Plant Pathology & Quarantine 8(1): 1–8 (2018) ISSN 2229-2217
www.ppqjournal.org **Article**

Doi 10.5943/ppq/8/1/1

Isolation of *Calonectria sulawesiensis* from soil in Thailand and its pathogenicity against *Eucalyptus camaldulensis*

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Jessadarom H, Phetruang W, Haitook S, Cheewangkoon R 2018 – Isolation of *Calonectria sulawesiensis* from soil in Thailand and its pathogenicity against *Eucalyptus camaldulensis*. Plant Pathology & Quarantine 8(1), 1–8, Doi 10.5943/ppq/8/1/1

Abstract

Calonectria species are abundant and widely distributed in tropical and subtropical countries. In our survey four isolates of *Calonectria sulawesiensis* were isolated from soil collected in Chiang Mai Province, Thailand. The identification was based on morphological characteristics and the phylogenetic analyses of translation elongation factor 1-alpha gene (*tef1-α*) regions. This is the first report of this fungus in Thailand. In vitro pathogenicity showed *Ca. sulawesiensis* activity against eucalyptus.

Key words – *Cylindrocladium* – pathogenicity

Introduction

The genus *Calonectria* was introduced in 1867 by De Notaris, to accommodate *Ca. daldiniana* from dead leaves of *Magnolia grandiflora* (Rossman 1979) and resides in the Nectriaceae (Rossman et al. 1999, Hibbett et al. 2007, Schoch et al. 2009). *Calonectria* is distinguished from other genera of the family Nectriaceae by its *Cylindrocladium* asexual state (Rossman et al. 1999). *Calonectria* shows a wide range of pathogenicity on a broad range of host plants worldwide (Crous 2002). In agriculture and forestry, *Calonectria* species have been reported to cause important diseases in several plant families such as Fabaceae, Myrtaceae, Pinaceae and Solanaceae (Crous & Kang 2001, Crous 2002, Lombard et al. 2009, 2010a). Currently 248 species are listed in Index Fungorum (Wijayawardene et al. 2017). Nonetheless, information on diversity of *Calonectria* in Thailand is inadequate, and presently only eight species have been reported namely: *Ca. asiatica* (*Cy. asiaticum*) (Crous et al. 2004, Lombard et al. 2015, 2016), *Ca. colhounii* (*Cy. colhounii*) (Henricot 2002, Crous et al. 2006), *Ca. kyotensis* (*Cy. floridanum*) (Henricot 2002), *Ca. malesiana* (*Cy. malesianum*) (Lombard et al. 2015, 2016), *Ca. monticola* (Lombard et al. 2016), *Ca. quinqueseptata* (*Cy. quinqueseptatum*) (Crous et al. 2002, Henricot 2002), *Ca. reteaudii* (*Cy. reteaudii*) (Crous et al. 2005, 2006) and *Ca. variabilis* (*Cy. pacificum*) (Kang et al. 2001, Henricot 2002). The main objective of the present study was to collect and identify species of *Calonectria* from soil in Thailand to provide information concerning the fungal diversity of this genus in Thailand.

Submitted 20 December 2017, Accepted 5 January 2018, Published 12 January 2018
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Materials & Methods

Fungal isolation and morphological studies

Soil samples were collected from different locations in Chiang Mai Province, northern Thailand including natural forest and commercial locations and the samples were baited with sterilized leaf discs of Eucalyptus as described by Crous (2002). Developing fungi were observed daily and isolated onto potato dextrose agar (PDA). The cultures were incubated for 10 days at room temperature (25–28ºC). Morphological characteristics were studied by mounting fungal tissues in 85% lactic acid. Thirty measurements were made of conidiophores, conidiogenus apparatus, and conidia at 630× magnification using a Plan-Apochromat 100x/1.4 oil immersion lens (Carl Zeiss, Germany) mounted on a Zeiss1090 Axioscope A1 microscope, with differential interference contrast (DIC) illumination. Colony colour was assessed using 7-day-old cultures grown on PDA incubated at room temperature using the colour charts of Rayner (1970). Living cultures were deposited in the working collection of Pedro Crous (CPC), housed at Westerdijk Fungal Biodiversity Institute (WI), Utrecht, Netherlands**.**

