Calonectria species, including four novel taxa, associated with _Eucalyptus_ in Malaysia

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Abstract:

The genus *Calonectria* accommodates many important pathogens of agricultural, horticultural and forestry crops, including *Eucalyptus*. During 2017 surveys of *Eucalyptus* diseases in Sabah, Malaysia, typical symptoms of Calonectria leaf blight were observed. A large number of *Calonectria* isolates were collected from diseased leaves and soils associated with symptomatic trees. The aim of this study was to identify and resolve the phylogenetic relationships between these isolates using morphological characters and DNA

sequence comparisons for six gene regions. From a collection of 73 isolates, eight species residing in three species complexes were identified. Among these, four undescribed species were characterized, and are named here as *Ca. borneana, Ca. ladang, Ca. pseudomalesiana* and *Ca. tanah*. Results of this study support the view that planted *Eucalyptus* in tropical and subtropical areas of the world represent niches remarkably rich in *Calonectria* spp. This also has implications for the management of diseases on these important trees.

Keywords: *Cylindrocladium*, Nectriaceae, multigene phylogeny, soil-borne, taxonomy.

Introduction

To meet the needs of rapidly growing economies, Southeast Asian countries have increased the establishment of commercial forest plantations. Importantly, they provide one of the most significant sources of fiber for the local pulp and paper industry. These planted forests, especially of *Eucalyptus* species, have been established relatively rapidly over the last two decades and are based mostly on short rotation cycles. There are presently at least 4.3 million ha of eucalypts planted, predominantly in different regions of South China, Indonesia, Malaysia, Thailand and Vietnam (Harwood and Nambiar 2014).

Pests and pathogens have emerged as a substantial threat to *Eucalyptus* plantations as forestry based on these trees expands (Wingfield et al. 2008, 2015; Paine et al. 2011). In Southeast Asia, some of the more important diseases of planted *Eucalyptus* include *Ganoderma* and *Phellinus* root rot (Old et al. 2003; Coetzee et al. 2011; Agustini et al. 2014), stem canker caused by species of Cryphonectriaceae (Chen et al. 2010, 2011; Rauf et al. 2020), leaf spot and leaf blight caused by various Mycosphaerellaceae and Teratosphaeriaceae (Wingfield et al. 1996; Old et al. 2003; Andjic et al. 2019; Havenga et al. 2021) and the recently reported Eucalyptus scab and shoot malformation (Pham et al. 2021). In addition to these, Calonectria leaf blight (CLB) is amongst the most prevalent diseases found in *Eucalyptus* plantations in the region (Lombard et al. 2015a; Li et al. 2017; Pham et al. 2019; Wang and Chen 2020).

Calonectria (Nectriaceae, Hypocreales) was first introduced by De Notaris (1867) to accommodate a nectrioid fungus collected from *Magnolia grandiflora* foliage in Italy. The genus now includes many important fungi that are pathogenic to a wide range of

agricultural, horticultural and forestry plants, especially in tropical and subtropical regions (Crous 2002; Lombard et al. 2010a; Marin-Felix et al. 2017). Species in this genus are characterized by their yellow to dark red, warty and uniloculate ascomata, clavate asci, and septate, hyaline and fusiform ascospores (Crous 2002; Lombard et al. 2015b). They produce asexual states with branched conidiophores, hyaline, cylindrical and septate conidia, and stipe extensions terminating in characteristic vesicles (Crous 2002; Lombard et al. 2015b). *Calonectria* species are also capable of producing microsclerotia that facilitate a soil-borne lifestyle (Crous 2002).

During the course of the last decade, considerable attention has been paid to the species boundaries in *Calonectria* (Li et al. 2017; Liu and Chen 2017; Lombard et al. 2010b, 2015a, 2016; Marin-Felix et al. 2017; Pham et al. 2019). As a result, the number of described species in the genus has increased significantly. Lack of consistency in taxonomic approaches such as the variable use of different markers/gene regions, or introduction of new taxa based on limited DNA sequence data, has resulted in a number of species being described that are synonyms of existing species. This has also, in some cases, resulted in an overestimation of species diversity. Liu et al. (2020) provided the most recent standardized and intensive approach to delineating species for *Calonectria* based on phylogenetic inference from eight gene regions. Consequently, the genus now accommodates 120 legitimate species residing in 11 well-defined species complexes (Liu et al. 2020).

Despite the growing importance of *Eucalyptus* plantation forestry in Malaysia, little is known regarding the presence of fungal diseases affecting this resource. During 2017 surveys of *Eucalyptus* diseases in Sabah (Borneo), typical symptoms of CLB were observed. The aims of this study were to collect a large number of fungal isolates from symptomatic leaves as well as soil samples associated with the diseased trees and to determine their identity based on multigene sequence analyses and morphological characteristics.

Materials and methods

Sampling and fungal isolations

Field surveys of *Eucalyptus* plantations were conducted in Tawau, Sabah, Indonesia during 2017. Ten leaves, displaying typical symptoms of CLB were randomly collected from each of

20 trees in a single heavily infected plantation. Samples were also taken randomly from the soil beneath each of these trees. These samples were placed in plastic bags and transferred to the laboratory for isolations to be made. Symptomatic leaves were incubated in moist chambers at 25 °C and examined daily for 7 days for fungal sporulation. Soil samples were baited with germinating alfalfa seeds (*Medicago sativa*) following the method described by Crous (2002). Conidiophores and condia typical of *Calonectria* spp. on the infected alfalfa seedlings were located with a dissection microscope and transferred using a sterile hypodermic needle to Petri dishes containing 2% (w/v) malt extract agar (MEA; 20 g malt extract (Biolab, Midrand, South Africa), 20 g Difco® agar (Becton Dickinson, Maryland, USA), 1 L deionized water). Primary isolations were incubated for 3–7 d at 25 °C to allow for fungal growth. Single hyphal tips of emerging fungal colonies were transferred to fresh MEA plates to obtain pure cultures. These cultures were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative cultures including the ex-type strains were deposited in the culture collection (CBS) of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands. Dried-down sporulating cultures were deposited in the dried herbarium collection (PREM) of the National Collection of Fungi, Roodeplaat, Pretoria, South Africa.

DNA extraction, PCR amplification and sequencing

DNA was extracted from 7-d-old isolates grown on 2% MEA, using Prepman® Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocols. A fragment of the actin (*ACT*) gene region was amplified using primers ACT-512F and ACT-783R (Carbone and Kohn 1999), a part of the calmodulin (*CMDA)* gene region with primers CAL-228F and CAL-2Rd (Carbone and Kohn 1999; Groenewald et al. 2013), a part of the histone H3 (*HIS3)* gene region with primers CYLH3F and CYLH3R (Crous et al. 2004), a fragment of translation elongation factor 1-alpha (*TEF1*) with primers EF1-728F and EF2 (O'Donnell et al. 1998; Carbone and Kohn 1999), a fragment of β-tubulin (*TUB2*) gene region with primers T1 and CYLTUB1R (O'Donnell and Cigelnik 1997; Crous et al. 2004) and a part of the DNA-directed RNA polymerase II second largest subunit (*RPB2*) with primer pair fRPB2-5F and fRBP2-7cR (Liu et al. 1999). Initially, the *TEF1* and *TUB2* gene regions were amplified for all isolates. Based on the preliminary sequencing results, isolates

representing the range of genotypes revealed by these two loci were chosen for further study.

The PCR reactions were conducted using an Applied Biosystems ProFlex PCR System (Thermo Fisher Scientific, Waltham, MA, USA) following the methods described by Pham et al. (2019) and conditions for PCR were as recommended by Liu et al. (2020). Amplified fragments were purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Amplicons were sequenced in both directions using an ABI PRISM™ 3100 DNA sequencer (Thermo Fisher Scientific, Waltham, MA, USA) at the Sequencing Facility of the Faculty of Natural and Agricultural Sciences, University of Pretoria. Geneious v. 7.0 was used to assemble and edit the raw sequences (Kearse et al. 2012). Sequences obtained in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov).

Phylogenetic analyses

Sequences of previously published species of *Calonectria* were obtained from GenBank database (http://www.ncbi.nlm.nih.gov/) to compare with those generated in this study. Alignments of all sequences were assembled using MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/) (Katoh and Standley 2013), then confirmed manually in MEGA v. 7 (Kumar et al. 2016). Bayesian inference (BI) and maximum likelihood (ML) analyses were performed on data sets for each separate gene region and the combined data set. The most appropriate model was obtained using the software jModeltest v. 1.2.5. (Posada, 2008). BI analyses were performed using MrBayes v. 3.2.6 (Ronquist et al. 2012) on the CIPRES Science Gateway v. 3.3. Four Markov chain Monte Carlo (MCMC) chains were run from a random starting tree for five million generations and trees were sampled every 100th generation. The first 25% of trees sampled were eliminated as burn-in and the remaining trees were used to determine the posterior probabilities. ML analyses were conducted using RaxML v. 8.2.4 on the CIPRES Science Gateway v. 3.3 (Stamatakis, 2014) with default GTR substitution matrix and 1,000 rapid bootstraps. Two isolates of *Curvicladiella cignea* (CBS 109167 and CBS 109168) were used as the outgroup taxa in all phylogenetic analyses. Final consensus trees were viewed using MEGA v. 7 (Kumar et al., 2016).

Morphology

The isolates were grown on synthetic nutrient-poor agar (SNA; Nirenberg 1981) to induce the production of the asexual structures. The fruiting structures that emerged were initially mounted in water that was later replaced with 85% lactic acid for observation and in which measurements were taken and images captured. Ascomata were induced by performing crosses between single hyphal tip isolates on minimal salt agar (MSA) as described by Pham et al. (2019). Vertical sections through the ascomata were prepared using a CM1520 cryostat (Leica, IL, USA). The sections were cut in 10–12 µm thickness and mounted in 85% lactic acid for further study. Nikon microscopes (Eclipse Ni, SMZ 18, Nikon, Tokyo, Japan) were used to study the characteristic morphological structures. Images were captured using a Nikon DS-Ri2 camera mounted on the microscopes using the NIS-Elements BR program. Up to fifty measurements were made of all characteristic structures whenever possible. Dimensions were presented in minimum-maximum and with average ± standard deviation in the case of spores.

