Plant Disease • 2023 • 107:730-742 • https://doi.org/10.1094/PDIS-01-22-0196-RE

Calonectria queenslandica: Causal Agent of *Eucalyptus* Leaf Blight in Southern China

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Abstract

Calonectria leaf blight caused by *Calonectria* spp. is among the most serious diseases affecting the health and sustainability of *Eucalyptus* plantations in southern China. Recent outbreaks of this disease in GuangDong Province prompted a need to identify the species involved. Typical symptoms of Calonectria leaf blight were observed on 2-year-old *Eucalyptus urophylla* × *E. grandis* trees in a plantation in the ZhaoQing region. In total, 38 *Calonectria* isolates were collected from 32 diseased trees. All isolates were identified using DNA sequence analyses of the translation elongation factor 1-α (*tef1*), β-tubulin (*tub2*), calmodulin (*cmdA*), and histone H3 (*his3*) gene regions. Phylogenetic analyses revealed that *Calonectria queenslandica* was the dominant species, accounting for 81.6% of the isolates collected. Other species isolated included *C. pseudoreteaudii* (10.5%), *C. reteaudii* (5.3%), and *C. aconidialis* (2.6%). This is the first report of *C. queenslandica* in China and all isolates had identical sequences in all four gene regions. PCR amplification using primers targeting the *MAT1-1-1* and *MAT1-2-1* genes in all *C. queenslandica* isolates revealed that only the *MAT1-2* idiomorph was present. The results suggest that *C. queenslandica* was introduced into the sampled area with very limited genetic diversity. Pathogenicity tests were conducted on two *Eucalyptus* genotypes widely planted in the GuangDong Province using isolates representing all species collected. The results showed that these species could all cause disease but the predominance of *C. queenslandica* on infected trees suggests that it is the major driver of the disease problem studied. Different *Eucalyptus* genotypes used in the pathogenicity tests differed in susceptibility to infection by the *Calonectria* spp. tested, providing opportunities to avoid leaf blight by deploying disease-tolerant planting stock.

Keywords: *Calonectria aconidialis*, *C. pseudoreteaudii*, *C. reteaudii*, mating type, pathogenicity

Eucalyptus spp. (Myrtaceae, Myrtales) and their hybrids have been extensively cultivated in many countries of the world due to their rapid growth, ability to grow under different environmental conditions, and many applications in wood-based products (Coppen 2002). In China, *Eucalyptus* trees have been widely planted in FuJian, GuangDong, GuangXi, HaiNan, and YunNan Provinces since they were first introduced in 1890 (Qi 2002). Currently, more than 5.4 million ha of *Eucalyptus* plantations have been established in China, accounting for 2.5% of the national forest area, and the annual production of *Eucalyptus* timber exceeds one-third of the national wood supply (Xu et al. 2019). However, in recent years, diseases caused by bacteria and fungi as well as insects have emerged to threaten the sustainability of these plantations in many parts of the world where these trees are planted (Li et al. 2022; Wingfield et al. 2008, 2015).

Many diseases caused by various pathogens impact negatively on the health of *Eucalyptus* plantations in China. These include stem cankers caused by species of Botryosphaeriaceae (Li et al. 2018), the Cryphonectriaceae (Wang et al. 2020), and *Teratosphaeria zuluensis* (Chen et al. 2011a); wilt caused by *Ceratocystis* spp. (S. F. Chen et al. 2013) and *Ralstonia pseudosolanacearum* (Carstensen et al. 2017); as well as leaf and shoot disease caused by species of Mycosphaerellaceae (Burgess et al. 2007), Teratosphaeriaceae (Burgess et al. 2006), *Calonectria* (Q. Z. Chen et al. 2013; Li et al. 2017; Wang and Chen 2020; Wu and Chen 2021), and *Quambalaria* (Chen et al. 2017). Of these, leaf blight caused by *Calonectria* spp. is considered one of the most important diseases affecting *Eucalyptus* plantations in southern China (Chen et al. 2011b; Wang and Chen 2020; Wu and Chen 2021).