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from 7-day-old cultures established from single conidial propagules, grown on malt extract agar (MEA) at room temperature, using the UltraClean™ microbial DNA isolation kit (Mo Bio Laboratories, California, USA) following the protocols provided by the manufacturer. Partial gene sequences were determined for the translation elongation factor 1-alpha (*tef1-α*) region using the primers and protocols described by Lombard et al. (2010b).

Phylogeny

The phylogenetic study was based upon translation elongation factor 1-alpha (*tef1-α*). Twelve sequences were download from Genbank and four sequences from this study to supplement the data set. *Ca. densa* and *Ca. humicola* were selected as outgroup taxa. The sequences were determined for translation elongation factor 1-alpha (*tef1-α*) and primers EF1-728F (Carbone & Kohn 1999) and EF2 (O'Donnell et al. 1998) were used in the phylogenetic analysis to determine species placement (Table 1). The data set, including the first record of *Calonectria sulawesiensis* from Thailand, were aligned by using MAFFT v. 7 (Katoh & Standley 2013) and checked manually using Bioedit (Hall 1999). Maximum likelihood analysis (ML) was carried out in PAUP* v. 4.0b10 (Swofford 2002). The search strategy was set to bootstrapping and the analysis performed using the HKY85+G model and compared with MrBayes v. 3.2.1. (Ronquist & Huelsenbeck 2003). The bootstrap values expressed from 500 replications by PAUP* v. 4.0b10 analysis which were equal or greater than 50% are given to the left of each node (Fig 2). Model of substitution used for Baysian analyses was using MrModeltest 2.2 (Nylander 2004). The Bayesian analyses were performed in MrBayes v. 3.2.1. (Ronquist & Huelsenbeck 2003). Bayesian analyses were conduct with the Markov chains run from random starting tree for 1000000 generations and trees were sampled every 100 generations. The Markov Chain Monte Carlo (MCMC) algorithm was used to estimate posterior probabilities (PP). Tree was visualized in Tree View (Page 1996).

Pathogenicity test

Four isolates of *Calonectria sulawesiensis* (CPC 33441, CPC 33442, CPC 33443 and CPC 33444) collected from natural forest were used to confirm Koch's postulates. Colonized mycelial plugs (5 mm in diameter) from 10-day-old PDA cultures were used to inoculate *Eucalyptus camaldulensis* leaves (three replicates per treatment) because eucalyptus has been reported to be a susceptible host of *Calonectria* spp. (Lombard et al. 2010b, 2016). Control leaves were inoculated with uncolonized agar plugs. Healthy leaves were surface disinfested with 70% ethanol. Two wounds were made 3 cm apart with a sterile needle in the left side of the leaves. The right side of leaves remained unwounded. Agar plugs were placed on each wound, and two plugs were also

placed 3 cm apart on the unwounded side. Inoculated leaves were incubated at room temperature (28–30 ºC) in a humidified chamber and observed daily.

Table 1 Isolates of *Calonectria* spp. studied in phylogenetic analyses with translation elongation factor1-alpha gene region.

Species	Isolate number	GenBank accession number	Reference
Ca. brasiliensis	CBS 230.51^{T}	GQ267328	Lombard et al. (2009)
	CBS 114257	GQ267329	Lombard et al. (2009)
Ca. cerciana	CBS 123693^{T}	FJ918559	Lombard et al. (2010c)
	CBS 123695	FJ918560	Lombard et al. (2010c)
Ca. densa	CBS 125249	GQ267350	Lombard et al. (2010b)
Ca. humicola	CBS 125251^{T}	GQ267353	Lombard et al. (2010b)
Ca. insularis	CBS 114558^{T}	FJ918556	Crous (2002)
	CBS 114559	FJ918555	Crous (2002)
Ca. sulawesiensis	CBS 125248	GQ267343	Lombard et al. (2010b)
	CBS 125253	GQ267340	Lombard et al. (2010b)
Ca. sulawesiensis	CPC 33441	-	This study
Ca. sulawesiensis	CPC 33442	-	This study
Ca. sulawesiensis	CPC 33443	-	This study
Ca. sulawesiensis	CPC 33444	-	This study
Ca. variabilis	CBS 112691	GQ267335	Crous (2002)
	CBS 114677	GQ267334	Crous (2002)

^{T} Ex-type cultures.