Colony characteristics were observed on 7-d-old cultures on MEA growing at 25 °C and colours were described using the charts of Rayner (1970). To determine the optimum growth temperature, five replicates for each species were prepared by transferring 5 mm diameter taken from the margins of actively growing cultures to the centres of Petri dishes containing MEA. These cultures were grown at temperatures ranging from 10–35 °C at 5 °C intervals. Two measurements of colony diameter perpendicular to each other were made for the cultures after 7 d and averages were computed.

Results

Fungal isolates

Collectively, 73 isolates having a morphology typical of *Calonectria* spp. were isolated from collected samples. Of these, 21 were from leaves having CLB symptoms and 52 were obtained from germinating alfalfa seeds used as baits for the soil samples (Fig. 1). 60% of the isolates were of a single species, and of these approximately 52% were from the soil and 48% were from leaves. The remaining isolates represented a maximum of 10% of any one species and all were from soil samples. Only one species occurred in both the soil and on

leaves. All isolates were fast growing on MEA and many produced microsclerotia in culture after 3–4 weeks.

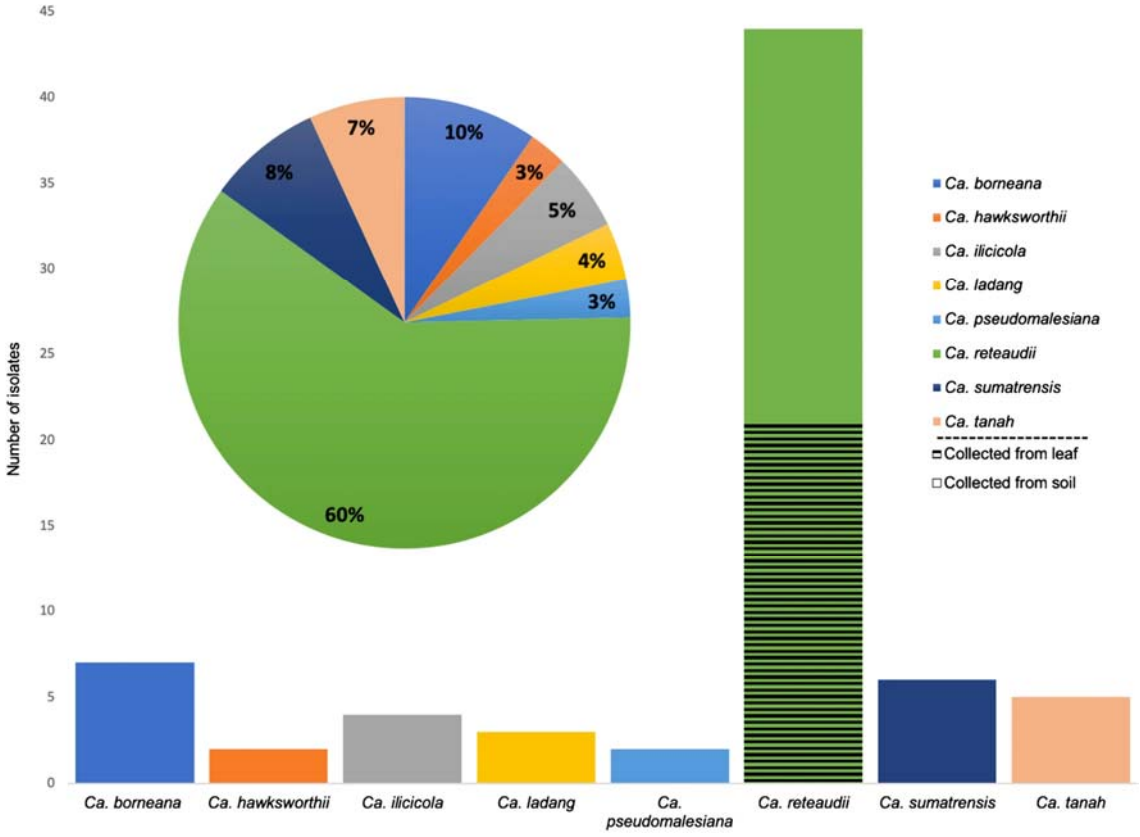

Fig. 1 Relative occurrence of *Calonectria* species associated with *Eucalyptus* plantations in Sabah, Malaysia. Different species are represented by different colours. Isolates obtained from leaves are represented by stripes in the bar chart.

Phylogenetic analyses

Based on the preliminary sequencing results of the *TEF1* and *TUB2* gene regions, 24 representative isolates were chosen for further study (Table 1). Amplicons of approximately 250 bp were generated for the *act* gene region, 660 bp for the *CMDA*, 430 bp for the *HIS3*, 1000 bp for the *RPB2,* 500 bp for the *TEF1* and 560 bp for the *TUB2*. For the phylogenetic analyses of each individual data set, the TPM2+I model was selected for *ACT*, the TrN+G model for *CMDA*, the TIM2+I+G for *HIS3*, the TIM1ef+I+G for *RPB2*, the TPM3uf+I+G for *TUB2*, and the GTR+I+G for *TEF1*. The ML tree for each individual gene region with bootstrap support values of ML and posterior probabilities of BI are presented in Figs. S1–6.

Table 1 Collection details and GenBank accessions of isolates included in the phylogenetic analyses

Species	Isolate number	Host/substrate	Locality	GenBank accessions						References
				ACT	*CMDA*	*HIS3*	*RPB2*	*TEF1*	*TUB2*	
Calonectria acaciicola	CMW 47173 ^{T} = CBS 143557	Soil	Vietnam	MT334933	MT335160	MT335399	MT412474	MT412690	MT412930	Liu et al. (2020)
Calonectria acaciicola	CMW 47174 = CBS 143558	Soil	Vietnam	MT334934	MT335161	MT335400	MT412475	MT412691	MT412931	Liu et al. (2020)
Calonectria acicola	CMW 30996 ^{T}	*Phoenix canariensis*	New Zealand	MT334935	MT335162	MT335401	MT412476	MT412692	MT412932	Liu et al. (2020)
Calonectria acicola	CBS 114812 = CMW 51216	*Phoenix canariensis*	New Zealand	MT334936	MT335163	MT335402	MT412477	MT412693	MT412933	Liu et al. (2020)
Calonectria aconidialis	CMW 35174 ^{T} = CBS 136086 = CERC 1850	Soil	China	MT334938	MT335165	MT335404	MT412479	MT412695	N/A	Liu et al. (2020)
Calonectria aconidialis	CMW 35384 = CBS 136091 = CERC 1886	Soil	China	MT334939	MT335166	MT335405	N/A	MT412696	N/A	Liu et al. (2020)
Calonectria aeknauliensis	CMW 48253 ^{T} = CBS 143559	Soil	Indonesia	MT334953	MT335180	MT335419	MT412486	MT412710	N/A	Liu et al. (2020)
Calonectria aeknauliensis	CMW 48254 = CBS 143560	Soil	Indonesia	MT334954	MT335181	MT335420	MT359641	MT359401	N/A	Liu et al. (2020)
Calonectria asiatica	CBS 114073 ^{T} = CMW 23782 = CPC 3900	Leaf litter	Thailand	GQ280428	AY725741	AY725658	N/A	AY725705	AY725616	Lombard et al. (2010b)
Calonectria auriculiformis	CMW 47178 ^{T} = CBS 143561	Soil	Vietnam	MT334964	MT335190	MT335430	MT412494	MT412721	MT412944	Liu et al. (2020)
Calonectria auriculiformis	CMW 47179 = CBS 143562	Soil	Vietnam	N/A	MT335191	MT335431	MT412495	MT412722	MT412945	Liu et al. (2020)
Calonectria australiensis	CMW 23669 ^{T} = CBS 112954 = CPC 4714	*Ficus pleurocarpa*	Australia	MT334965	MT335192	MT335432	MT412496	MT412723	MT412946	Liu et al. (2020)
Calonectria borneana	**CMW 50832 = CBS 144551**	Soil	Malaysia	OL635113	OL635065	OL635041	OL635089	OL635017	N/A	This study
Calonectria borneana	**CMW 50833 = CBS 144552**	Soil	Malaysia	OL635114	OL635066	OL635042	OL635090	OL635018	N/A	This study
Calonectria borneana	**CMW 50782 ^{T} = CBS 144553**	Soil	Malaysia	OL635115	OL635067	OL635043	OL635091	OL635019	N/A	This study
Calonectria brasiliensis	CBS 230.51 ^{T} = IMI 299576	*Eucalyptus* sp.	Brazil	MT334970	MT335200	MT335440	MT412504	MT412731	MT412953	Liu et al. (2020)
Calonectria brasiliensis	CMW 32949 = CBS 114257 = CPC 1944	*Eucalyptus* sp.	Brazil	MT334971	MT335201	MT335441	MT412505	MT412732	MT412954	Liu et al. (2020)
Calonectria brassicicola	CBS 112841 ^{T} = CMW 51206 = CPC 4552	Soil	Indonesia	N/A	KX784561	N/A	N/A	KX784689	KX784619	Lombard et al. (2016)
Calonectria bumicola	CMW 48257 ^{T} = CBS 143575	Soil	Indonesia	MT334975	MT335205	MT335445	MT412509	MT412736	N/A	Liu et al. (2020)
Calonectria canadiana	CMW 23673 ^{T} = CBS 110817 = STE-U 499	*Picea* sp.	Canada	MT334976	MT335206	MT335446	MT412510	MT412737	MT412958	Liu et al. (2020)
Calonectria canadiana	CERC 8952	Soil	China	MT335058	MT335290	MT335530	MT412587	MT412821	MT413035	Liu et al. (2020)
Calonectria canadiana	CERC 8957	Soil	China	MT335059	MT335291	MT335531	MT412588	MT412822	MT413036	Liu et al. (2020)
Calonectria cerciana	CMW 25309 ^{T} = CBS 123693	*Eucalyptus urophylla* × *Eucalyptus grandis*	China	MT334981	MT335211	MT335451	MT412515	MT412742	MT412963	Liu et al. (2020)
Calonectria cerciana	CMW 25290 = CBS 123695	*Eucalyptus urophylla* × *Eucalyptus grandis*	China	MT334982	MT335212	MT335452	MT412516	MT412743	MT412964	Liu et al. (2020)
Calonectria chinensis	CMW 23674 ^{T} = CBS 114827 = CPC 4101	Soil	China	MT334990	MT335220	MT335460	MT412524	MT412751	MT412972	Liu et al. (2020)
Calonectria chinensis	CMW 30986 = CBS 112744 =									