Calonectria spp. have a wide distribution in tropical and subtropical regions of the world (Crous 2002). There are approximately 126 formally described species based on DNA sequence data, of which 51 have been isolated from *Eucalyptus* seedlings, or from trees and soils in the understory of *Eucalyptus* plantations (Crous et al. 2018, 2019, 2021a, b; Liu et al. 2020; Mohali and Stewart 2021; Wang et al. 2019). Twenty-six species identified using phylogenetic inference of DNA sequence data have been reported from China. Of these, eight have been isolated from diseased *Eucalyptus* trees or seedlings, and these include *Calonectria aciculata, C. cerciana, C. crousiana, C. eucalypti, C. fujianensis*, *C. hawksworthii*, *C. pauciramosa*, and *C. pseudoreteaudii* (Feng et al. 2007; Liu et al. 2020; Liu et al. 2021; Lombard et al. 2015a; Yang et al. 2014; Wang et al. 2019; Wu and Chen 2021). Most of these species are associated with disease symptoms that include leaf spots, shoot blight, stem cankers, and root disease on plantation trees and cutting rot, damping-off, and stem cankers on nursery seedlings (Crous 2002).

Disease surveys in *Eucalyptus* nurseries and plantations of southern China have suggested that *C. pseudoreteaudii* is the only dominant species causing disease. Furthermore, it is widely distributed on different *Eucalyptus* genotypes in the Leizhou Peninsula, ZhanJiang Region (GuangDong Province) and in one experimental plantation in the BeiHai Region of GuangXi Province (Wang and Chen 2020; Wu and Chen 2021).

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Funding: This study was initiated through the bilateral agreement between the Governments of South Africa and China and supported by the National Key R&D Program of China (China-South Africa Forestry Joint Research Centre Project; project number 2018YFE0120900), the National Ten-Thousand Talents Program (project number W03070115), and the GuangDong Top Young Talents Program (project number 20171172).

e-Xtra: Supplementary material is available online.

The author(s) declare no conflict of interest.

Accepted for publication 27 July 2022.

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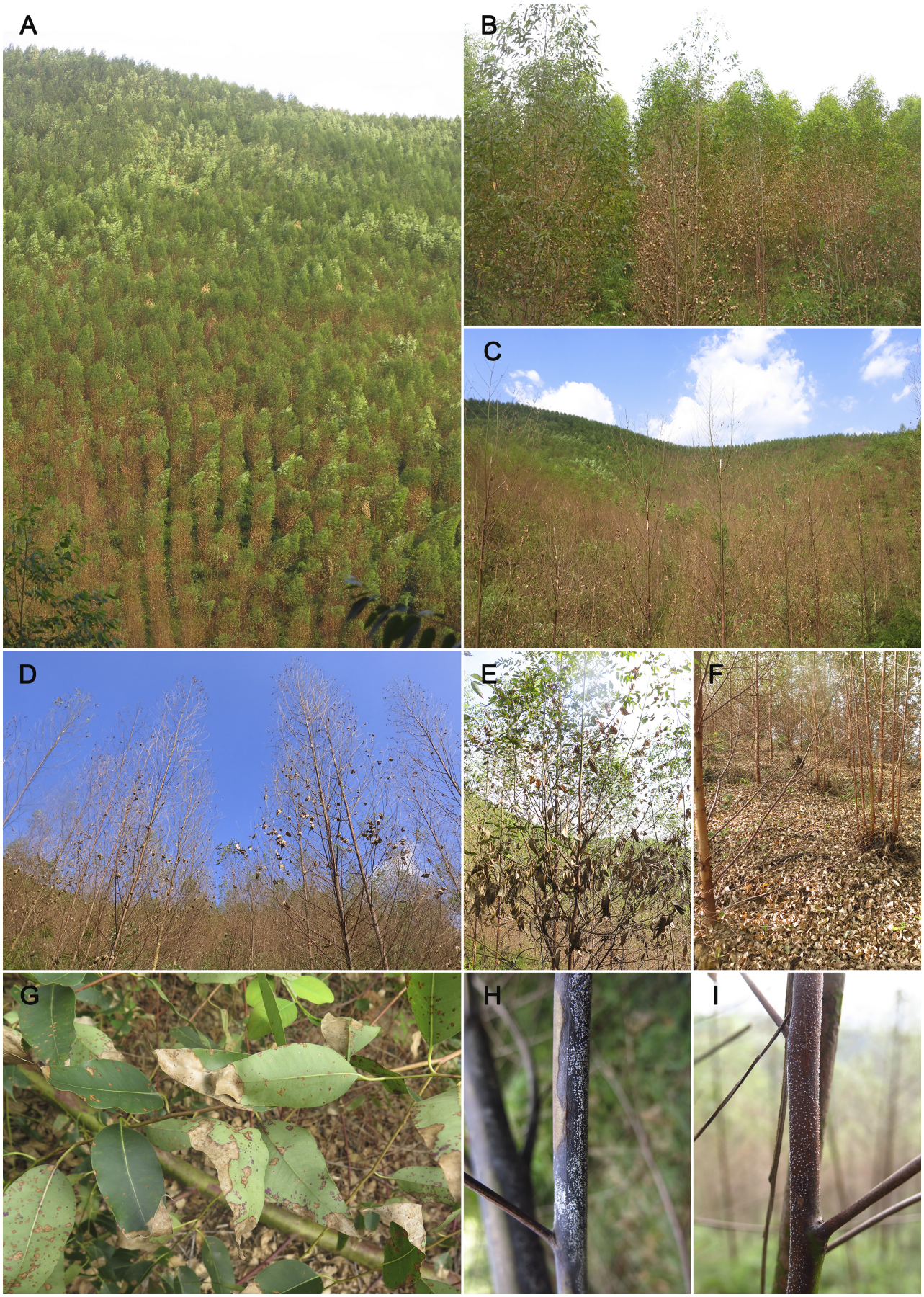