Results

Isolates

Four isolates of *Calonectria sulawesiensis* were collected from soil in Chiang Mai Province. Identification was based on a phylogenetic tree supported by morphological observations.

Morphology

Fig 1

The characteristics of *Calonectria sulawesiensis* included: Mycelium hyaline, smooth 1.5–4 µm wide. Conidiophores macronematous consisting of a stipe, a penicillate arrangement of fertile branches, a stipe extension, and terminal vesicle; stipe 2–5 septate, hyaline, smooth, 70–250 × 4–7.5 µm; stipe extension 3–6 septate, straight, hyaline, smooth, 100–250 µm long, 2–5 µm wide, terminating in a broadly clavate to ellipsoidal vescicle, 10–40×2.5–7.5 µm. Conidiogenous apparatus 40–120 µm long, 250–100 µm wide; primary branches 0–1 septate, 15–30 × 3.5–15 µm; secondary branches aseptate,7–30 × 3–5 µm; tertiary branches aseptate, 8–13 × 2.5–4 µm, each terminal branch producing 2–4 phiallides; phialides doliiform to reniform, hyaline, aseptate, 6–10 × 2.5–4.5 µm. Conidia cylindrical, rounded at the both ends, straight, 0–1 septate, lacking an evident abscission scar, 35–45 × 2.5–4 µm. Cultural characteristics: Colony buff (face), sienna to brick (reverse) mycelial growth on PDA, chlamydospores extensive, dense, forming numerous microsclerotia, sienna. (Fig 1).