Table 1 (continued)

Species	Isolate number	Host/substrate	Locality	GenBank accessions						References
				ACT	*CMDA*	*HIS3*	*RPB2*	*TEF1*	*TUB2*	
Calonectria cochinchinensis	CMW 47186 = CBS 143568	Soil	Vietnam	MT334996	MT335226	MT335466	MT412530	MT412757	MT412978	Liu et al. (2020)
Calonectria cochinchinensis	CMW 49915 ^{T} = CBS 143567	Soil	Vietnam	MT334995	MT335225	MT335465	MT412529	MT412756	MT412977	Liu et al. (2020)
Calonectria colombiensis	CMW 23676 ^{T} = CBS 112220 = CPC 723	Soil	Colombia	MT334998	MT335228	MT335468	MT412532	MT412759	MT412980	Liu et al. (2020)
Calonectria colombiensis	CMW 30985 = CBS 112221 = CPC 724	Soil	Colombia	MT334999	MT335229	MT335469	MT412533	MT412760	MT412981	Liu et al. (2020)
Calonectria crousiana	CMW 27249 ^{T} = CBS 127198	*Eucalyptus grandis*	China	MT335000	MT335230	MT335470	MT412534	MT412761	MT412982	Liu et al. (2020)
Calonectria crousiana	CMW 27253 = CBS 127199	*Eucalyptus grandis*	China	MT335001	MT335231	MT335471	MT412535	MT412762	MT412983	Liu et al. (2020)
Calonectria curvispora	CMW 48245 = CBS 143565	Soil	Indonesia	MT335003	MT335233	MT335473	MT412537	MT412764	N/A	Liu et al. (2020)
Calonectria curvispora	CMW 48246 = CBS 143566	Soil	Indonesia	MT335004	MT335234	MT335474	MT412538	MT412765	N/A	Liu et al. (2020)
Calonectria curvispora	CMW 23693 ^{T} = CBS 116159 = CPC 765	Soil	Madagascar	MT335002	MT335232	MT335472	MT412536	MT412763	N/A	Liu et al. (2020)
Calonectria cylindrospora	CMW 30978 = CBS 110666 = STE-U 497	*Ilex vomitoria*	USA	MT335007	MT335237	MT335477	MT412541	MT412768	MT412986	Liu et al. (2020)
Calonectria cylindrospora	CBS 119670 = CMW 51310 = CPC 12766	*Pistacia lentiscus*	Italy	MT335006	MT335236	MT335476	MT412540	MT412767	MT412985	Liu et al. (2020)
Calonectria hawksworthii	CMW 14878 ^{T} = CBS 125277	*Eucalyptus* sp.	Indonesia	MT335141	MT335378	MT335618	MT412670	MT412909	MT413119	Liu et al. (2020)
Calonectria hawksworthii	CMW 14879 = CBS 125,253	*Eucalyptus* sp.	Indonesia	MT335142	MT335379	MT335619	MT412671	MT412910	MT413120	Liu et al. (2020)
Calonectria hawksworthii	CMW 31395	*Eucalyptus urophylla* × *Eucalyptus grandis*	China	MT335018	MT335248	MT335488	MT412550	MT412779	MT412997	Liu et al. (2020)
Calonectria hawksworthii	CMW 31393 = CBS 136641	*Eucalyptus urophylla* × *Eucalyptus grandis*	China	MT335017	MT335247	MT335487	MT412549	MT412778	MT412996	Liu et al. (2020)
Calonectria hawksworthii	CBS 111870 ^{T} = CMW 51194 = CPC 2405	*Nelumbo nucifera*	Mauritius	MT335024	MT335254	MT335494	MT412556	MT412785	MT413003	Liu et al. (2020)
Calonectria hawksworthii	**CMW 50823 = CBS 144544**	Soil	Malaysia	OL635116	OL635068	OL635044	OL635092	OL635020	OL635135	This study
Calonectria hawksworthii	**CMW 50824 = CBS 144545**	Soil	Malaysia	OL635117	OL635069	OL635045	OL635093	OL635021	OL635136	This study
Calonectria heveicola	CMW 49913 ^{T} = CBS 143570	Soil	Vietnam	MT335025	MT335255	MT335495	N/A	MT412786	MT413004	Liu et al. (2020)
Calonectria heveicola	CMW 49928 = CBS 143571	Soil	Vietnam	MT335048	MT335280	MT335520	MT412577	MT412811	MT413025	Liu et al. (2020)
Calonectria hongkongensis	CBS 114828 ^{T} = CMW 51217 = CPC 4670	Soil	China	MT335028	MT335258	MT335498	MT412559	MT412789	MT413007	Liu et al. (2020)
Calonectria hongkongensis	CMW 31383	Soil	China	MT335029	MT335259	MT335499	MT412560	MT412790	MT413008	Liu et al. (2020)
Calonectria ilicicola	CMW 30998 ^{T} = CBS 190.50 = IMI 299389 =									

Table 1 (continued)

Species	Isolate number	Host/substrate	Locality	GenBank accessions						References
				ACT	*CMDA*	*HIS3*	*RPB2*	*TEF1*	*TUB2*	
Calonectria ilicicola	**CMW 50837 = CBS 144554**	Soil	Malaysia	OL635118	OL635070	OL635046	OL635094	OL635022	N/A	This study
Calonectria ilicicola	**CMW 50838 = CBS 144555**	Soil	Malaysia	OL635119	OL635071	OL635047	OL635095	OL635023	N/A	This study
Calonectria ilicicola	**CMW 50840 = CBS 144554**	Soil	Malaysia	OL635120	OL635072	OL635048	OL635096	OL635024	N/A	This study
Calonectria indonesiae	CMW 23683 ^{T} = CBS 112823 = CPC 4508	*Syzygium aromaticum*	Indonesia	MT335037	MT335267	MT335507	MT412565	MT412798	MT413015	Liu et al. (2020)
Calonectria indonesiae	CBS 112840	*Syzygium aromaticum*	Indonesia	MT335038	MT335268	MT335508	MT412566	MT412799	MT413016	Liu et al. (2020)
Calonectria insularis	CMW 30991 ^{T} = CBS 114558 = CPC 768	Soil	Madagascar	N/A	MT335269	MT335509	MT412567	MT412800	MT413017	Liu et al. (2020)
Calonectria insularis	CMW 30992 = CBS 114559 = CPC 954	Soil	Mexico	N/A	MT335270	MT335510	MT412568	MT412801	MT413018	Liu et al. (2020)
Calonectria kyotensis	CBS 114525 ^{T} = ATCC 18834 = CMW 51824 = CPC 2367	*Robinia pseudoacacia*	Japan	MT335039	MT335271	MT335511	MT412569	MT412802	MT413019	Liu et al. (2020)
Calonectria kyotensis	CBS 114550	Soil	China	MT335016	MT335246	MT335486	MT412548	MT412777	MT412995	Liu et al. (2020)
Calonectria ladang	**CMW 50774 = CBS 144548**	Soil	Malaysia	N/A	OL635073	OL635049	OL635097	OL635025	N/A	This study
Calonectria ladang	**CMW 50775 = CBS 144549**	Soil	Malaysia	OL635121	OL635074	OL635050	OL635098	OL635026	N/A	This study
Calonectria ladang	**CMW 50776 ^{T} = CBS 144550**	Soil	Malaysia	OL635122	OL635075	OL635051	OL635099	OL635027	N/A	This study
Calonectria lantauensis	CERC 3301	Soil	China	MT335041	MT335273	MT335513	N/A	MT412804	N/A	Liu et al. (2020)
Calonectria lantauensis	CERC 3302 ^{T} = CBS 142888 = CMW 47252	Soil	China	MT335040	MT335272	MT335512	MT412570	MT412803	N/A	Liu et al. (2020)
Calonectria lateralis	CMW 31412 ^{T} = CBS 136629	Soil	China	MT335042	MT335274	MT335514	MT412571	MT412805	MT413020	Liu et al. (2020)
Calonectria malesiana	CMW 23687 ^{T} = CBS 112752 = CPC 4223	Soil	Indonesia	MT335054	MT335286	MT335526	MT412583	MT412817	MT413031	Liu et al. (2020)
Calonectria malesiana	CBS 112710 = CMW 51199 = CPC 3899	Leaf litter	Thailand	MT335055	MT335287	MT335527	MT412584	MT412818	MT413032	Liu et al. (2020)
Calonectria maranhensis	CBS 134811 ^{T} = LPF142	*Eucalyptus* sp.	Brazil	N/A	KM396035	KM396118	N/A	KM395861	KM395948	Alfenas et al. (2015)
Calonectria maranhensis	CBS 134812 = LPF143	*Eucalyptus* sp.	Brazil	N/A	KM396036	KM396119	N/A	KM395862	KM395949	Alfenas et al. (2015)
Calonectria multiseptata	CMW 23692 ^{T} = CBS 112682 = CPC 1589	*Eucalyptus grandis*	Indonesia	MT335067	MT335299	MT335539	MT412596	MT412830	MT413044	Liu et al. (2020)
Calonectria pacifica	CMW 16726 ^{T} = CBS 109063 = IMI 354528 = STE-U 2534	*Araucaria heterophylla*	USA	MT335079	MT335311	MT335551	MT412604	MT412842	N/A	Liu et al. (2020)
Calonectria pacifica	CMW 30988 = CBS 114038	*Ipomoea aquatica*	New Zealand	MT335080	MT335312	MT335552	MT412605	MT412843	N/A	Liu et al. (2020)
Calonectria pluriliteralis	CBS 111401 ^{T} = CMW 51178 = CPC 1637	Soil	Ecuador	N/A	MT335340	MT335580	MT412632	MT412870	MT413082	Liu et al. (2020)
Calonectria propaginicola	CBS 134815 ^{T} = LPF220	*Eucalyptus* sp.	Brazil	N/A	KM396040	KM396123	N/A	KM395866	KM395953	Alfenas et al. (2015)
Calonectria propaginicola	CBS 134816 = LPF222	*Eucalyptus* sp.	Brazil	N/A	KM396041	KM396124	N/A	KM395867	KM395954	Alfenas et al. (2015)
Calonectria pseudomalesiana	**CMW 50821 T =**									