Fig. 1. Disease symptoms on *Eucalyptus* plantation caused by *Calonectria* spp. **A,** Leaf infections occurred in the basal and middle part of *Eucalyptus* trees; **B and C,** 30 to 80% of leaves of trees were infected; **D,** whole leaves of the trees were infected; **E,** leaves of *Eucalyptus* trees dried out as a consequence of pathogen infection; **F,** leaf drying and defoliation caused by *Calonectria*; **G,** necrotic lesions of leaves caused by *Calonectria*; and **H and I,** masses of conidiophores of *Calonectria* on the stem.

In this study, disease surveys were conducted in a *Eucalyptus* plantation in ZhaoQing Region, GuangDong Province where leaf blight had emerged as a serious problem. Isolates of *Calonectria* spp. were collected from the affected trees and these were identified using multigene phylogenetic analyses. Additionally, the mating types of the isolated of *Calonectria* spp. were determined and their pathogenicity was assessed on two commonly planted *Eucalyptus* genotypes.

Materials and Methods

Disease symptoms, samples, and fungal isolations

In September 2018, a serious disease outbreak was observed in a 2-year-old *Eucalyptus urophylla* × *E. grandis* plantation near the town of HuoDao, GaoYao District, ZhaoQing Region (22°51′26.69″ N, 112°25′01.08″ E). Leaf infections occurred from the bases and moved upward into the crowns of the trees. The area of this *Eucalyptus* plantation is about 50 ha with around 80,000 trees planted. Approximately 60% of the trees in this plantation were affected, with up to 100% of leaves on a single tree infected, especially on trees in low-lying areas (Fig. 1A to G). White masses of conidiophores were commonly seen on branches, shoots, twigs, and leaves of affected trees (Fig. 1H and I).

We adopted a random sampling pattern, collecting symptomatic leaves and twigs from a diseased *Eucalyptus* tree every 100 m along a transect throughout the plantation. Samples were collected from 32 symptomatic *E. urophylla* × *E. grandis* hybrid trees. For 15 of the trees, only diseased leaves were collected; for 10 of these trees, only diseased twigs were collected; and both diseased leaves and twigs were collected from 7 of the trees. Leaves and twigs with conidiophore masses present were collected and transported to the laboratory for further study. The symptomatic leaves and twigs were transferred to Petri dishes kept at room temperature for 1 to 2 days until conidial masses characteristic of *Calonectria* spp. were abundant on the surface of infected tissues. At least one *Calonectria* isolate was obtained from either leaves or twigs of each sampled tree, with one exception where a diseased twig was sampled but no isolate could be recovered.

