analyses (Table 1).

Sequences in ITS and LSU data sets were aligned using the iterative refinement method (FFT-NS-i settings) of the online platform of MAFFT v. 5.667[34]. The alignments were further edited manually in MEGA version 4 software[33]. All alignments were deposited in TreeBASE.

The phylogenetic analyses were conducted using the maximum likelihood (ML) method, the ML tests were conducted with PHYML v. 3.0[35] and the best models of nucleotide substitution were established with MODELTEST v. 3.7[36]. The analyses were conducted using PHYML v. 3.0[35]. Additional ML parameters in PHYML included retention of the maximum number of 1000 trees and the determination of nodal support by nonparametric bootstrapping with 1000 replicates. The phylogenetic trees were viewed using MEGA version 4 software[33].

2.4 Morphology

Single hyphal tip cultures of each *Quambalaria* sp. identified using DNA sequence data were subculture on 2% MEA media for 2 weeks at 25°C for morphological analysis. Four isolates for each identified *Quambalaria* sp. were used for comparisons of colony morphology. Conidiogenous cells and conidia were mounted in sterile water on microscope slides for measurements to be made using a Zeiss Axio Imager A1 microscope and a Zeiss AxioCam MRc digital camera with Zeiss Axio Vision Rel. 4.8 software (Carl Zeiss Ltd., Munchen, Germany). For each isolate, 25 measurements were made of conidia and ten of conidiophores. These measurements were compared with those published for species of *Quambalaria*. Results are presented as (minimum–) (mean – standard deviation) – (mean + standard deviation) (–maximum).

Table 1 Isolates of *Quambalaria* species collected from eucalypt trees in southern China in 2015 and 2016 and used for phylogenetic and morphological analysis

Identity	Isolate No.[a]	GenBank accession No.[b]		Host	Location	Collector	Reference
		ITS	LSU				
Quambalaria coyrecup	WAC12947[cde]	DQ823431	DQ823444	*Corymbia calophylla*	Western Australia, Australia	T Paap	Paap et al.[23]
Q. coyrecup	WAC12948[de]	DQ823433	DQ823446	*C. calophylla*	Western Australia, Australia	T Paap	Paap et al.[23]
Q. coyrecup	WAC12949[e]	DQ823432	DQ823445	*C. calophylla*	Western Australia, Australia	T Paap	Paap et al.[23]
Q. coyrecup	WAC12950[de]	DQ823429	DQ823447	*C. calophylla*	Western Australia, Australia	T Paap	Paap et al.[23]
Q. coyrecup	WAC12951[de]	DQ823430	DQ823448	*C. calophylla*	Western Australia, Australia	T Paap	Paap et al.[23]
Q. coyrecup	BRIP48338[d]	EF444877	N/A[g]	*C. polycarpa*	Darwin, Northern Territory, Australia	R Pitkethley	Pegg et al.[24]
Q. coyrecup	BRIP48339[d]	EF444878	N/A	*C. polycarpa*	Darwin, Northern Territory, Australia	R Pitkethley	Pegg et al.[24]
Q. cyanescens	CBS357.73[cde] = CMW5583	DQ317622	DQ317615	*skin of man*	Netherlands	TF Visser	de Beer et al.[22]
Q. cyanescens	CBS876.73[de] = CMW5584	DQ317623	DQ317616	*Eucalyptus pauciflora*	New South Wales, Australia	VF Brown	de Beer et al.[22]
Q. cyanescens	WAC12952[de]	DQ823419	DQ823440	*C. calophylla*	Western Australia, Australia	T Paap	Paap et al.[23]
Q. cyanescens	WAC12953[de]	DQ823422	DQ823443	*C. ficifolia*	Western Australia, Australia	T Paap	Paap et al.[23]
Q. cyanescens	WAC12954[e]	DQ823420	DQ823442	*C. calophylla*	Western Australia, Australia	T Paap	Paap et al.[23]
Q. cyanescens	WAC12955[de]	DQ823421	DQ823441	*C. calophylla*	Western Australia, Australia	T Paap	Paap et al.[23]
Q. cyanescens	BRIP48396[d]	EF444874	N/A	Native *C. citriodora*	Beaudesert, Queensland, Australia	GS Pegg	Pegg et al.[24]
Q. cyanescens	BRIP48398[d]	EF444875	N/A	Native *C. citriodora*	Beaudesert, Queensland, Australia	GS Pegg	Pegg et al.[24]
Q. cyanescens	BRIP48403[d]	EF444876	N/A	Native *C. citriodora*	Beaudesert, Queensland, Australia	GS Pegg	Pegg et al.[24]
Q. eucalypti	CBS118844[cde] = CMW1101	DQ317625	DQ317618	*Eucalyptus grandis*	Kwambonambi, South Africa	MJ Wingfield	de Beer et al.[22]
Q. eucalypti	CBS 119680[de] = CMW11678	DQ317626	DQ317619	*E. grandis* clone NH58	Kwambonambi, South Africa	L Lombard	de Beer et al.[22]
Q. eucalypti	CMW14329	DQ317614	N/A	*E. grandis* × *E. camaldulensis* clone	Kwambonambi, South Africa	J Roux	Roux et al.[30]
Q. eucalypti	CBS118615 = CMW17252	DQ317609	N/A	*E. nitens*	Rooihoogte, South Africa	ZL Mthalane & J Roux	Roux et al.[30]
Q. eucalypti	CMW17253	DQ317610	N/A	*E. nitens*	Rooihoogte, South Africa	ZL Mthalane & J Roux	Roux et al.[30]
Q. eucalypti	CMW17254	DQ317611	N/A	*E. nitens*	Rooihoogte, South Africa	ZL Mthalane & J Roux	Roux et al.[30]
Q. eucalypti	CMW17255	DQ317612	N/A	*E. nitens*	Rooihoogte, South Africa	ZL Mthalane & J Roux	Roux et al.[30]
Q. eucalypti	CBS118616 = CMW17771	DQ317613	N/A	*E. grandis* clone	Kwambonambi, South Africa	J Roux	Roux et al.[30]
Q. eucalypti	UY1036	EU439922	N/A	*Myrceugenia glaucescens*	Uruguay	C. A. Pérez	Pérez et al.[28]

(*Continued*)