Table 1 (continued)

Species	Isolate number	Host/substrate	Locality	GenBank accessions						References
				ACT	*CMDA*	*HIS3*	*RPB2*	*TEF1*	*TUB2*	
Calonectria pseudomalesiana	**CMW 50779 = CBS 144668**	Soil	Malaysia	OL635124	OL635077	OL635053	OL635101	OL635029	OL635138	This study
Calonectria pseudoreteaudii	CMW 25310 ^{T} = CBS 123694	*Eucalyptus urophylla* × *Eucalyptus grandis*	China	MT335119	MT335354	MT335594	MT412647	MT412885	MT413096	Liu et al. (2020)
Calonectria pseudoreteaudii	CMW 25292 = CBS 123696	*Eucalyptus urophylla* × *Eucalyptus grandis*	China	MT335120	MT335355	MT335595	MT412648	MT412886	MT413097	Liu et al. (2020)
Calonectria queenslandica	CMW 30604 ^{T} = CBS 112146 = CPC 3213	*Eucalyptus urophylla*	Australia	MT335132	MT335367	MT335607	MT412660	MT412898	MT413108	Liu et al. (2020)
Calonectria queenslandica	CMW 30603 = CBS 112155 = CPC 3210	*Eucalyptus pellita*	Australia	MT335133	MT335368	MT335608	MT412661	MT412899	MT413109	Liu et al. (2020)
Calonectria reteaudii	CMW 30984 ^{T} = CBS 112144 = CPC 3201	*Eucalyptus camaldulensis*	Vietnam	MT335135	MT335370	MT335610	MT412663	MT412901	MT413111	Liu et al. (2020)
Calonectria reteaudii	CMW 16738 = CBS 112143 = CPC 3200	*Eucalyptus* sp.	Vietnam	MT335136	MT335371	MT335611	MT412664	MT412902	MT413112	Liu et al. (2020)
Calonectria reteaudii	CMW 47410 = CBS 143563	*Eucalyptus urophylla*	Vietnam	MT334966	MT335193	MT335433	MT412497	MT412724	N/A	Liu et al. (2020)
Calonectria reteaudii	CMW 47433 = CBS 143564	*Eucalyptus pellita*	Vietnam	MT334967	MT335194	MT335434	MT412498	MT412725	MT412947	Liu et al. (2020)
Calonectria reteaudii	**CMW 50797**	*Eucalyptus* sp.	Malaysia	OL635125	OL635078	OL635054	OL635102	OL635030	OL635139	This study
Calonectria reteaudii	**CMW 50814 = CBS 144667**	Soil	Malaysia	N/A	OL635079	OL635055	OL635103	OL635031	OL635140	This study
Calonectria reteaudii	**CMW 50780 = CBS 144546**	Soil	Malaysia	OL635126	OL635080	OL635056	OL635104	OL635032	OL635141	This study
Calonectria reteaudii	**CMW 50836**	Soil	Malaysia	OL635127	OL635081	OL635057	OL635105	OL635033	OL635142	This study
Calonectria reteaudii	**CMW 50841 = CBS 144547**	Soil	Malaysia	OL635128	OL635082	OL635058	OL635106	OL635034	OL635143	This study
Calonectria sumatrensis	CMW 23698 ^{T} = CBS 112829 = CPC 4518	Soil	Indonesia	MT335145	MT335382	MT335622	MT412674	MT412913	N/A	Liu et al. (2020)
Calonectria sumatrensis	CMW 30987 = CBS 112934 = CPC 4516	Soil	Indonesia	MT335146	MT335383	MT335623	MT412675	MT412914	N/A	Liu et al. (2020)
Calonectria sumatrensis	CBS 112826 = CMW 51202 = CPC 4519	Soil	Indonesia	MT335144	MT335381	MT335621	MT412673	MT412912	MT413121	Liu et al. (2020)
Calonectria sumatrensis	CBS 112936 = CMW 51207 = CPC 4504	Soil	Indonesia	MT335143	MT335380	MT335620	MT412672	MT412911	N/A	Liu et al. (2020)
Calonectria sumatrensis	**CMW 50783 = CBS 144557**	Soil	Malaysia	OL635129	OL635083	OL635059	OL635107	OL635035	N/A	This study
Calonectria sumatrensis	**CMW 50784 = CBS 144558**	Soil	Malaysia	OL635130	OL635084	OL635060	OL635108	OL635036	N/A	This study
Calonectria sumatrensis	**CMW 50785 = CBS 144559**	Soil	Malaysia	OL635131	OL635085	OL635061	OL635109	OL635037	N/A	This study
Calonectria syzygiicola	CBS 112831 ^{T} = CMW 51204 = CPC 4511	*Syzygium aromaticum*	Indonesia	N/A	N/A	N/A	N/A	KX784736	KX784663	Lombard et al. (2016)
Calonectria tanah	**CMW 50771 = CBS 144560**	Soil	Malaysia	OL635132	OL635086	OL635062	OL635110	OL635038	OL635144	This study
Calonectria tanah	**CMW 50772 = CBS 144561**	Soil	Malaysia	OL635133	OL635087	OL635063	OL635111	OL635039	OL635145	This study
Calonectria tanah	**CMW 50777 ^{T} = CBS 144562**	Soil	Malaysia	OL635134	OL635088	OL635064	OL635112	OL635040	OL635146	This study
Calonectria tonkinensis	CMW 47430 ^{T} =									

Table 1 (continued)

Species	Isolate number	Host/substrate	Locality	GenBank accessions						References
				ACT	***CMDA***	***HIS3***	***RPB2***	***TEF1***	***TUB2***	
Calonectria uniseptata	CBS 413.67 = CMW 23678 = CPC 2391 = IMI 299577	*Paphiopedilum callosum*	Germany	GQ280451	GQ267379	GQ267248	N/A	GQ267307	GQ267208	Lombard et al. (2016)
Calonectria variabilis	CMW 2914 = CBS 112691 = CPC 2506	*Theobroma grandiflorum*	Brazil	N/A	MT335393	MT335633	MT412684	MT412924	MT413131	Liu et al. (2020)
Calonectria variabilis	CMW 3187 ^{T} = AR2675 = CBS 114677 = CPC 2436	*Schefflera morototoni*	Brazil	N/A	MT335392	MT335632	MT412683	MT412923	MT413130	Liu et al. (2020)
Calonectria yunnanensis	CERC 5337	Soil	China	MT335158	MT335397	MT335637	MT412688	MT412928	MT413135	Liu et al. (2020)
Calonectria yunnanensis	CERC 5339 ^{T}	Soil	China	MT335157	MT335396	MT335636	MT412687	MT412927	MT413134	Liu et al. (2020)
Curvicladiella cignea	CBS 109167 ^{T} = CPC 1595 = MUCL 40269	Decaying leaf	French Guiana	KM231122	KM231287	KM231461	KM232311	KM231867	KM232002	Lombard et al. (2015b)
Curvicladiella cignea	CBS 109168 = CPC 1594 = MUCL 40268	Decaying seed	French Guiana	KM231121	KM231286	KM231460	KM232312	KM231868	KM232003	Lombard et al. (2015b)

N/A represents information that is not available. Isolates obtained in this study are indicated in **bold**. T denotes ex-type strain

AR, Amy Y. Rossman working collection; *ATCC*, American Type Culture Collection, VA, USA; *CBS*, the culture collection of Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; *CERC*, China Eucalypt Research Centre, Zhanjiang, Guangdong Province, China; *CMW*, the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; *CPC*, Pedro Crous working collection housed at Westerdijk Fungal Biodiversity Institute; *IMI*, International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, UK; *LPF*, Laboratório de Patologia Florestal, Universidade Federal de Viçosa, Viçosa, Brazil; *MUCL*, Mycotheque, Laboratoire de Mycologie Systematique st Appliqee, I'Universite, Louvain-la-Neuve, Belgium; *STE-U*, Department of Plant Pathology, University of Stellenbosch, South Africa; *UFV*, Universidade Federal de Viçosa, Viçosa, Brazil