Conidial masses were lifted from the infected tissues with sterile syringe needles and transferred to 2% malt extract agar (MEA) plates (20 g of malt extract, 20 g of agar, and 1 liter of water) under a Stemi 2000-C stereomicroscope (Carl Zeiss Ltd., Munchen, Germany) and incubated for 3 to 5 days. Single hyphal tips were cut from the edges of the resulting cultures, transferred to clean 2% MEA plates, and incubated at room temperature for 7 days to obtain pure cultures. The pure cultures obtained were

Table 1. Isolates of *Calonectria* reported in this study[a]

Species	Genotype[b]	Isolate[c]	Host, substrate	Mating type	GenBank accession number					
					MAT1-1-1	*MAT1-2-1*	*tef1*	*tub2*	*cmdA*	*his3*
Calonectria queenslandica	AAAA	CSF12027	*Eucalyptus urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803057	OM802900	OM802938	OM802976	OM803014
C. queenslandica	AAAA	CSF12028	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803058	OM802901	OM802939	OM802977	OM803015
C. queenslandica	AAAA	CSF12029	*E. urophylla* × *E. grandis*, twig	MAT1-2	NA	OM803059	OM802902	OM802940	OM802978	OM803016
C. queenslandica	AAAA	CSF12030	*E. urophylla* × *E. grandis*, twig	MAT1-2	NA	OM803060	OM802903	OM802941	OM802979	OM803017
C. queenslandica	AAAA	CSF12031[d,e]	*E. urophylla* × *E. grandis*, twig	MAT1-2	NA	OM803061	OM802904	OM802942	OM802980	OM803018
C. queenslandica	AAAA	CSF12032	*E. urophylla* × *E. grandis*, twig	MAT1-2	NA	OM803062	OM802905	OM802943	OM802981	OM803019
C. queenslandica	AAAA	CSF12033	*E. urophylla* × *E. grandis*, twig	MAT1-2	NA	OM803063	OM802906	OM802944	OM802982	OM803020
C. queenslandica	AAAA	CSF12034[d,e]	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803064	OM802907	OM802945	OM802983	OM803021
C. queenslandica	AAAA	CSF12035	*E. urophylla* × *E. grandis*, twig	MAT1-2	NA	OM803065	OM802908	OM802946	OM802984	OM803022
C. queenslandica	AAAA	CSF12036	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803066	OM802909	OM802947	OM802985	OM803023
C. queenslandica	AAAA	CSF12037[d,e]	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803067	OM802910	OM802948	OM802986	OM803024
C. queenslandica	AAAA	CSF12038	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803068	OM802911	OM802949	OM802987	OM803025
C. queenslandica	AAAA	CSF12039	*E. urophylla* × *E. grandis*, twig	MAT1-2	NA	OM803069	OM802912	OM802950	OM802988	OM803026
C. queenslandica	AAAA	CSF12042	*E. urophylla* × *E. grandis*, twig	MAT1-2	NA	OM803070	OM802913	OM802951	OM802989	OM803027
C. queenslandica	AAAA	CSF12043	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803071	OM802914	OM802952	OM802990	OM803028
C. queenslandica	AAAA	CSF12044	*E. urophylla* × *E. grandis*, twig	MAT1-2	NA	OM803072	OM802915	OM802953	OM802991	OM803029
C. queenslandica	AAAA	CSF12045	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803073	OM802916	OM802954	OM802992	OM803030
C. queenslandica	AAAA	CSF12046	*E. urophylla* × *E. grandis*, twig	MAT1-2	NA	OM803074	OM802917	OM802955	OM802993	OM803031
C. queenslandica	AAAA	CSF12047	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803075	OM802918	OM802956	OM802994	OM803032
C. queenslandica	AAAA	CSF12048	*E. urophylla* × *E. grandis*, twig	MAT1-2	NA	OM803076	OM802919	OM802957	OM802995	OM803033

(*Continued on next page*)

[a] All isolates were collected by S. F. Chen, G. Q. Li, and W. W. Li. NA = not available.
[b] Genotype assignment based on sequences of the translation elongation factor 1-α (*tef1*), β-tubulin (*tub2*), calmodulin (*cmdA*), and histone H3 (*his3*) regions.
[c] CSF = Culture Collection of the Research Institute of Fast-growing Trees (RIFT)/China Eucalypt Research Centre (CERC), Chinese Academy of Forestry, ZhanJiang, GuangDong Province, China.
[d] Isolates used for phylogenetic analyses.
[e] Isolates used for pathogenicity tests.

deposited in the Culture Collection (CSF) at the Research Institute of Fast-Growing Trees (RIFT)/China Eucalypt Research Centre (CERC) of the Chinese Academy of Forestry (CAF) in ZhanJiang, GuangDong Province, China.