Identity	Isolate No.[a]	GenBank accession No.[b]		Host	Location	Collector	Reference
		ITS	LSU				
Q. eucalypti	UY1718	EU439923	N/A	*M. glaucescens*	Uruguay	C. A. Pérez	Pérez et al.[28]
Q. eucalypti	PE3/MEAN 996	JX297605	N/A	*E. globulus*	Portugal	N/A	Braganca et al.[27]
Q. eucalypti	PE6/MEAN 997	JX297603	N/A	*E. globulus*	Portugal	N/A	Braganca et al.[27]
Q. eucalypti	PE27/MEAN 998	JX297604	N/A	*E. globulus*	Portugal	N/A	Braganca et al.[27]
Q. eucalypti	PE28/MEAN 999	JX297600	N/A	*E. globulus*	Portugal	N/A	Braganca et al.[27]
Q. eucalypti	PE29/MEAN 1000	JX297602	N/A	*E. globulus*	Portugal	N/A	Braganca et al.[27]
Q. eucalypti	PE30/MEAN 1001	JX297601	N/A	*E. globulus*	Portugal	N/A	Braganca et al.[27]
Q. eucalypti	PE52/MEAN 1002	JX297606	N/A	*E. globulus*	Portugal	N/A	Braganca et al.[27]
Q. eucalypti	PE53/MEAN 1003	JX297598	N/A	*E. globulus*	Portugal	N/A	Braganca et al.[27]
Q. eucalypti	PE54/MEAN 1004	JX297599	N/A	*E. globulus*	Portugal	N/A	Braganca et al.[27]
Q. eucalypti	PE93/MEAN 1006	KR336802	N/A	*E. globulus*	Portugal	N/A	Braganca et al.[27]
Q. eucalypti	PE96/MEAN 1009	KR336803	N/A	*E. globulus*	Portugal	N/A	Braganca et al.[27]
Q. eucalypti	PE151/MEAN 1012	KR336804	N/A	*E. globulus*	Portugal	N/A	Braganca et al.[27]
Q. eucalypti	PE152/MEAN 1013	KR336805	N/A	*E. globulus*	Portugal	N/A	Braganca et al.[27]
Q. eucalypti	PE153/MEAN 1014	KR336806	N/A	*E. globulus*	Portugal	N/A	Braganca et al.[27]
Q. eucalypti	PE154/MEAN 1015	KR336807	N/A	*E. globulus*	Portugal	N/A	Braganca et al.[27]
Q. eucalypti	BRIP48367	EF444823	N/A	*C. torelliana* × *C. citriodora* subsp. *variegata*	Walkamin, Queensland, Australia	GS Pegg	Pegg et al.[24]
Q. eucalypti	BRIP48422[d]	EF444832	N/A	*E. dunnii*	New South Wales, Australia	AJ Carnegie	Pegg et al.[24]
Q. eucalypti	BRIP48498[d]	EF444844	N/A	*E. grandis*	New South Wales, Australia	AJ Carnegie	Pegg et al.[24]
Q. eucalypti	BRIP48507[d]	EF444822	N/A	*E. grandis*	Moggill, Queensland, Australia	GS Pegg	Pegg et al.[24]
Q. eucalypti	**CERC8476**[d]	**KY615009**	N/A	*E. grandis*	Guangdong, China	SF Chen & JQ Li	This study
Q. eucalypti	**CERC8477**[g]	**KY615010**	N/A	*E. grandis*	Guangdong, China	SF Chen & JQ Li	This study
Q. eucalypti	**CERC8478**	**KY615011**	N/A	*E. grandis*	Guangdong, China	SF Chen & JQ Li	This study
Q. eucalypti	**CERC8479**[g]	**KY615012**	**KY615050**	*E. grandis*	Guangdong, China	SF Chen & JQ Li	This study
Q. eucalypti	**CERC8480**[g]	**KY615013**	N/A	*E. grandis*	Guangdong, China	SF Chen & JQ Li	This study
Q. eucalypti	**CERC8481**	**KY615014**	**KY615051**	*E. grandis*	Guangdong, China	SF Chen & JQ Li	This study
Q. eucalypti	**CERC8482**[g]	**KY615015**	N/A	*E. grandis*	Guangdong, China	SF Chen & JQ Li	This study
Q. eucalypti	**CERC8483**	**KY615016**	N/A	*E. grandis*	Guangdong, China	SF Chen & JQ Li	This study
Q. pitereka	DAR19773[cde]	DQ823423	DQ823438	*C. eximia*	New South Wales, Australia	J Walker & AL Bertus	Paap et al.[23]
Q. pitereka	CMW 6707[de]	DQ317627	DQ317620	*Corymbia maculata*	New South Wales, Australia	MJ Wingfield	de Beer et al.[22]

(*Continued*)