ACT

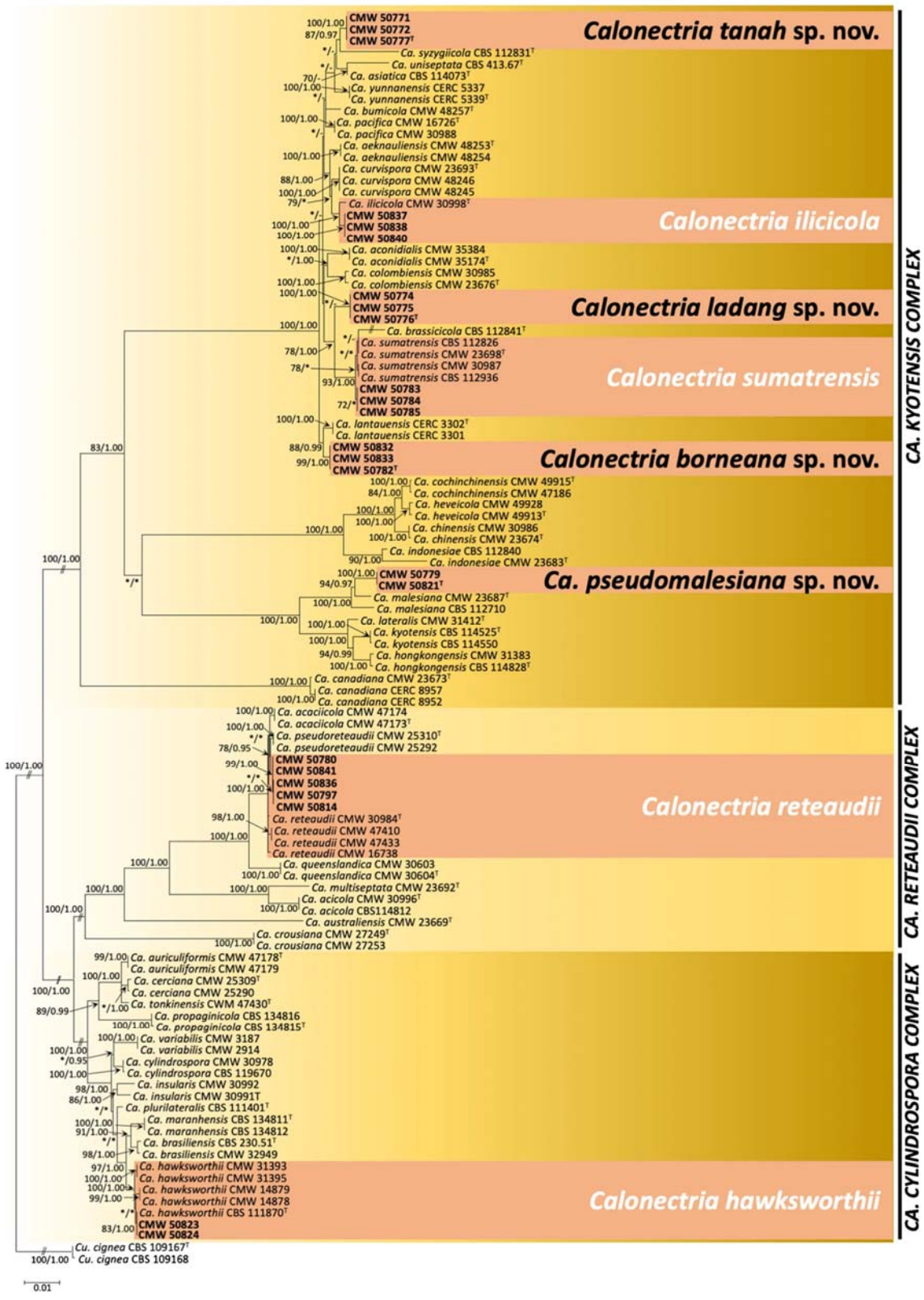
ACT+CMDA+HIS3+RPB2+TEF1+TUB2
Calonectria tanah sp. nov.
CMW 50771
CMW 50772
CMW 50777T
Ca. syzygiicola CBS 112831T
Ca. uniseptata CBS 413.67T
Ca. asiatica CBS 114073T
Ca. yunnanensis CERC 5337
Ca. yunnanensis CERC 5339T
Ca. bumicola CMW 48257T
Ca. pacifica CMW 16726T
Ca. pacifica CMW 30988
Ca. aeknauliensis CMW 48253T
Ca. aeknauliensis CMW 48254
Ca. curvispora CMW 23693T
Ca. curvispora CMW 48246
Ca. curvispora CMW 48245
Calonectria ilicicola
Ca. ilicicola CMW 30998T
CMW 50837
CMW 50838
CMW 50840
Ca. aconidialis CMW 35384
Ca. aconidialis CMW 35174T
Ca. colombiensis CMW 30985
Ca. colombiensis CMW 23676T
Calonectria ladang sp. nov.
CMW 50774
CMW 50775
CMW 50776T
Ca. brassicicola CBS 112841T
Calonectria sumatrensis
Ca. sumatrensis CBS 112826
Ca. sumatrensis CMW 23698T
Ca. sumatrensis CMW 30987
Ca. sumatrensis CBS 112936
CMW 50783
CMW 50784
CMW 50785
Ca. lantauensis CERC 3302T
Ca. lantauensis CERC 3301
Calonectria borneana sp. nov.
CMW 50832
CMW 50833
CMW 50782T
Ca. cochinchinensis CMW 49915T
Ca. cochinchinensis CMW 47186
Ca. heveicola CMW 49928
Ca. heveicola CMW 49913T
Ca. chinensis CMW 30986
Ca. chinensis CMW 23674T
Ca. indonesiae CBS 112840
Ca. indonesiae CMW 23683T
Ca. pseudomalesiana sp. nov.
CMW 50779
CMW 50821T
Ca. malesiana CMW 23687T
Ca. malesiana CBS 112710
Ca. lateralis CMW 31412T
Ca. kyotensis CBS 114525T
Ca. kyotensis CBS 114550
Ca. hongkongensis CMW 31383
Ca. hongkongensis CBS 114828T
Ca. canadiana CMW 23673T
Ca. canadiana CERC 8957
Ca. canadiana CERC 8952
Ca. acaciicola CMW 47174
Ca. acaciicola CMW 47173T
Ca. pseudoreteaudii CMW 25310T
Ca. pseudoreteaudii CMW 25292
Calonectria reteaudii
CMW 50780
CMW 50841
CMW 50836
CMW 50797
CMW 50814
Ca. reteaudii CMW 30984T
Ca. reteaudii CMW 47410
Ca. reteaudii CMW 47433
Ca. reteaudii CMW 16738
Ca. queenslandica CMW 30603
Ca. queenslandica CMW 30604T
Ca. multiseptata CMW 23692T
Ca. acicola CMW 30996T
Ca. acicola CBS114812
Ca. australiensis CMW 23669T
Ca. crousiana CMW 27249T
Ca. crousiana CMW 27253
Ca. auriculiformis CMW 47178T
Ca. auriculiformis CMW 47179
Ca. cerciana CMW 25309T
Ca. cerciana CMW 25290
Ca. tonkinensis CWM 47430T
Ca. propaginicola CBS 134816
Ca. propaginicola CBS 134815T
Ca. variabilis CMW 3187
Ca. variabilis CMW 2914
Ca. cylindrospora CMW 30978
Ca. cylindrospora CBS 119670
Ca. insularis CMW 30992
Ca. insularis CMW 30991T
Ca. plurilateralis CBS 111401T
Ca. maranhensis CBS 134811T
Ca. maranhensis CBS 134812
Ca. brasiliensis CBS 230.51T
Ca. brasiliensis CMW 32949
Calonectria hawksworthii
Ca. hawksworthii CMW 31393
Ca. hawksworthii CMW 31395
Ca. hawksworthii CMW 14879
Ca. hawksworthii CMW 14878
Ca. hawksworthii CBS 111870T
CMW 50823
CMW 50824
Cu. cignea CBS 109167T
Cu. cignea CBS 109168
0.01
CA. KYOTENSIS COMPLEX
CA. RETEAUDII COMPLEX
CA. CYLINDROSPORA COMPLEX

Fig. 2 Phylogenetic tree based on maximum likelihood (ML) analysis of a combined DNA data set of *ACT, CMDA, HIS3, RPB2, TEF1* and *TUB2* sequences for *Calonectria* spp. Bootstrap values ≥70% for ML analyses and posterior probabilities values ≥0.95 obtained from Bayesian inference (BI) are indicated at the nodes as ML/BI. Bootstrap values <70% or probabilities values <0.95 are marked with "*", and nodes lacking the support values are marked with "–". Isolates representing ex-type material are marked with "T". *Curvicladiella cignea* (isolate CBS 109167 and CBS 109168) represents the outgroup.

The combined sequence data set used in the phylogenetic analyses included 106 ingroup taxa and 3 383 characters. Concatenated sequence alignments of the six gene regions together with closely related *Calonectria* species were deposited in TreeBASE (28786). Optimal substitution models were applied to individual loci in the concatenated dataset for the BI analyses. ML and BI analyses resulted in phylogenetic trees with concordant topologies and showed similar phylogenetic relationships between taxa. The ML tree with bootstrap support values of the ML and the posterior probabilities obtained from BI is presented in Fig. 2. Isolates obtained from this study resided in three species complexes including the *Ca. kyotensis* (Sphaero-Naviculate Group) complex, the *Ca. cylindrospora* complex and the *Ca. reteaudii* complex (Prolate Group).

Seventeen isolates in the *Ca. kyotensis* complex clustered in six clades. Of these, three isolates grouped with *Ca. ilicicola* and three with *Ca. sumatrensis*. The remaining 11 isolates resided in four clades distinct from any known species and represent novel taxa. Five isolates resided in the *Ca. reteaudii* complex and were identified as *Ca. reteaudii*. The remaining isolates resided in the *Ca. cylindrospora* complex of which two isolates were identified as *Ca. hawksworthii*, grouping together with the ex-type isolate of that species.