DNA extraction, PCR amplification, and sequencing

All isolates obtained in this study were used in DNA sequence analyses. These isolates were transferred to 2% MEA plates and incubated at room temperature for 7 days before DNA extraction was carried out. Mycelium was scraped from the surface of the plates with a sterilized scalpel, transferred to 2-ml Eppendorf tubes, and crushed with a Tissue Lyser (Qiagen, Hilden, Germany). Total genomic DNA was extracted using the cetyltrimethylammonium bromide method, as described by van Burik et al. (1998). The extracted DNA was dissolved in 30 μl of Tris-EDTA buffer (10 mM Tris-HCL and 1 mM EDTA, pH 8.0) to which 2.5 μl of RNase (10 mg/ml) was added and incubated at 37°C for 1 h to degrade RNA. DNA concentration was quantified using a Nano-Drop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and diluted to a concentration of 50 to 100 ng/μl.

Four loci, including the translation elongation factor 1-α (*tef1*), β-tubulin (*tub2*), calmodulin (*cmdA*), and histone H3 (*his3*), were amplified using primers described by Liu et al. (2020), including EF1-728F and EF2 for the *tef1* gene region, T1 and CYLTUB1R for the *tub2* gene region, CAL-228F and CAL-2Rd for the *cmdA* gene region, and CYLH3F and CYLH3R for the *his3* gene region. The PCR mixtures and amplification protocols were the same as those used by Liu et al. (2020). PCR products were visualized using 2% (wt/vol) agarose gel electrophoresis and submitted to the Beijing Genomics Institute (GuangZhou, China) for bidirectional sequencing with the same primers used for PCR amplifications. Raw sequences were curated and consensus sequences were generated using Geneious v.9.1.4 (Kearse et al. 2012) and were deposited in GenBank (Table 1) (https://www.ncbi.nlm.nih.gov/genbank/).

Multigene phylogenetic analyses

To identify the species complex in which the isolates resided, nucleotide BLAST searches were conducted using the sequences of the four gene regions generated in this study. The *tef1*, *tub2*, *cmdA*, and *his3* sequences generated were analyzed together with sequences for the ex-type strains of all of the published species in the relevant species complexes. The datasets of Liu et al. (2020) were used as basal data for phylogenetic analyses (Table 2). All datasets were aligned using an online version of MAFFT v. 7 (https://mafft.cbrc.jp/alignment/server) with FFT-NS-i option (Katoh and Standley 2013). The sequence alignments were manually edited where necessary using MEGA v. 7.0 software (Kumar et al. 2016).

Phylogenetic analyses were performed separately for each of the *tef1*, *tub2*, *cmdA*, and *his3* sequence datasets as well as on the concatenated dataset of all four gene regions. Maximum-parsimony (MP) and maximum-likelihood (ML) were used for phylogenetic analyses. The MP and ML analyses were performed using the methods described by Liu and Chen (2017). The phylogenetic trees obtained were viewed using MEGA v. 7.0 software (Kumar et al. 2016). Two isolates of *Curvicladiella cignea* (CBS 109167 and CBS 109168) were used as outgroup taxa in the analyses.

MAT gene amplification and mating type assignment

Mating types of all *Calonectria* isolates obtained in this study were identified using PCR amplification targeting the *MAT1-1-1* and *MAT1-2-1* genes. This was achieved using the primers Cal_MAT111_F/Cal_MAT111_R and Cal_MAT121_F/Cal_MAT121_R for *MAT1-1-1* and *MAT1-2-1* respectively, following protocols described by Li et al. (2020).

Pathogenicity tests

To determine and compare the pathogenicity of the *Calonectria* spp. identified in this study, representative isolates of all species were

Table 1. (*Continued from previous page*)