Identity	Isolate No.[a]	GenBank accession No.[b]		Host	Location	Collector	Reference
		ITS	LSU				
Q. pitereka	CBS118828[de] = CMW5318	DQ317628	DQ317621	*C. citriodora* subsp. *variegata*	Queensland, Australia	M Ivory	de Beer et al.[22]
Q. pitereka	CMW23610	EF427372	N/A	*C. citriodora*	Guangdong, China	YJ Xie	Zhou et al.[17]
Q. pitereka	CMW23611	EF427373	N/A	*C. citriodora*	Guangdong, China	YJ Xie	Zhou et al.[17]
Q. pitereka	CMW23612	EF427374	N/A	*C. citriodora*	Guangdong, China	YJ Xie	Zhou et al.[17]
Q. pitereka	CMW23613[d]	EF427375	N/A	*C. citriodora*	Guangdong, China	YJ Xie	Zhou et al.[17]
Q. pitereka	BRIP48325	EF427366	N/A	*C. citriodora* subsp. *variegata*	Queensland, Australia	GS Pegg	Zhou et al.[17]
Q. pitereka	BRIP48361[d]	EF427367	N/A	*C. citriodora* subsp. *variegata*	Queensland, Australia	GS Pegg	Zhou et al.[17]
Q. pitereka	BRIP48370[d]	EF427368	N/A	*C. citriodora* subsp. *variegata*	Queensland, Australia	GS Pegg	Zhou et al.[17]
Q. pitereka	BRIP48384[d]	EF427369	N/A	*C. citriodora* subsp. *variegata*	Queensland, Australia	GS Pegg	Zhou et al.[17]
Q. pitereka	BRIP48386[ed]	EF427370	N/A	*C. citriodora* subsp. *variegata*	Queensland, Australia	GS Pegg	Zhou et al.[17]
Q. pitereka	BRIP48531[d]	EF427371	N/A	*C. citriodora* subsp. *variegata*	Queensland, Australia	GS Pegg	Zhou et al.[17]
Q. pitereka	WAC12957[e]	DQ823426	DQ823437	*C. ficifolia*	Western Australia, Australia	T Paap	Paap et al.[23]
Q. pitereka	WAC12958[e]	DQ823427	DQ823436	*C. calophylla*	Western Australia, Australia	T Paap	Paap et al.[23]
Q. pitereka	QP26[e]	DQ823424	DQ823434	*C. citriodora* subsp. *variegata*	Queensland, Australia	GS Pegg	Paap et al.[23]
Q. pitereka	QP45[de]	DQ823425	DQ823439	*C. citriodora* subsp. *variegata*	Queensland, Australia	GS Pegg	Paap et al.[23]
Q. pitereka	BRIP48346[d]	EF444845	N/A	*C. citriodora* subsp. *citriodora*	Davies Creek, Queensland, Australia	GS Pegg	Pegg et al.[24]
Q. pitereka	BRIP48317	EF444854	N/A	*C. henryi*	Coolabunia, Queensland, Australia	GS Pegg	Pegg et al.[24]
Q. pitereka	BRIP48381[d]	EF444858	N/A	*C. citriodora* subsp. *citriodora*	Silkwood, Queensland, Australia	GS Pegg	Pegg et al.[24]
Q. pitereka	BRIP48383[d]	EF444859	N/A	*C. citriodora* subsp. *variegata*	Beaudesert, Queensland, Australia	GS Pegg	Pegg et al.[24]
Q. pitereka	WAC12956[d]	DQ823428	N/A	*C. ficifolia*	Western Australia, Australia	T Paap	Paap et al.[23], Pegg et al.[24]
Q. pitereka	BRIP48349[d]	EF444860	N/A	*C. torelliana* × *C. citriodora* subsp. *variegata*	Mareeba, Queensland, Australia	GS Pegg	Pegg et al.[24]
Q. pitereka	BRIP48325[d]	EF427366	N/A	*C. citriodora* subsp. *variegata*	Binjour, Queensland, Australia	GS Pegg	Pegg et al.[24]
Q. pitereka	BRIP48328[d]	EF444872	N/A	Native *C. citriodora* subsp. *variegata*	Dilkoon, New South Wales, Australia	GS Pegg	Pegg et al.[24]
Q. pitereka	BRIP48432[d]	EF444873	N/A	*C. citriodora* subsp. *variegata*	Grafton, New South Wales, Australia	GS Pegg	Pegg et al.[24]
Q. pitereka	**CERC8486**[de]	**KY615017**	**KY615052**	*C. citriodora provenance* CERC10	Guangdong, China	SF Chen & GQ Li	This study
Q. pitereka	**CERC8488**[e]	**KY615018**	**KY615053**	*C. citriodora provenance* CERC12	Guangdong, China	SF Chen & GQ Li	This study

(*Continued*)

Identity	Isolate No.[a]	GenBank accession No.[b]		Host	Location	Collector	Reference
		ITS	LSU				
Q. pitereka	**CERC8489**	**KY615019**	N/A	*C. citriodora provenance* CERC13	Guangdong, China	SF Chen & GQ Li	This study
Q. pitereka	**CERC8491**	**KY615020**	N/A	*C. citriodora provenance* CERC15	Guangdong, China	SF Chen & GQ Li	This study
Q. pitereka	**CERC8494**[eg]	**KY615021**	**KY615054**	*C. citriodora provenance* CERC17	Guangdong, China	SF Chen & GQ Li	This study
Q. pitereka	**CERC9093**	**KY615022**	N/A	*C. citriodora* provenance CR76	Guangdong, China	SF Chen & Y Lin	This study
Q. pitereka	**CERC9094**	**KY615023**	N/A	*C. citriodora* provenance N371	Guangdong, China	SF Chen & Y Lin	This study
Q. pitereka	**CERC9095**	**KY615024**	N/A	*C. citriodora* provenance N28	Guangdong, China	SF Chen & Y Lin	This study
Q. pitereka	**CERC9096**	**KY615025**	N/A	*C. citriodora* provenance N411	Guangdong, China	SF Chen & Y Lin	This study
Q. pitereka	**CERC9097**[eg]	**KY615026**	**KY615055**	*C. citriodora* provenance N223	Guangdong, China	SF Chen & Y Lin	This study
Q. pitereka	**CERC9098**[g]	**KY615027**	N/A	*C. citriodora* provenance N322	Guangdong, China	SF Chen & Y Lin	This study
Q. pitereka	**CERC9099**[eg]	**KY615028**	**KY615056**	*C. citriodora provenance* CR033	Guangdong, China	SF Chen & Y Lin	This study
Q. pitereka	**CERC9100**	**KY615029**	N/A	*C. citriodora provenance* CR039	Guangdong, China	SF Chen & Y Lin	This study
Q. pitereka	**CERC9101**	**KY615030**	N/A	*C. citriodora* provenance CR92	Guangdong, China	SF Chen & Y Lin	This study
Q. pitereka	**CERC9102**	**KY615031**	N/A	*C. citriodora* provenance CR36	Guangdong, China	SF Chen & Y Lin	This study
Q. pitereka	**CERC9103**[e]	**KY615032**	**KY615057**	*C. citriodora* provenance N601	Guangdong, China	SF Chen & Y Lin	This study
Q. pitereka	**CERC9104**	**KY615033**	N/A	*C. citriodora* provenance N28	Guangdong, China	SF Chen & Y Lin	This study
Q. simpsonii	CBS 124772[de]	**GQ303290**	**GQ303321**	*Eucalyptus tintinnans*	Edith Falls, Australia	BA Summerell	Cheewangkoon et al.[26]
Q. simpsonii	CBS 124773[de]	**GQ303291**	**GQ303322**	*Eucalyptus* sp.	Lamphoon, Thailand	R Cheewangkoon	Cheewangkoon et al.[26]
Q. simpsonii	**CERC8496**[dg]	**KY615034**	N/A	*E. urophylla* × *E. grandis*	Hainan, China	SF Chen & QL Liu	This study
Q. simpsonii	**CERC8499**	**KY615035**	N/A	*E. urophylla* × *E. grandis*	Hainan, China	SF Chen & QL Liu	This study
Q. simpsonii	**CERC8505**[d]	**KY615036**	N/A	*E. urophylla* × *E. grandis*	Hainan, China	SF Chen & QL Liu	This study
Q. simpsonii	**CERC8507**[de]	**KY615037**	**KY615058**	*E. urophylla* × *E. grandis*	Hainan, China	SF Chen & QL Liu	This study
Q. simpsonii	**CERC8512**[d]	**KY615038**	N/A	*E. urophylla* × *E. grandis*	Hainan, China	SF Chen & QL Liu	This study
Q. simpsonii	**CERC8514**	**KY615039**	N/A	*E. urophylla* × *E. grandis*	Hainan, China	SF Chen & QL Liu	This study
Q. simpsonii	**CERC8516**	**KY615040**	N/A	*E. urophylla* × *E. grandis*	Hainan, China	SF Chen & QL Liu	This study
Q. simpsonii	**CERC8517**[e]	**KY615041**	**KY615059**	*E. urophylla* × *E. grandis*	Hainan, China	SF Chen & QL Liu	This study
Q. simpsonii	**CERC8519**[dg]	**KY615042**	N/A	*E. urophylla* × *E. grandis*	Hainan, China	SF Chen & QL Liu	This study