Taxonomy

Based on phylogenetic analyses and morphological examination, isolates collected in this study represented four previously described, *Ca. hawksworthii*, *Ca. ilicicola*, *Ca. reteaudii* and *Ca. sumatrensis,* and four novel species. The undescribed taxa included four species in the *Ca. kyotensis* complex. The descriptions for these species are provided as follows:

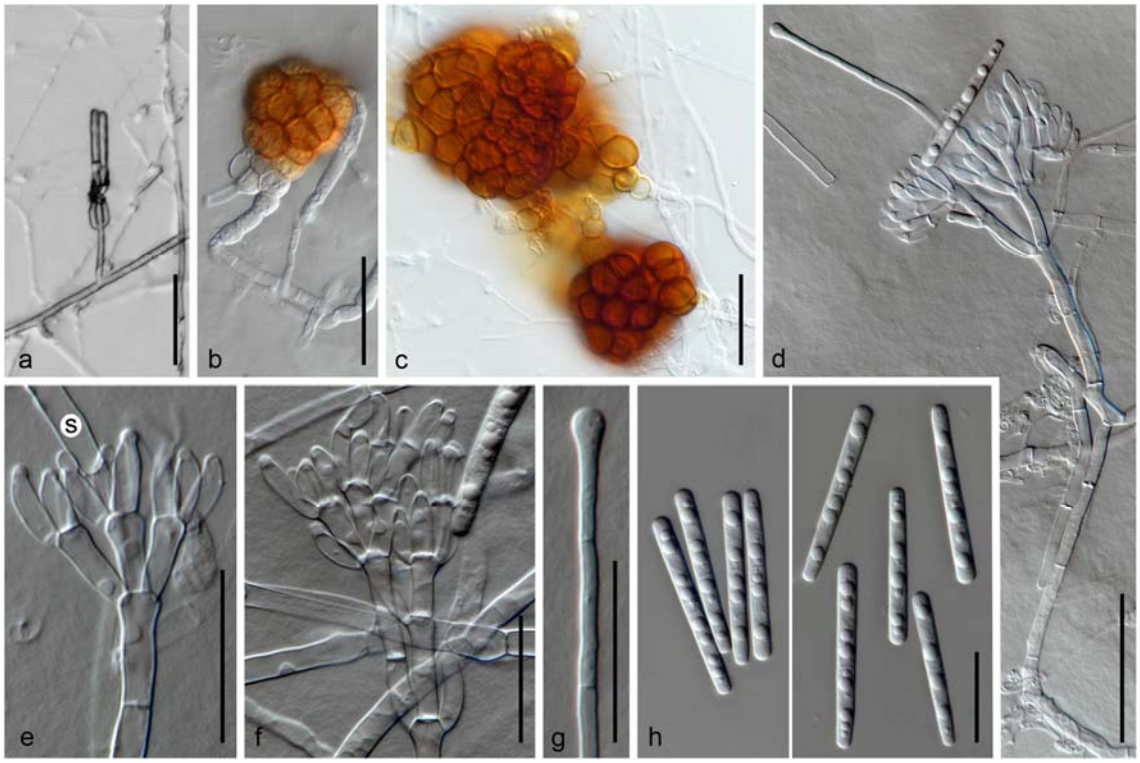

Fig. 3 Micrographs of *Calonectria borneana* sp. nov. (ex-holotype CMW 50782 = CBS 144553). **a** Conidiophore on SNA. **b, c** Microsclerotia. **d** Conidiophore and stipe. **e, f** Conidiogenous apparatus (S = stipe). **g** Terminal vesicle of stipe. **h** Conidia. Scale bars: a–d = 50 µm; e–h = 25 µm

Calonectria borneana N.Q. Pham, Marinc. & M.J. Wingf., **sp. nov.** Fig. 3

MycoBank MB841985

Etymology. Name refers to Borneo island, where this fungus was first isolated.

Type material. MALAYSIA. Sabah, Tawau, Brumas, from soil in *Eucalyptus* sp. plantation, Aug. 2017, M.R.B.A Rauf, PREM 63260, holotype, cultures ex-holotype CMW 50782, CBS 144553.

Description. *Sexual state* not observed. *Asexual state* present but scarce on SNA.. *Macroconidiophores* formed on surface of SNA, consisting of conidiogenous apparatus and occasionally elongated stipes. *Stipes* originating within conidiogenous apparatus,

terminating in inflated apex, 31–74 µm long, 3–4 µm wide at base, 2–2.5 µm wide at middle, terminal vesicles subglobose to globose, 3.5–5 µm wide. *Conidiogenous apparatuses* hyaline, branched in 2–4 tiers, primary branches cylindrical, aseptate, rarely 1-septate 11–34 × 4–5 µm, secondary branches 11–16 × 3.5–5 µm, tertiary branches 10–13 × 3–4.5 µm. *Conidiogenous cells* blastic, cylindrical to ovoid. *Macroconidia* hyaline, cylindrical, round at both ends, gradually tapering towards base, 1-septate, gelatinous sheath visible at both ends or throughout the length, 42–55 × 4–5 (49.4 ± 2.97 × 4.8 ± 0.3) µm. *Sclerotia* present, in clumps or in chains. *Mega*- and *microconidia* not observed.

Culture characteristics. Colonies on 2% MEA after 7 d in the dark, white to salmon to saffron on the surface, ochreous to umber in reverse, aerial mycelium sparse. Optimal growth temperature at 25 °C reaching an average of 61.6 mm, followed by 20 °C (53.4 mm), 30 °C (44.6 mm), 15 °C (24.7 mm) and no growth at 10 and 35 °C after 7 d.

Distribution. Sabah, Malaysia.

Notes. *Calonectria borneana* is a member of the *Ca. kyotensis* complex. This species is phylogenetically closely related to *Ca. lantauensis*. The macroconidia of *Ca. borneana* (avg. 49.4 × 4.8 µm) are smaller than those of *Ca. lantauensis* (avg. 55 × 5 µm). It can be differentiated from its most closely related species by sequences of *CMDA*, *HIS*3, *RPB2* and *TEF1* gene regions.

Additional material examined. MALAYSIA. Sabah, Tawau, Brumas, from soil in *Eucalyptus* sp. plantation, Aug. 2017, M.R.B.A Rauf, PREM 63258, cultures CMW 50832, CBS 144551; PREM 63259, cultures CMW 50833, CBS 144552.

Calonectria ladang N.Q. Pham, Marinc. & M.J. Wingf., **sp. nov.** Fig. 4

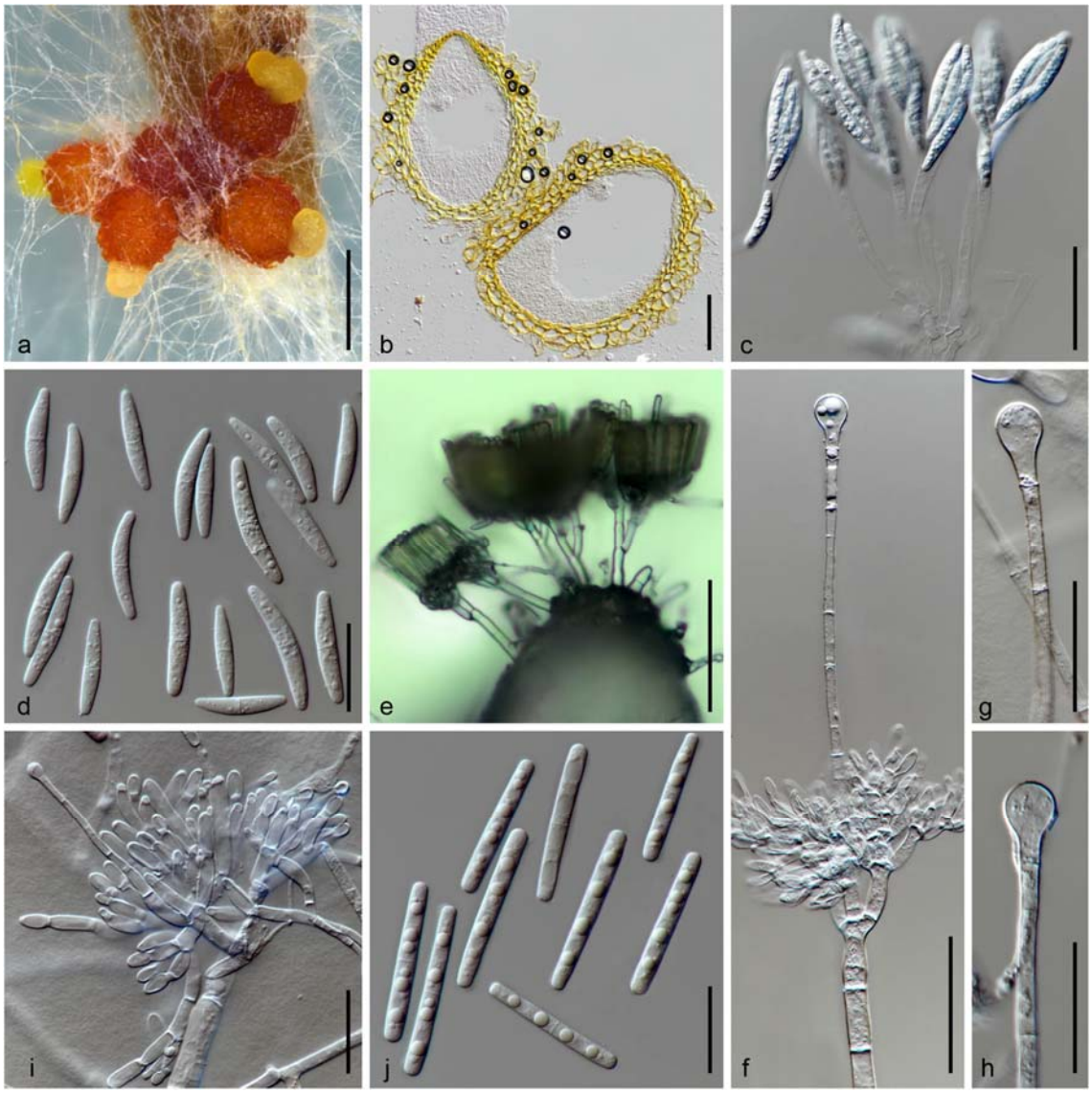

Fig. 4 Micrographs of *Calonectria ladang* sp. nov. (ex-holotype CMW 50776 = CBS 144550). **a** Ascomata on toothpick exudating yellow mass of ascospores. **b** Vertical section of ascomata. **c** Asci. **d** Ascospores. **e** Conidiophores on SNA showing cylindrical conidial cluster. **f** Conidiogenous apparatus with extended stipe. **g, h** Terminal vesicle of stipe. **i** Conidiogenous apparatus. **j** Conidia. Scale bars: a = 500 µm, b, e = 100 µm; c, f = 50 µm; d, g–j = 25 µm

MycoBank MB841987

Etymology. Name refers to the Malay word for plantations ("ladang"), the environment where this fungus was isolated.