Species	Genotype[b]	Isolate[c]	Host, substrate	Mating type	GenBank accession number					
					MAT1-1-1	*MAT1-2-1*	*tef1*	*tub2*	*cmdA*	*his3*
C. queenslandica	AAAA	CSF12049	*E. urophylla* × *E. grandis*, twig	MAT1-2	NA	OM803077	OM802920	OM802958	OM802996	OM803034
C. queenslandica	AAAA	CSF12050	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803078	OM802921	OM802959	OM802997	OM803035
C. queenslandica	AAAA	CSF12052	*E. urophylla* × *E. grandis*, twig	MAT1-2	NA	OM803079	OM802922	OM802960	OM802998	OM803036
C. queenslandica	AAAA	CSF12053	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803080	OM802923	OM802961	OM802999	OM803037
C. queenslandica	AAAA	CSF12055	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803081	OM802924	OM802962	OM803000	OM803038
C. queenslandica	AAAA	CSF12057[d,e]	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803082	OM802925	OM802963	OM803001	OM803039
C. queenslandica	AAAA	CSF12059[d,e]	*E. urophylla* × *E. grandis*, twig	MAT1-2	NA	OM803083	OM802926	OM802964	OM803002	OM803040
C. queenslandica	AAAA	CSF12060	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803084	OM802927	OM802965	OM803003	OM803041
C. queenslandica	AAAA	CSF12063	*E. urophylla* × *E. grandis*, twig	MAT1-2	NA	OM803085	OM802928	OM802966	OM803004	OM803042
C. queenslandica	AAAA	CSF12064	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803086	OM802929	OM802967	OM803005	OM803043
C. queenslandica	AAAA	CSF12065	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803087	OM802930	OM802968	OM803006	OM803044
C. pseudoreteaudii	AAAA	CSF12040[d,e]	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803088	OM802931	OM802969	OM803007	OM803045
C. pseudoreteaudii	AAAA	CSF12041[d,e]	*E. urophylla* × *E. grandis*, twig	MAT1-1	OM803052	NA	OM802932	OM802970	OM803008	OM803046
C. pseudoreteaudii	AAAA	CSF12054[d]	*E. urophylla* × *E. grandis*, leaf	MAT1-1	OM803053	NA	OM802933	OM802971	OM803009	OM803047
C. pseudoreteaudii	AAAA	CSF12061[d,e]	*E. urophylla* × *E. grandis*, leaf	MAT1-1	OM803054	NA	OM802934	OM802972	OM803010	OM803048
C. reteaudii	AAAA	CSF12051[d,e]	*E. urophylla* × *E. grandis*, leaf	MAT1-2	NA	OM803089	OM802935	OM802973	OM803011	OM803049
C. reteaudii	AAAA	CSF12058[d,e]	*E. urophylla* × *E. grandis*, leaf	MAT1-1	OM803055	NA	OM802936	OM802974	OM803012	OM803050
C. aconidialis	AAAA	CSF12056[d,e]	*E. urophylla* × *E. grandis*, leaf	Homothallic	OM803056	OM803090	OM802937	OM802975	OM803013	OM803051