(*Continued*)

Identity	Isolate No.[a]	GenBank accession No.[b]		Host	Location	Collector	Reference
		ITS	LSU				
Q. simpsonii	**CERC8526**	**KY615043**	N/A	*E. urophylla* × *E. grandis*	Hainan, China	SF Chen & QL Liu	This study
Q. simpsonii	**CERC8532**	**KY615044**	N/A	*E. urophylla* × *E. grandis*	Guangdong, China	SF Chen & JQ Li	This study
Q. simpsonii	**CERC8534**[deg]	**KY615045**	**KY615060**	*E. urophylla* × *E. grandis*	Guangdong, China	SF Chen & JQ Li	This study
Q. simpsonii	**CERC8536**[e]	**KY615046**	**KY615061**	*E. urophylla* × *E. grandis*	Guangdong, China	SF Chen & JQ Li	This study
Q. simpsonii	**CERC8539**[eg]	**KY615047**	**KY615062**	*E. urophylla* × *E. grandis*	Guangdong, China	SF Chen & JQ Li	This study
Q. simpsonii	**CERC8541**[d]	**KY615048**	N/A	*E. urophylla* × *E. grandis*	Guangdong, China	SF Chen & JQ Li	This study
Q. simpsonii	**CERC8543**[d]	**KY615049**	N/A	*E. urophylla* × *E. grandis*	Guangdong, China	SF Chen & JQ Li	This study
Microstroma juglandis	R.B. 2042[de]	DQ317634	DQ317617	*Juglans regia*	Germany	R Bauer	de Beer et al.[22]

Note: [a] Designation of isolates and culture collections: WAC, Department of Agriculture Western Australia Plant Pathogen Collection, Perth, Australia; BRIP, the plant pathology herbarium for Queensland Department of Primary Industries and Fisheries, Australia; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW, Tree Protection Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CERC, China Eucalypt Research Centre (CERC), Chinese Academy of Forestry (CAF), ZhanJiang, GuangDong, China; DAR, the plant pathology herbarium for the Department of Agriculture in NSW, Australia; MEAN, fungal collection of Instituto Nacional de Investigação Agrária e Veterinária – INIAV, Oeiras, Portugal; R.B., Herbarium R. Bauer, Tübingen, Germany; Isolate numbers in boldface were collected in this study; [b] GenBank numbers in boldface were sequenced in this study; [c] Holotype specimens or ex-type isolates; [d] Isolates used in phylogenetic analyses by ITS sequence; [e] Isolates used in phylogenetic analyses by LSU sequence; [f] N/A = not available; [g] Isolates used in morphological studies.

3 Results

3.1 Collections of fungal isolates

A total of 41 fungal isolates showing typical morphology of *Quambalaria* species were isolated. Seventeen isolates were from leaves or shoots on 17 *C. citriodora* trees of 16 provenances in two experimental plantations in Guangdong Province, eight isolates were from leaves of one *E. urophylla* × *E. grandis* clone in one plantation in Guangdong Province, and 16 isolates were from cankers caused by *T. zuluensis* on the stems of *E. urophylla* × *E. grandis* clones in four plantations in Guangdong and Guangxi. Each of the 41 isolates was from a single tree and all were included in the DNA sequence comparisons and phylogenetic analyses (Table 1).

3.2 Phylogenetic analyses

The aligned ITS sequence data set consisted of 65 taxa and 634 characters (TreeBASE No. 20574). For the ML analyses, the Model Test analysis recommended a HKY + I + G model [Lset Base = (0.2639, 0.2186, 0.2071); Nst (number of substitution rate categories) = 2; Transition/transversion ratio = 2.6045; Rate matrix = (1.0000, 4.3151, 2.9747, 2.9747, 8.1747); Rates = gamma; Shape = 0.7544]. The phylogenetic analyses showed that isolates sequenced in this study resided in three clades that represent *Q. pitereka*, *Q. eucalypti* and *Q. simpsonii* (Fig. 2).

For the ITS sequences, all Chinese and all those from previous studies represented 32 haplotypes. These included three, seven, four, 12 and six haplotypes of *Q. coyrecup*, *Q. cyanescens*, *Q. eucalypti*, *Q. pitereka* *Q. simpsonii,* respectively (Tables 2–4, S1). The Chinese isolates collected in this study represented six haplotypes including one of Q. *pitereka*, one of *Q. eucalypti,* and four newly designated haplotypes of *Q. simpsonii* (Table S1).

The aligned LSU sequence data set consisted of 37 taxa and 561 characters (TreeBASE No. 20574). For ML analyses, model test analysis recommended a TrN + G model [Lset Base = (0.2492, 0.1916, 0.3025); Nst = 6; Rate matrix = (1.0000, 7.7487, 1.0000, 1.0000, 31.1002); Rates = equal]. The phylogenetic analyses showed that isolates sequenced in this study resided in three clades of *Q. pitereka*, *Q. eucalypti* and *Q. simpsonii*, respectively (Fig. 3).