Type material. MALAYSIA. Sabah, Tawau, Brumas, from soil in *Eucalyptus* sp. plantation, Aug. 2017, M.R.B.A. Rauf, holotype, PREM 63257, cultures ex-holotype CMW 50776, CBS 144550.

Description. *Sexual* and *asexual state* present. *Ascomata* on MSA orange tinted with rough surface, becoming darker with age, obpyriform, single or clustered, 325–520 × 235–450 µm. *Ascomatal walls* yellow in 85% lactic acid, composed of a few layers of cells, *textura globulosa*, becoming compressed and colourless towards inside, 45–78 µm thick, 13–27 µm thick near ostiole. *Asci* clavate with long stipe, 134–181 µm long, 13.5–22 µm wide at spore-bearing part. *Ascospores* bright yellow in mass when oozing at tip of ascomata, hyaline, fusoid, curved, 1(–2)-septate, slightly constricted at septum, 26–44 × 4–6 (32.5 ± 4.45 × 5.2 ± 0.43) µm. *Macroconidiophores* formed on surface of SNA or aerial hyphae, consisting of conidiogenous apparatus and elongated stipes terminating in subglobose apical cell. *Stipes* extended as part of conidiogenous apparatus, tapering towards inflated apex, 66.5–195 µm long, 3.5–6.5 µm wide at base, 2–3.5 µm wide at middle, terminal vesicles subglobose to globose, 4–13 µm wide. *Conidiogenous apparatuses* hyaline, branched in 3–5 tiers, rarely in 6 tiers, primary branches cylindrical, 12.5–25 × 4–8 µm, secondary branches 9–25.5 × 3.5–8 µm, tertiary branches 9–17 × 3.5–7 µm, quarternary branches 10–16 × 3.5–5.5 µm. *Conidiogenous cells* blastic, cylindrical to ovoid, 8.5–17 × 3–5.5 µm. *Macroconidia* hyaline, cylindrical, 1-septate, rounded at both ends, gradually tapering to base, 39–52 × 4–6 (45.8 ± 2.75 × 4.9 ± 0.35) µm. *Mega-* and *microconidia* not observed. *Sclerotia* present, in chains.

Culture characteristics. Colonies on 2% MEA after 7 d in the dark, white to salmon to rosy buff on the surface, ochreous to umber in reverse, aerial mycelium sparse. Optimal growth temperature at 25 °C reaching 61.6 mm, followed by 30 °C (48.5 mm), 20 °C (44.2 mm), 15 °C (26.4 mm) and no growth at 10 and 35 °C after 7 d.

Distribution. Sabah, Malaysia.

Notes. *Calonectria ladang* is a member of the *Ca. kyotensis* complex. This species is phylogenetically closely related to *Ca. sumatrensis*. The macroconidia of *Ca. ladang* (avg. 45.8 × 4.9 µm) are smaller than those of *Ca. sumatrensis* (avg. 58 × 5 µm). It can be differentiated from its most closely related species by sequences of *ACT*, *CMDA*, *HIS*3, *RPB2* and *TEF1* gene regions.

Additional material examined. MALAYSIA. Sabah, Tawau, Brumas, from soil in *Eucalyptus* sp. plantation, Aug. 2017, M.R.B.A Rauf, PREM 63255, cultures CMW 50774, CBS 144548; PREM 63256, cultures CMW 50775, CBS 144549.

Calonectria pseudomalesiana N.Q. Pham, Marinc. & M.J. Wingf., **sp. nov.** Fig. 5

MycoBank MB841988

Etymology. Name refers to the fact that this fungus closely resembles *Calonectria malesiana.*

Type material. MALAYSIA. Sabah, Tawau, Brumas, from soil in *Eucalyptus* sp. plantation, Aug. 2017, M.J. Wingfield, holotype, PREM 63261, cultures ex-holotype CMW 50821, CBS 144563.

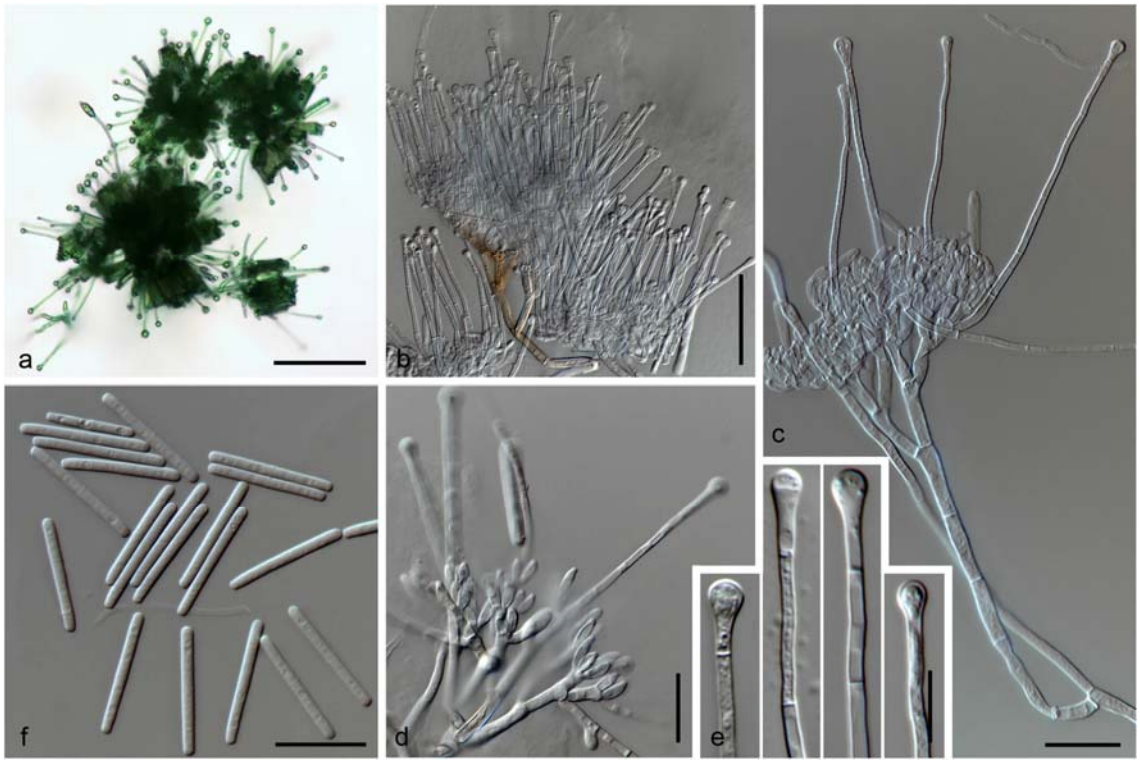

Fig. 5 Micrographs of *Calonectria pseudomalesiana* sp. nov. (ex-holotype CMW 50821 = CBS 144563). **a** Conidiophores on SNA showing conidial clusters and elongated stipes. **b, c, d** Conidiogenous apparatus with conidiogenous cells and stipes. **e** Terminal vesicles of stipe. **f** Conidia. Scale bars: a = 100 µm; b = 50 µm; c, d, f = 25 µm; e = 10 µm

Description. *Sexual state* not observed. *Macroconidiophores* on SNA consisting of conidiogenous apparatus and stipes. *Stipes* 235–301 µm long, or emerging from conidiogenous apparatus on already formed conidiophores, 67–112 µm long, 3–5 µm wide at base, tapering towards apex, 2–4 µm wide at middle, apex ellipsoidal to subglobose, 3–6 µm wide at apex. *Conidiogenous apparatuses* branching in 2–5 tiers, primary branches 11–25 × 3–5 µm, secondary branches 8–20 × 3–5 µm, tertiary branches 8–14 × 2–4 µm. *Conidiogenous cells* cylindrical to ovoid, becoming narrower at apex, 7–12 × 2–4 µm. *Macroconidia* hyaline, cylindrical, 1-septate, gradually tapering towards base, round at apex,

with gelatinous sheath at both ends, 24.5–38 × 3 (33.6 ± 2.51 × 3 ± 0.16) µm. *Mega*- and *microconidia* not observed. *Sclerotia* present, in chains.

Culture characteristics. Colonies on 2% MEA after 7 d in the dark, white to buff on the surface and sienna to umber in reverse, aerial mycelium sparse to moderate. Optimal growth temperature at 30 °C reaching 43.9 mm, followed by 25 °C (34.8 mm), 20 °C (21.9 mm), 15 °C (15.1 mm) and no growth at 10 and 35 °C after 7 d.

Distribution. Sabah, Malaysia.

Additional material examined. MALAYSIA. Sabah, Tawau, Brumas, from soil in *Eucalyptus* sp. plantation, Aug. 2017, M.R.B.A Rauf, PREM 63262, cultures CMW 50779, CBS 144668.

Notes. *Calonectria pseudomalesiana* is a member of the *Ca. kyotensis* complex. This species is phylogenetically closely related to *Ca. malesiana*. The macroconidia of *Ca. pseudomalesiana* (avg. 33.6 × 3 µm) are smaller than those of *Ca. sumatrensis* (avg. 47.5 × 4 µm). It can be differentiated from its most closely related species by sequences of *ACT*, *CMDA*, *HIS*3, *RPB2*, *TEF1* and *TUB*2 gene regions.

Calonectria tanah N.Q. Pham, Marinc. & M.J. Wingf., **sp. nov.** Fig. 6

MycoBank MB841989

Etymology. Name refers to the Malay word for soil (“tanah”), the substrate from which this fungus was first isolated.