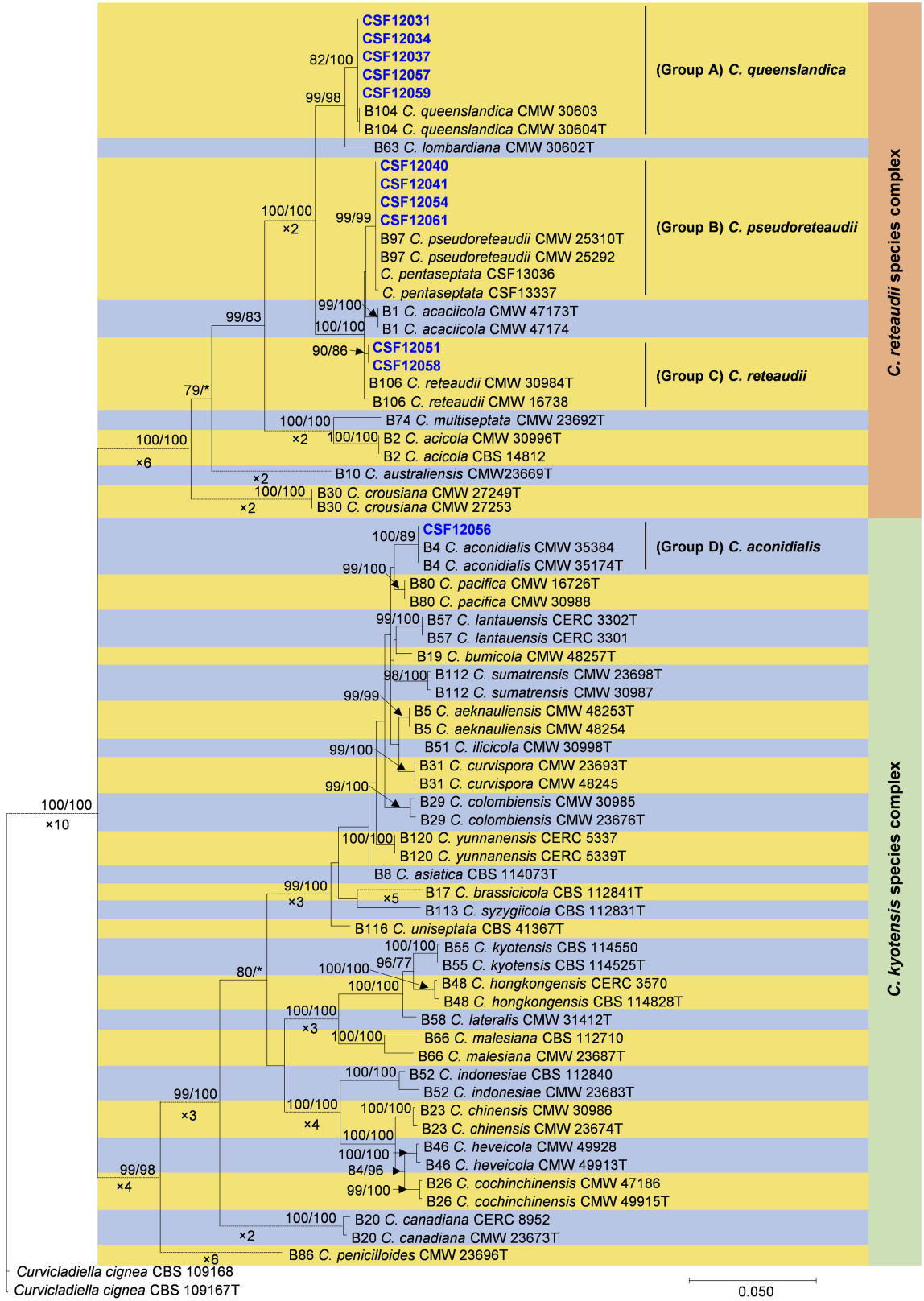

Fig. 2. Phylogenetic trees obtained from maximum-likelihood (ML) analyses of the combined *tef1*, *tub2*, *cmdA*, and *his3* gene sequences for species in the *Calonectria reteaudii* species complex and *C. kyotensis* species complex. Bootstrap support values ≥70% for ML and maximum-parsimony (MP) are indicated above the branches as ML/MP. Bootstrap support values <70% are marked with an asterisk (*). Isolates obtained in this study are highlighted in blue and in bold type; "T" represents ex-type isolates and "B" species codes are consistent with the published results in Liu et al. (2020). *Curvicladiella cignea* (CBS 109167 and CBS 109168) was used as the outgroup taxon.

selected for inoculation trials. Potted plants, between 30 and 40 cm in height of the *E. urophylla* × *E. tereticornis* hybrid (genotype CEPT1876) and *E. urophylla* × *E. grandis* hybrid (genotype CEPT1877) were used in the inoculations.

Two methods were used in the inoculation tests. These included tests using mycelial plugs for all isolates investigated and conidia where these could be induced to form. For the mycelial inoculations, plugs (5 mm in diameter) were taken from the actively growing margins of 7-day-old cultures on MEA and were placed on the back of the leaves, with the mycelial surface in direct contact with the leaf tissues. Plugs of sterile and uninoculated MEA were used as the negative controls. In all, 10 leaves on three trees (3 to 4 leaves per tree) of each *Eucalyptus* genotype were used per isolate and 10 were used for the negative controls.

For the conidial inoculations, suspensions were prepared using the methods described by Wang and Chen (2020). The concentrations of the conidia were determined using a hemocytometer and adjusted to around 5×10^4 conidia/ml. Eight seedlings of each *Eucalyptus* genotype were used per isolate. The inoculation was carried out by spraying the conidial suspensions onto the surface of leaves until run-off. Similar numbers of plants were sprayed with sterile water as negative controls. All of the plants inoculated with mycelial plugs or conidia were kept in plastic chambers to maintain a stable condition of temperature (26 to 28°C) and humidity (60 to 70%) for 3 days. The entire experiments using either mycelial plugs or conidia were repeated once under the same conditions.

The results of the inoculations were evaluated after 3 days. For the mycelial plug inoculations, lesion diameters perpendicular to each other were measured and the averages were calculated. For the inoculations using conidial suspensions, a disease index (DI) was established. This involved determining the area of the lesions using the software "Leaf Doctor" (Pethybridge and Nelson 2015). Disease severity was assessed by estimating the proportion of the leaf area covered by the lesion and assessing this using a 0-to-5 scale, where 0 represented no lesions and 1 to 5 represented 1 to 10, 11 to 25, 26 to 50, 51 to 75, and 76 to 100% of the leaf area diseased, respectively. The DI [DI = $\sum$ (representative rating scale × number of diseased leaves)/(maximum rating scale [5] × total number of leaves examined)] was calculated using the method described by Mishra et al. (2009).