For the LSU sequences, 13 Chinese isolates which included all six haplotypes determined based on ITS sequences were used for phylogenetic analyses. These isolates and all of those sequenced in previous studies represented six haplotypes. These included two haplotypes of *Q. pitereka* and one each of *Q. coyrecup*, *Q. cyanescens*, *Q. eucalypti* and *Q. simpsonii* (Table S1). The Chinese isolates included in this study represented three haplotypes including one newly designated haplotype of

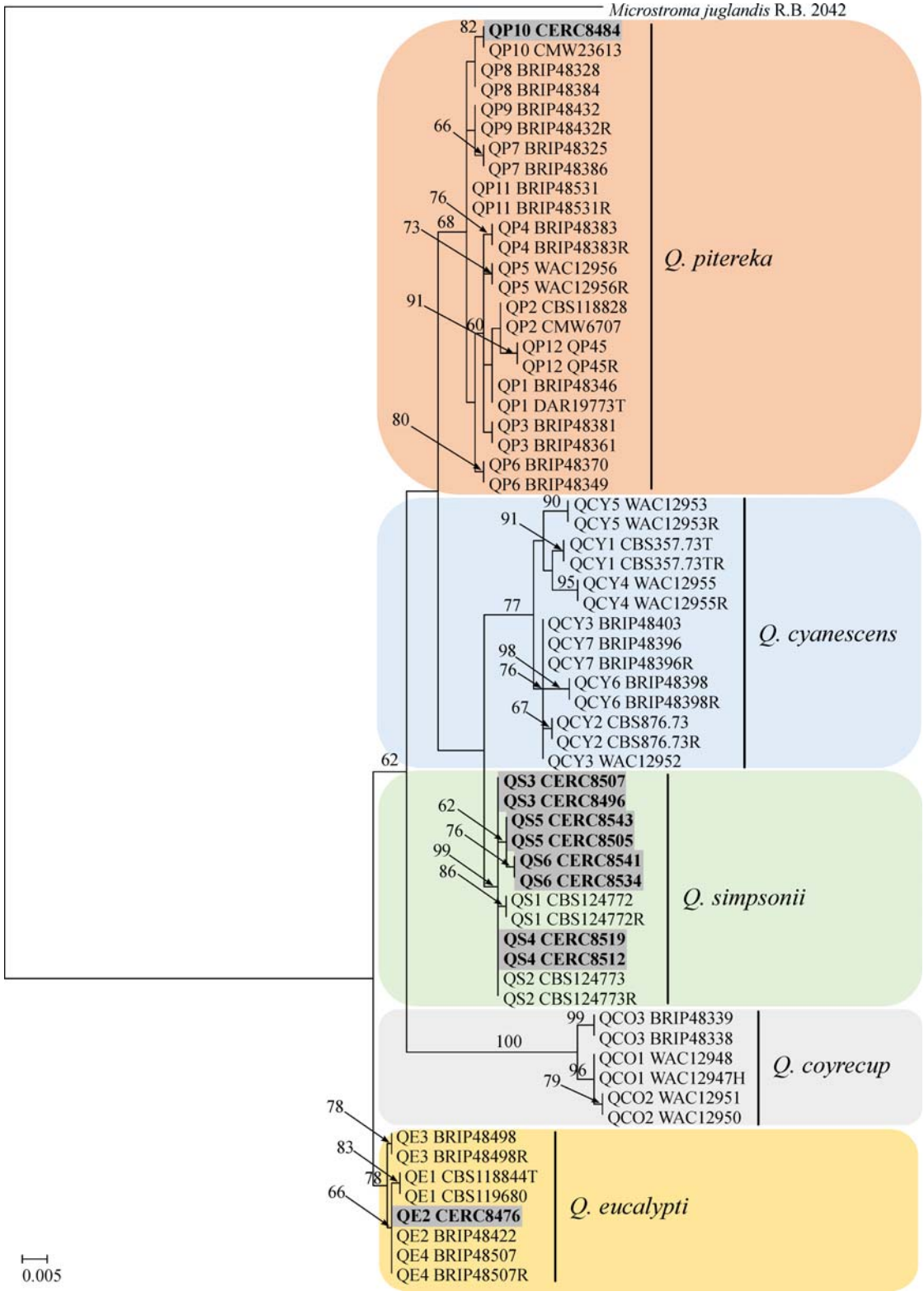

Fig. 2 Phylogenetic tree based on maximum likelihood analysis of ITS sequence data of haplotypes of five *Quambalaria* species, *Q. coyrecup* (QCO), *Q. cyanescens* (QCY), *Q. eucalypti* (QE), *Q. pitereka* (QP) and *Q. simpsonii* (QS). Bootstrap values > 60% are presented at branches, bootstrap values < 60% or absent values are not shown. Haplotypes and isolates from eucalypts in this study are in boldface and highlighted. Isolates representing ex-type are marked with T, isolates repeated are marked with R. The tree is rooted to *Microstroma juglandis*.

Table 2 Four haplotypes of *Q. eucalypti* as determined from the polymorphic nucleotides within the aligned sequence data of ITS region for isolates collected from species of *Eucalyptus*, *C. torelliana* × *C. citriodora* subsp. *variegate* and *M. glaucescens*

Haplotype	121[a]	158	159	160	161	162	558
QE1	T	–	–	–	–	–	**T**[b]
QE2	T	–	–	–	–	–	C
QE3	**C**	–	–	–	–	–	C
QE4	T	**T**	**T**	**A**	**T**	**A**	C

Note: [a] Base pair (bp) positions in aligned data; [b] Nucleotides that are different from the majority consensus sequence are underlined and highlighted in bold.

Table 3 Twelve haplotypes of *Q. pitereka* as determined from the polymorphic nucleotides within the aligned sequence data of ITS region for isolates collected from species of *Corymbia*

Haplotype	24[a]	54	107	112	214	219	233	236	390	451	606	614
QP1	T	A	G	G	T	**G**[b]	T	C	C	C	C	A
QP2	T	A	G	**A**	T	**G**	T	C	C	C	C	A
QP3	T	A	G	**A**	T	A	T	C	C	C	C	A
QP4	T	A	G	G	T	A	T	C	**T**	C	C	A
QP5	T	A	G	G	T	A	T	C	C	**A**	C	A
QP6	T	A	G	G	T	A	T	C	C	C	**T**	G
QP7	**A**	G	G	**G**	**G**	A	T	C	C	C	C	G
QP8	**A**	G	G	G	T	A	T	C	C	C	C	G
QP9	T	G	G	**G**	**G**	A	T	C	C	C	C	G
QP10	**A**	G	**A**	G	T	A	T	C	C	C	C	G
QP11	T	G	G	G	T	A	T	C	C	C	C	G
QP12	T	G	G	**A**	T	**G**	**C**	**G**	C	C	C	A

Note: [a] Base pair (bp) positions in aligned data; [b] Nucleotides that are different from the majority consensus sequence are underlined and highlighted in bold.