Type material. MALAYSIA. Sabah, Tawau, Brumas, from soil in *Eucalyptus* sp. plantation, Aug. 2017, M.R.B.A Rauf, holotype PREM 63265, cultures ex-holotype CMW 50777, CBS 144562.

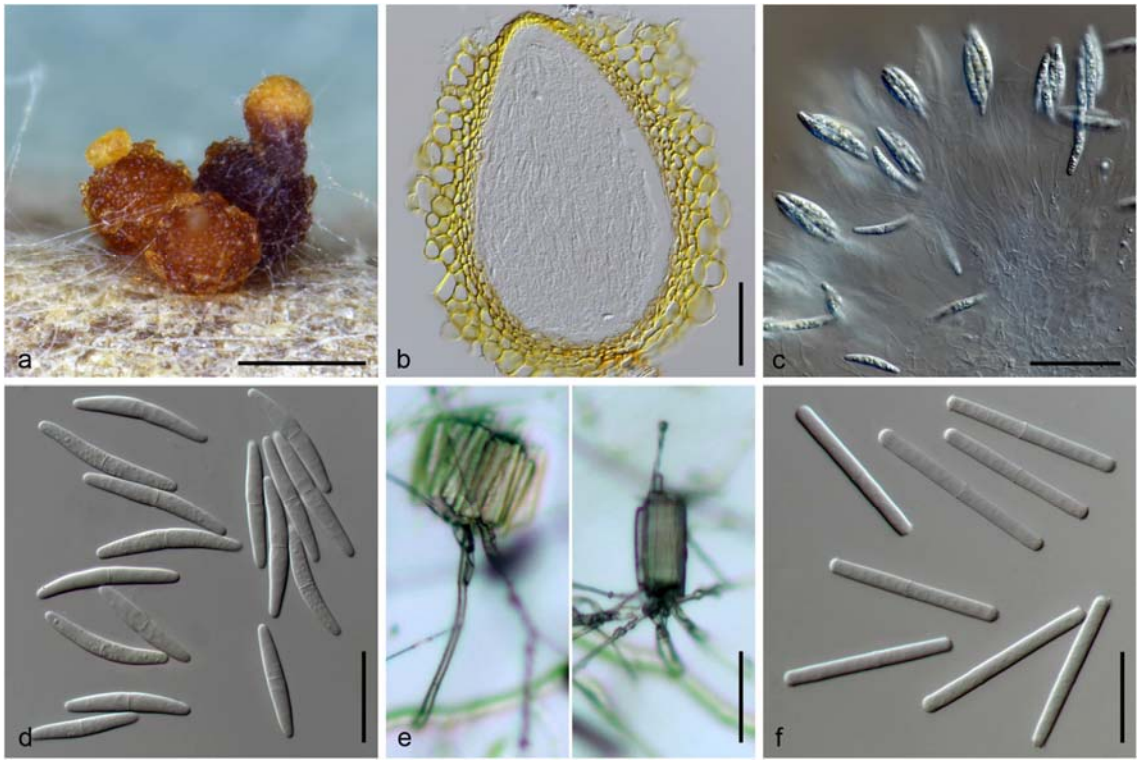

Fig. 6 Micrographs of *Calonectria tanah* sp. nov. (ex-holotype CMW 50777 = CBS 144562). **a** Ascomata on a toothpick exhudating yellow ascospore mass. **b** Vertical section of ascoma (in 85% lactic acid). **c** Asci. **d** Ascospores. **e** Conidiophores on SNA showing cylindrical conidial cluster and stipe. **f** Conidia. Scale bars: a = 500 µm; b = 100 µm; c, e = 50 µm; **d**, **f** = 25 µm

Description. *Sexual state* present on MSA. *Asexual state* present but scarce on SNA. *Ascomata* single or gregarious, subglobose to ovoid, 370–500 × 260–470 µm. *Ascomatal walls* composed of a few layers of cells, *textura globulosa*, becoming compressed toward inside, 55–110 µm wide, 15–24 µm wide near ostiole. *Asci* clavate with elongated stipe. *Ascospores* hyaline, fusoid, slightly curved, 1-septate, slightly constricted at septum, 26–47 × 4–6 (35.9 ± 4.14 × 5 ± 0.43 µm). *Macroconidiophores* on SNA composed of conidiogenous apparatus and stipes. *Stipes* part of conidiogenous apparatus, elongated, tapering towards apex, apex ellipsoidal to subglobose. *Conidiogenous apparatus* and *conidiogenous cells* not described due to lack of sufficient material. *Macroconidia* hyaline, cylindrical, round at apex,

tapering towards base, 1–2-septate, with gelatinous sheath at both ends, 42–57 × 4–5 (48 ± 3.33 × 4.4 ± 0.26) µm. *Mega*- and *microconidia* not observed.

Culture characteristics. Colonies on 2% MEA after 7 d in the dark, white to salmon to saffron on the surface, ochreous to umber in reverse, aerial mycelium moderate. Optimal growth temperature at 25 °C reaching 62.1mm, followed by 30 °C (49.5mm), 20 °C (36.9 mm), 15 °C (20.8 mm) and no growth at 10 and 35 °C after 7d.

Distribution. Sabah, Malaysia.

Additional material examined. MALAYSIA. Sabah, Tawau, Brumas, from soil in *Eucalyptus* sp. plantation, Aug. 2017, M.R.B.A Rauf, PREM 63263, cultures CMW 50771, CBS 144560; PREM 63264, cultures CMW 50772, CBS 144561.

Notes. *Calonectria tanah* is a member of the *Ca. kyotensis* complex. This species is phylogenetically closely related to *Ca. syzygiicola*. It can be differentiated from its most closely related species by sequences of *CMDA*, *HIS*3, *TEF1* and *TUB2* gene regions.

Discussion

This study represents the most intensive investigation of *Calonectria* species associated with *Eucalyptus* plantation forestry in Malaysia. In total, 73 isolates of *Calonectria* were characterized from infected *Eucalyptus* leaf tissues or on germinating alfalfa seeds used as baits for the soil samples associated with symptomatic trees. Based on phylogenetic analyses and morphological characteristics, eight species residing in three different species complexes were identified. These include four previously described species i.e. *Ca. hawksworthii, Ca. ilicicola, Ca. reteaudii* and *Ca. sumatrensis*. In addition, four novel taxa were introduced in this study as *Ca. borneana, Ca. ladang, Ca. pseudomalesiana* and *Ca. tanah.*

A combination of a morphological species concept and multigene phylogenetic analyses utililising informative DNA barcodes provides the most robust view of species boundaries in *Calonectria* (Liu et al. 2020). In the case of *Calonectri*a, where ex-type strains and barcode sequences have been designated for the majority of described species, these provide a reliable foundation to describe new species with confidence (Liu et al. 2020). In this study, phylogenetic analysis of six loci (*ACT, CMDA, HIS3, RPB2, TEF1* and *TUB2*) individually, as well as in combination, made it possible to distinguish all species collected in Malaysia. The data also provided strong support revealing the novelty of the four new taxa, which had robust bootstrap and posterior probability values.

This study represents the first report of *Ca. hawksworthii* from South East Asia and it is the first species residing in *Ca. cylindrospora* complex to be reported from Malaysia. Liu et al. (2020) recently reduced *Ca. foliicola* and *Ca. sulawesiensis* to synonymy with *Ca. hawksworthii* based on phylogenetic inference. This species was previously found to be associated with leaf spots on water-lilies in Mauritius (Crous 2002), and on *Eucalyptus* in Indonesia and China (Lombard et al. 2010b, 2015a). In the present study, *Ca. hawksworthii* was obtained only from soils samples and its possible role as a plant pathogen is unknown.

The majority of the isolates (60%) obtained in this study were those of *Ca. reteaudii*. This was the only species recovered from both infected leaves (21 isolates) and soil (23 isolates) (Fig. 1). Most species in *Ca. reteaudii* complex are well-known pathogens associated with CLB on *Eucalyptus* and they have predominantly been found in tropical and subtropical regions of Southeast Asia and Australasia (Crous 2002; Old et al. 2003; Crous et al. 2006; Lombard et al. 2010b; Li et al. 2017; Pham et al. 2019; Liu et al. 2020; Wang and Chen 2020). C*alonectria reteaudii* was dominant in most of the sampled plantations in this study and continues to represent a threat to the *Eucalyptus* plantation forestry in the region.

The *Ca. kyotensis* complex emerged as the most diverse species complex in this study. The results included two previously described species in the complex i.e. *Ca. ilicicola* and *Ca. sumatrensis*. In addition, the four novel taxa emerging from this study also resided in this complex. Consequently, the *Ca. kyotensis* complex now accommodates 29 species and is thus the largest species complex in *Calonectria*. The greatest species diversity for this complex appears to occur in different regions of Asia and the species predominantly been

isolated from soils (Crous 2002; Crous et al. 2004, 2021; Lombard et al. 2010b, 2015a; Li et al. 2017; Liu and Chen 2017; Pham et al. 2019; Wu and Chen 2021).

Together with those of previous investigations, results of this study supported the view that planted *Eucalyptus* in tropical and subtropical areas represent a niche that is remarkably rich in species of *Calonectria,* especially in the soils associated with these trees (Alfenas et al. 2015; Lombard et al. 2015a; Li et al. 2017; Pham et al. 2019; Wu and Chen 2021). With the exception of *Ca. ilicicola* and *Ca. reteaudii*, all of the other six species found in this study represent first reports for this region. However, it remains to be determined whether they have been introduced from other areas. Alternatively, whether the *Eucalyptus* environment could have influenced their presence in the soils. This is an intriguing question that might have an impact on the future health of *Eucalyptus* trees planted in the tropics and sub-tropics.

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Declarations

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Conflicts of interest/Competing interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Nam Q. Pham and Michael J. Wingfield contributed to the study conception and design. Michael J. Wingfield collected the samples. Nam Q. Pham and Seonju Marincowitz performed the laboratory work and data analysis. The first draft of the manuscript was written by Nam Q. Pham and all authors contributed to subsequent versions. All authors read and approved the final manuscript.

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