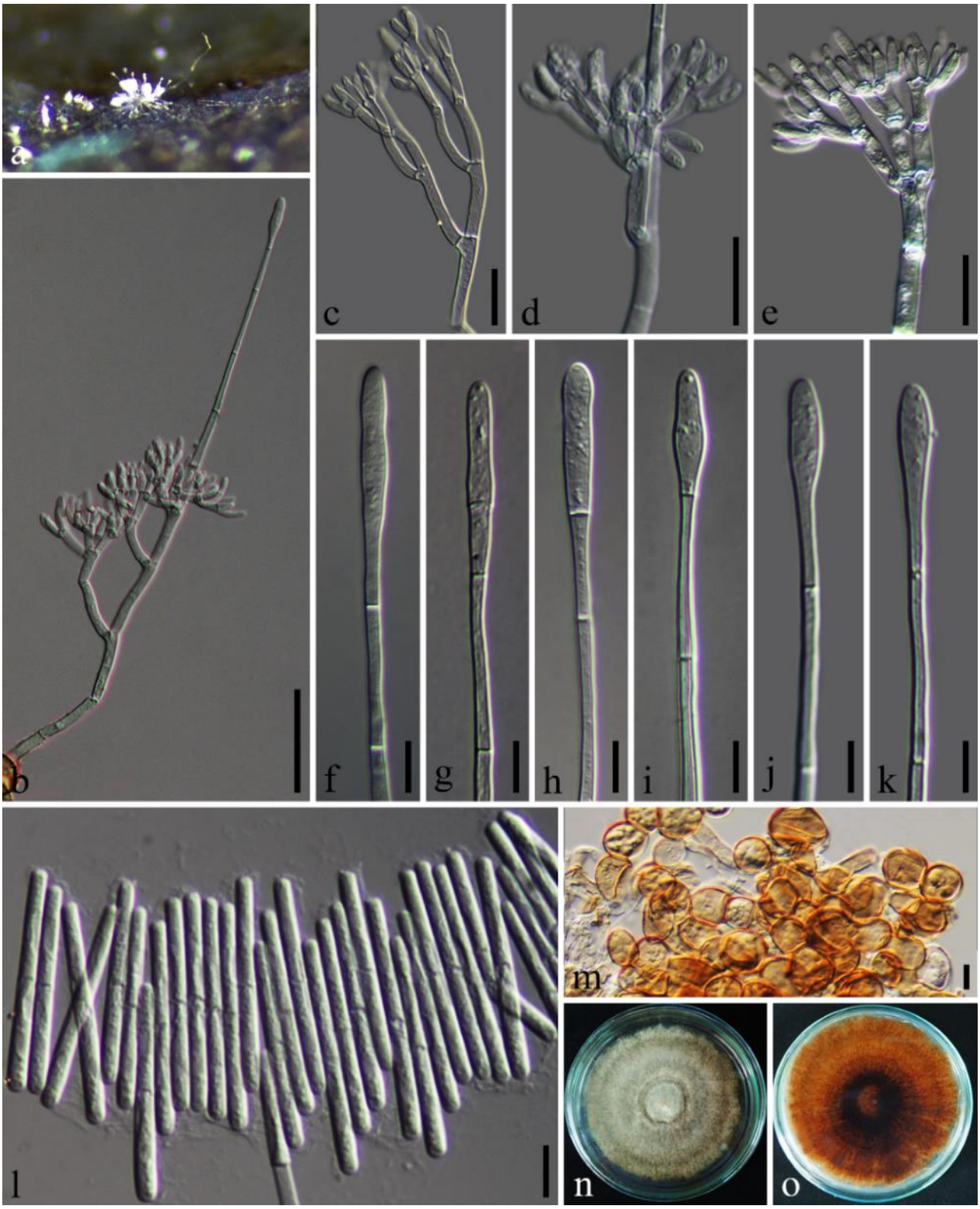

Fig 1 – Morphology of *Calonectria sulawesiensis* (asexual morph). a, Sporulation on *Eucalyptus* leaf. b, Conidiophores. c–e, Conidiogenous apparatus with conidiophore branches and doliiform to reniform phialides. f–k, Broadly clavate to ellipsoidal vescicles. l, Conidia covered with mucilage. m, Chlamydospores. n–o, Colonies on PDA (n face, o reverse). **–** Bars b =50 µm, c–e = 20 µm, f–m = 10 µm.

Phylogenetic analyses

The data set comprised 16 isolates of six species of *Calonectria*, including *Ca. sulawesiensis* (CBS 125277, ex-type). The phylogenetic tree derived from the *tef1-α* region showed that four isolates from Thailand belong to *Ca. sulawesiensis* as they clustered with *Ca. sulawesiensis* in a

strongly supported clade. The phylogenetic tree obtained from Baysian and ML analyses is in agreement with Lombard et al. (2010b) based on PAUP* v. 4.0b10 (Fig 2).