Table 4 Six haplotypes of *Q. simpsonii* as determined from the polymorphic nucleotides within the aligned sequence data of ITS region for isolates collected from species of *Eucalyptus*

Haplotype	4[a]	171	553	605	621
QS1	**A**[b]	A	**T**	T	–
QS2	**A**	A	C	T	–
QS3	G	A	C	T	**T**
QS4	G	A	C	T	–
QS5	G	A	C	**C**	–
QS6	G	**G**	C	**C**	–

Note: [a] Base pair (bp) positions in aligned data; [b] Nucleotides that are different from the majority consensus sequence are underlined and highlighted in bold.

Q. pitereka, and one haplotype for each of *Q. eucalypti* and *Q. simpsonii* (Table S1).

3.3 Morphology

Four isolates of *Q. pitereka* (CERC8494, CERC9097, CERC9098 and CERC9099), *Q. eucalypti* (CERC8477, CERC8479, CERC8480 and CERC8482) and *Q. simpsonii* (CERC8496, CERC8519, CERC8534 and CERC8539) were used in the morphological analysis. Colonies of these species were finely floccose becoming powdery and white (Figs. 4a, 4c and 4e). The morphological characteristics of the fruiting structures of these species are summarized in Table 5 and illustrated in Figs. 4b, 4d and 4f. Conidiogenous cells of *Q. pitereka*, *Q. eucalypti* and *Q. simpsonii* were (7.4–89.6) µm× (1.4–2.6) µm (av. 46.0 µm × 2.0 µm), (8.4–77.1) µm × (1.3–2.8) µm (av. 37.4 µm × 2.2 µm), and (7.0–82.1) µm × (1.5–2.9) µm (av. 25.6 µm × 2.4 µm), respectively. The conidia of *Q. pitereka* (primary conidia narrow fusiform, av. 10.9 µm × 3.4 µm, length/width = 3.2; secondary conidia narrow fusiform, av. 6.0 µm × 2.7 µm, length/width = 2.2) are longer and narrower (by length/width) than that of

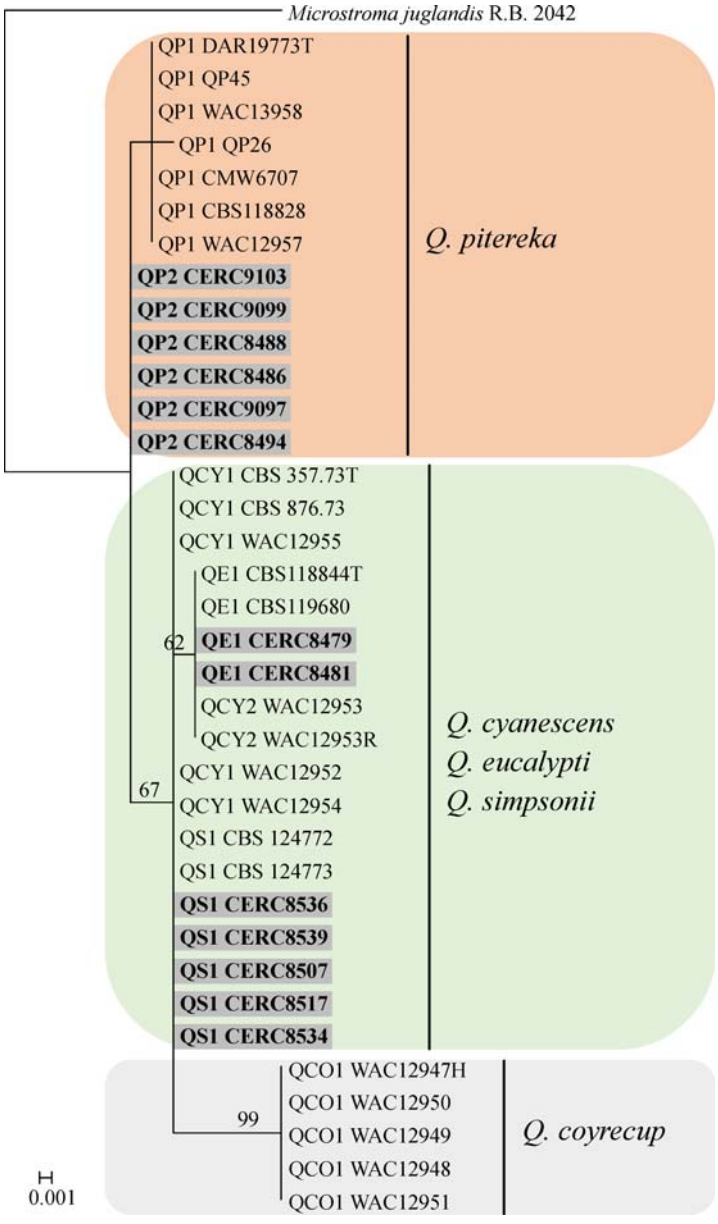

Fig. 3 Phylogenetic tree based on Maximum Likelihood analysis of large subunit sequence data of haplotypes of five *Quambalaria* species, *Q. coyrecup* (QCO), *Q. cyanescens* (QCY), *Q. eucalypti* (QE), *Q. pitereka* (QP) and *Q. simpsonii* (QS), respectively. Bootstrap values > 60% are presented at branches, bootstrap values < 60% or absent values are not shown. Haplotypes and isolates from eucalypts in this study are in boldface and highlighted. Isolates representing ex-type are marked with T, isolates repeated are marked with R. The tree is rooted to *Microstroma juglandis*.

Q. eucalypti (primary conidia ellipsoid, av. 6.2 µm × 3.8 µm, length/width = 1.6; secondary conidia obovoid, av. 3.3 µm × 2.6 µm, length/width = 1.3) and *Q. simpsonii* (primary conidia fusiform, av. 7.9 µm × 3.3 µm, length/width = 2.4; secondary conidia obovoid to ellipsoid, av. 3.7 µm × 2.4 µm, length/width = 1.5), the conidia of *Q. simpsonii* are slight longer and narrower than that of *Q. eucalypti*. The morphology of *Q. pitereka*, *Q. eucalypti* and *Q. simpsonii* identified in this study is similar to the results of previous studies[20,23,26].

4 Discussion

In this study, three species of *Quambalaria, Q. pitereka*, *Q. eucalypti* and *Q. simpsonii*, were identified from *Eucalyptus* and *Corymbia* plantations in Guangdong and Hainan Provinces in southern China. These *Quambalaria* spp. were identified and characterized based on phylogenetic analysis of sequence data for LSU and ITS regions, and morphology. This is the first report of *Q. eucalypti* in

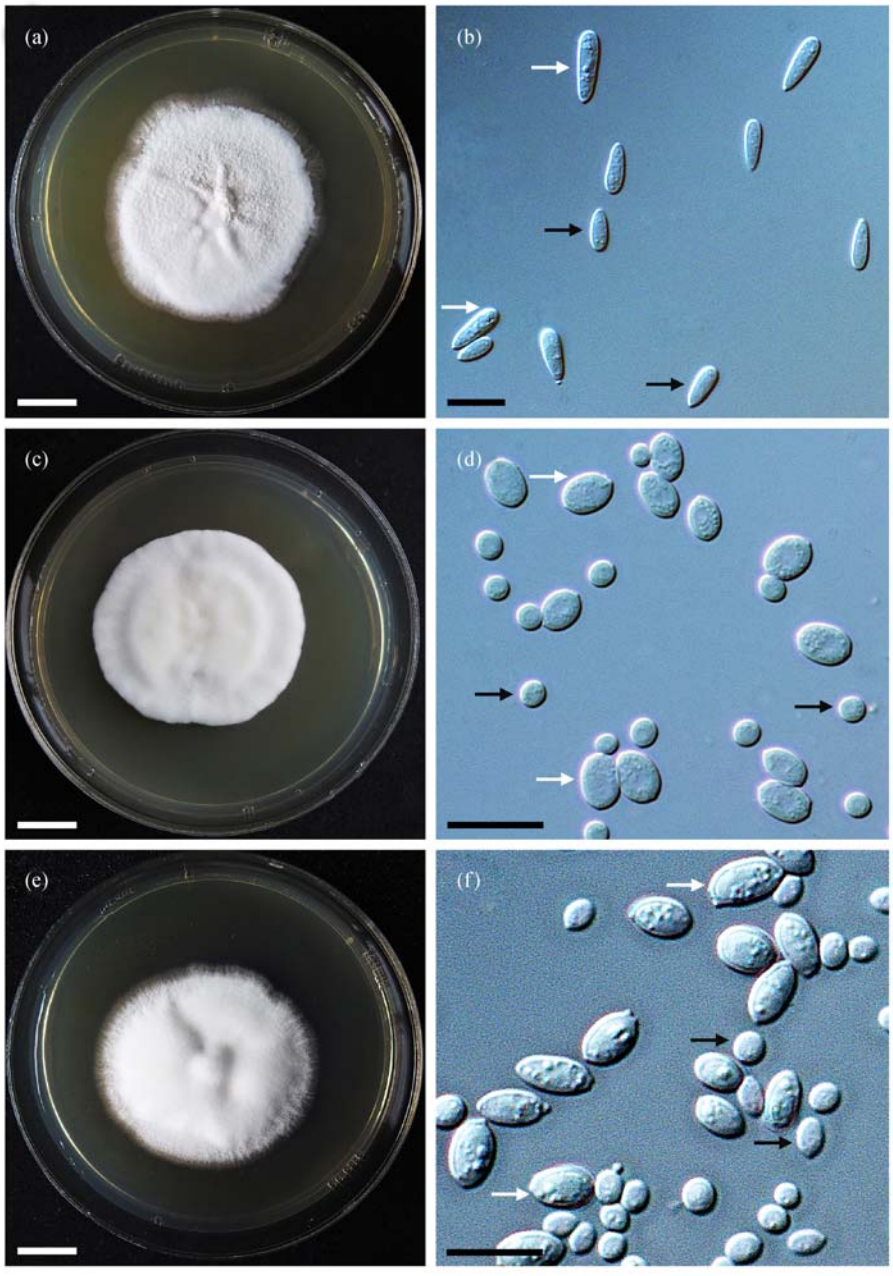

Fig. 4 Cultures grown on malt extract agar at 25°C after 2 weeks and the primary and secondary conidia. (a,b) *Quambalaria pitereka*; (c,d) *Q. eucalypti*; (e,f) *Q. simpsonii*.

Asia and the first report of *Q. simpsonii* on eucalypts in China.

Quambalaria pitereka is specific to eucalypts in the genus *Corymbia*. This fungus is widely distributed in different regions/sites on different species of *Corymbia* in Australia[24,25]. Outside Australia, *Q. pitereka* has previously been reported only on *C. citriodora* in one plantation in Guangdong Province[17]. The results of this study showed that the sequenced isolates of *Q. pitereka* include 12 haplotypes, only one of these was found in China and the remaining haplotypes were known only from Australia. This high level of genetic diversity for isolates from Australia supported the view[24] that *Q. pitereka* was native to that country. In the present study, *Q. pitereka* was isolated from 17 *C. citriodora* provenances in two experimental plantations. These are relatively distant from the site where *Q. pitereka* was first reported in 2007[17] and the ITS haplotype was the same as that found in the study of Zhou et al.[17]. These results suggest that *Q. pitereka* could spread actively between different regions and *C. citriodora* provenances in China.