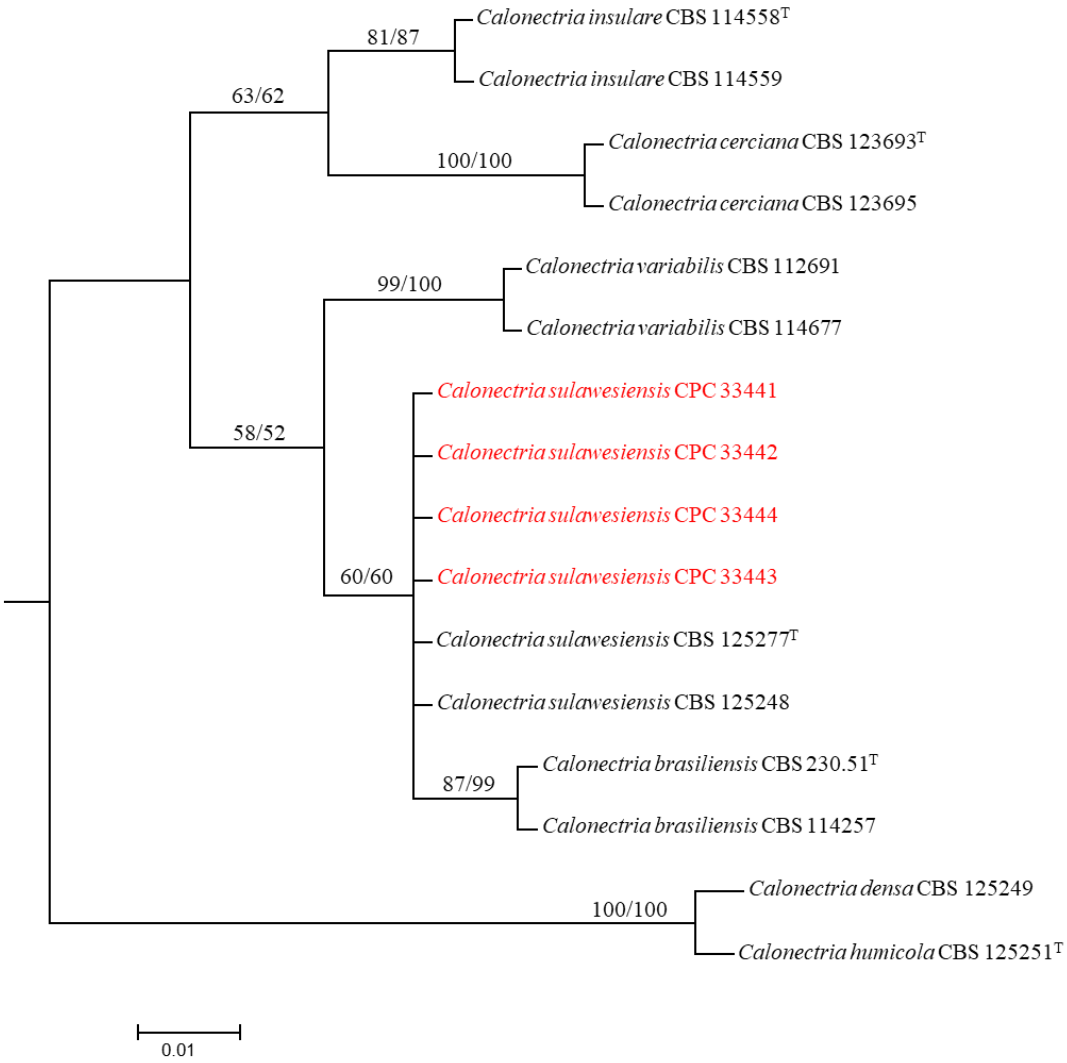

Fig 2 The ML tree inferred from the analyzed partial translation elongation factor 1-alpha gene region (*tef1-α*). The first set of numbers above the node are PAUP* v. 4.0b10 bootstrap values equal or greater than 50%. The second set of number above the node are Baysian posterior probabilities of more than 50% and expressed as percentages. Strain numbers are indicated after species names. Isolates in this study are red. The tree is rooted to *Ca. densa* CBS 125249 and *Ca. humicola* CBS 125251^{T}. ^{T} ex-type cultures. The scale bar indicates expected changes per site.

Pathogenicity

Three days after inoculation, small necrotic lesions developed around the points of inoculation of isolates CPC 33442 and CPC 33444 at the wounded sites, leading to necrosis and larger lesions. These two isolates also caused similar symptoms on non-wounded sites (Fig 3). The pathogens were successfully re-isolated from inoculated and symptomatic leaves, while control plants remained healthy. Leaves inoculated with isolates CPC 33441 and CPC 33443 remained healthy. To our knowledge, this is the first report of *Ca. sulawesiensis* as a pathogen under laboratory conditions.