Quambalaria eucalypti is considered to be one of the most important pathogens of eucalypts. Outside Australia, this fungus was first reported on *Eucalyptus* in nurseries in

Table 5 Primary conidial and secondary conidia measurements of three *Quambalaria* species identified in this study

Species	Isolate No.	Primary conidia			Secondary conidia		
		(L × W) size[a]/μm	(L × W) mean[b]/μm	L/W[c]	(L × W) sizea/μm	(L × W) mean[b]/μm	L/W[c]
Q. pitereka	CERC8494	(7.0–)7.5–13.0(–20.5) × (2.5–)3.0–3.5(–4.0)	10.2 × 3.3	3.1	(4.0–)4.5–6.0(–7.0) × 2.0–2.5(–3.0)	5.2 × 2.5	2.1
	CERC9097	(8.0–)8.5–12.5(–15.0) × (2.5–)2.5–3.5(–4.0)	10.5 × 3.0	3.5	(4.5–)5.0–6.5(–7.0) × 2.0–2.5(–3.0)	5.7 × 2.3	2.5
	CERC9098	(8.0–)10.5–14.0(–16.0) × (2.0–)3.0–4.5(–5.0)	12.2 × 3.6	3.4	6.0–7.5(–8.0) × (2.0–)2.5–3.5(–4.0)	6.8 × 3.0	2.3
	CERC9099	(8.0–)9.0–12.5(–15.0) × (3.0–)3.5–4.5(–5.5)	10.7 × 4.0	2.7	5.5–7.0(–7.5) × 2.5–3.5(–4.0)	6.3 × 3.1	2.2
	Average[d]	(7.0–)8.5–13.0(–20.5) × (2.0–)2.5–4.0(–5.5)	10.9 × 3.4	3.2	(4.0–)5.0–7.0(–78.0) × 2.0–3.0(–4.0)	6.0 × 2.7	2.2
Q. eucalypti	CERC8477	(4.5–)5.0–7.5(–8.0) × (3.0–)3.5–4.5(–4.5)	6.2 × 3.9	1.6	2.5–3.0(–3.5) × 2.5–3.0	3.0 × 2.5	1.2
	CERC8479	(5.5–)6.0–7.0(–7.5) × (2.5–)3.0–4.0(–4.5)	6.3 × 3.7	1.7	2.5–3.5(–4.0) × (2.0–)2.5–3.0	3.0 × 2.5	1.2
	CERC8482	(4.5–)5.0–6.5(–7.0) × (3.0–)3.5–4.0(–4.5)	5.7 × 3.9	1.5	3.0–4.0(–5.0) × 2.5–3.0	3.6 × 2.8	1.3
	CERC8480	(5.5–)6.0–7.0(–8.0) × (3.0–)3.5–4.0(–4.5)	6.4 × 3.8	1.7	3.0–3.6(–4.0) × 2.5–3.0	3.4 × 2.7	1.3
	Average[d]	(4.5–)5.5–7.0(–8.0) × (2.5–)3.5–4.0(–4.5)	6.2 × 3.8	1.6	(2.5–)3.0–4.0(–5.0) × (2.0–)2.5–3.0	3.3 × 2.6	1.3
Q. simpsonii	CERC8496	(6.0–)6.5–9.5(–11.0) × (2.0–)2.5–3.5(–4.0)	8.3 × 3.1	2.7	(3.0–)3.5–4.5(–5.0) × 2.0–2.5(–3.0)	4.0 × 2.4	1.7
	CERC8519	(5.5–)6.0–8.0(–9.0) × 3.0–4.0(–4.5)	7.1 × 3.6	2.0	(2.5–)3.0–3.5(–4.0) × 2.0–2.5	3.3 × 2.3	1.4
	CERC8534	(6.0–)7.0–9.0(–10.0) × (2.0–)3.0–3.5(–4.0)	7.9 × 3.2	2.5	(3.0–)3.5–4.5(–5.0) × 2.0–3.0	4.0 × 2.5	1.6
	CERC8539	(5.5–)6.5–10.5(–12.5) × (2.5–)3.0–4.0(–4.5)	8.4 × 3.4	2.5	3.0–4.0(–4.5) × 2.0–3.0	3.6 × 2.5	1.4
	Average[d]	(5.5–)6.5–9.5(–12.5) × (2.0–)3.0–4.0(–4.5)	7.9 × 3.3	2.4	(2.5–)3.0–4.0(–5.0) × 2.0–2.5(–3.0)	3.7 × 2.4	1.5

Note: [a] L × W = length × width, minimum–(average–standard deviation)–(average + standard deviation)–maximum; [b] L × W = length × width; [c] L/W = average length/average width; [d] average measurements of the *Quambalaria* species.

South Africa[20] and it was later found in Brazil[37] and Portugal[27] where it causes leaf spots, shoot infections and lesions on seedling stems. *Q. eucalypti* has also been recorded in *Eucalyptus* plantations in Brazil[38], South Africa[30], Australia[24] and Portugal[27] where it can result in severe shoot and leaf blight and stem cankers[24,27,30]. Other than on *Eucalyptus*, *Q. eucalypti* has been isolated from leaf lesions on native *M. glaucescens* trees in Uruguay[28] and *Corymbia* species in Australia[24]. In this study, *Q. eucalypti* was isolated from a diseased *E. urophylla* × *E. grandis* clone. It appears to be a pathogen of emerging importance in China.

The ITS haplotype determination showed that all four haplotypes of *Q. eucalypti* determined in this study are found in Australia. Only two of the four haplotypes have been found in other countries including China, Portugal, South Africa and Uruguay. Portugal, South Africa and Uruguay share the same haplotype, the other haplotype apart from Australia was only found in China. Results in this study support the view that *Q. eucalypti* is native to Australia and that this is the source of introductions to new areas[24].

Quambalaria simpsonii was first reported from species of *Eucalyptus* in Australia and Thailand, but it is unknown whether this is a pathogen[26]. In the present study, *Q. simpsonii* was consistently isolated with *T. zuluensis* from cankered *E. urophylla* × *E. grandis* stems in four sites in Guangdong and Guangxi, China. Whether *Q. simpsonii* is pathogenic to *Eucalyptus* trees, and the ecological interaction between *Q. simpsonii* and *T. zuluensis* remains to be clarified.

5 Conclusions

The genus *Quambalaria* presently includes six species. Most of these are pathogens that cause leaf and shoot blight, and cankers on *Eucalyptus* and *Corymbia*. They are considered native to Australia but have been inadvertently introduced into countries of Africa, Asia, Europe and South America. This has most likely occurred via the trade in eucalypt germplasm[39]. In the present study, three *Quambalaria* spp. were identified in China; *Q. pitereka* on *C. citriodora*, *Q. eucalypti* on clones *E. urophylla* × *E. grandis* and *Q. simpsonii* isolated from stem cankers of *E. urophylla* × *E. grandis* caused by *T. zuluensis*. These are widespread in areas of China where eucalypts are grown and they are likely to become more important to commercial forestry in the future.

Supplementary materials The online version of this articale at https:// doi.org/10.15302/J-FASE-2017173 contains supplementary material (Table S1).

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Compliance with ethics guidelines Shuaifei Chen, Qianli Liu, Guoqing Li, and Michael J. Wingfield declare they have no conflicts of interest or financial conflicts to disclose.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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