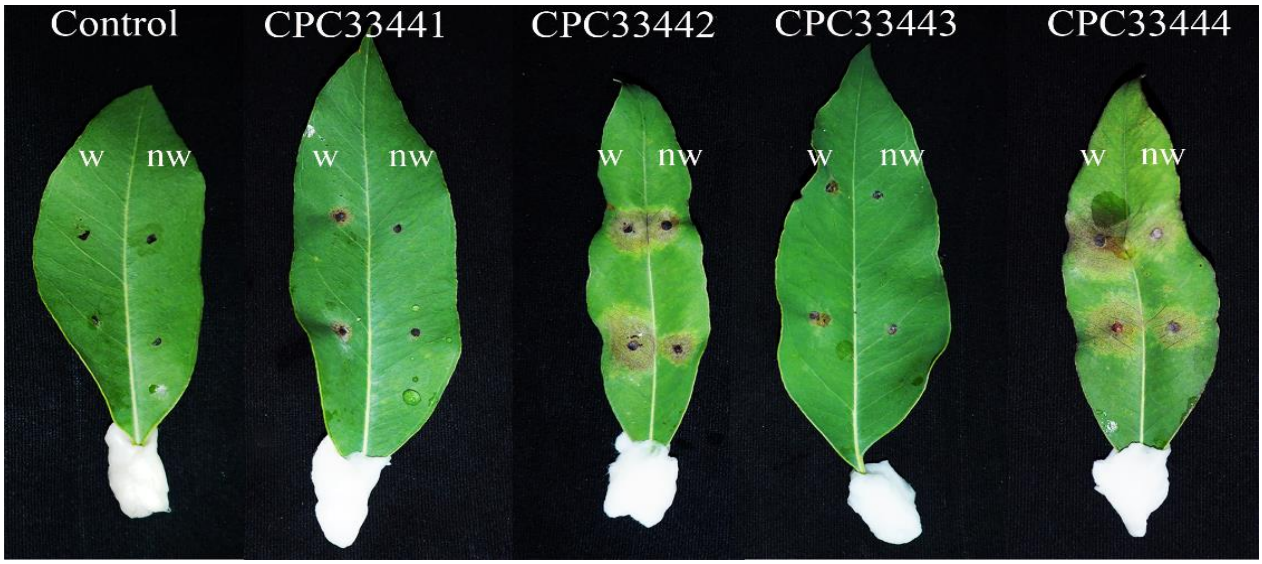

Fig 3 – Leaf blight symptoms on eucalyptus leaves from isolates CPC 33442 and CPC 33444. w = wounded, nw = non-wounded

Discussion

Species in the genus *Calonectria* represent an important group of plant pathogenic fungi that cause serious losses to plant crops in tropical and subtropical climates (Alfenas et al. 2015). Fungal pathogens, including species of *Calonectria*, cause a serious threat to the growth and sustainability of wood and pulp products globally (Crous 2002, Lombard et al. 2015, 2016).

Calonectria sulawesiensis was first described in 2010 from leaves of *Eucalyptus* sp., Sulawesi, Indonesia (Lombard et al. 2010b). *Ca. sulawesiensis* resided in the *Ca. cylindrospora* complex, closely related to *Ca. brasiliensis* (Lombard et al. 2010b, Alfenas et al. 2015). Phylogenetically, *Ca. sulawesiensis* can be distinguished from other species in the *Ca. morganii* complex based on DNA sequence data (Lombard et al. 2010b). Members of this complex are well known pathogens of various hosts worldwide (Crous 2002), however, data is inadequate regarding the pathogenicity of *Ca. sulawesiensis* (Lombard et al. 2010b). This study represents the first report of this fungus outside Indonesia and also demonstrates its pathogenicity on *Eucalyptus camaldulensis*. Future research will include a wider study of the potential host range of *Ca. sulaweseinsis.*

Acknowledgements

The first author offers particular thanks to the Graduate School, Chiang Mai University for partial financial support. We thank Prof. Dr. Robert J. McGovern, Dr. Lorenzo Lombard, Dr. Sajeewa Maharachchikumbura, Mr. Anuruddha Karunarathna, and Ms. Chanokned Senwanna for their valuable suggestions and help.

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