The inoculated fungi were reisolated by cutting small pieces of infected leaf tissue (about 0.04 cm^2) from the edges of the lesions and placing these on 2% MEA plates, after which they were incubated at room temperature. These isolations were made from four randomly selected plants of each *Eucalyptus* genotype inoculated with each test isolate, and the same number of isolations was also made from the negative controls. The reisolated fungi were identified based on morphological characteristics compared with those of the isolates used in the inoculations. The experimental data were analyzed by one-way analysis of variance using SPSS Statistics 22 software (IBM Corp., Armonk, NY, U.S.A.). The pathogenicity tests were performed during September 2020 at the experimental nursery of the China Eucalypt Research Centre, GuangDong Province, China.

Results

Fungal isolations

In total, 38 *Calonectria* isolates were obtained from 31 sampled trees, including 22 from diseased leaves and 16 from diseased twigs (Table 1). Based on their morphological characteristics, 37 isolates resided in the prolate group and 1 resided in the sphaero-naviculate group of *Calonectria* as defined by Lombard et al. (2010a).

Sequencing and multigene phylogenetic analyses

The *tef1*, *tub2*, *cmdA*, and *his3* gene regions were successfully amplified and sequenced for all 38 isolates obtained in this study (Table 1). The sequence fragments for the *tef1*, *tub2*, *cmdA*, and *his3* gene regions were approximately 500, 565, 685, and 435 bp, respectively. Based on the combination of sequences for the four different loci, the 38 isolates collected grouped into four sequence genotypes made up of 31 isolates, 4 isolates, 2 isolates, and 1 isolate, respectively. BLAST searches using the *tef1*, *tub2*, *cmdA*, and *his3* sequences for each genotype group showed that these isolates resided in either the *Calonectria reteaudii* species complex or the *C. kyotensis* species complex. Twelve isolates representing the four genotypes were selected for phylogenetic analyses (Table 1).

A partition homogeneity test on the combined dataset for the *tef1*, *tub2*, *cmdA*, and *his3* gene regions yielded a *P* value of 0.001 and, consequently, these gene regions were concatenated for phylogenetic analysis, as recommended by Cunningham (1997). Trees generated from MP and ML analyses of the four individual genes and the combined dataset were mostly consistent in the grouping of the isolates, although the relative positions of some species were different in the MP and ML trees. The ML tree obtained with the combined dataset is presented in Figure 2 and ML trees obtained with individual datasets are presented in Supplementary Figures S1 to S4. Detailed information on the datasets and parameters used in the MP and ML analyses are shown in Supplementary Table S1.

The 12 representative isolates selected for phylogenetic analysis clustered in four phylogenetic groups (designated as groups A to D) based on *tef1*, *tub2*, *cmdA*, *his3*, and analyses of the combined datasets analyses (Fig. 2; Supplementary Figs. S1 to S4). Isolates in groups A to C resided in the *C. reteaudii* species complex whereas a single isolate for group D resided in the *C. kyotensis* species complex.

Isolates in group A clustered with or were close to *C. queenslandica* and *C. lombardiana* in the *tef1*, *tub2*, *cmdA*, and *his3* trees (Supplementary Figs. S1 to S4). In the combined *tef1*/*tub2*/*cmdA*/*his3* tree, these isolates were most closely related to *C. queenslandica* (Fig. 2) and were identified as that species. Isolates in group B grouped with *C. pseudoreteaudii* in the *tef1*, *tub2*, and *his3* trees (Supplementary Figs. S1, S2, and S4) and with *C. pseudoreteaudii* and *C. reteaudii* in the *cmdA* tree (Supplementary Fig. S3). In the combined analysis, all of these isolates grouped with the ex-type isolate of *C. pseudoreteaudii* (Fig. 2) and were consequently identified as that species. The two isolates in group C grouped with or were close to *C. reteaudii* and *C. acaciicola* in the *tef1*, *tub2*, *cmdA*, and *his3* trees (Supplementary Figs. S1 to S4). These isolates were most closely related to *C. reteaudii* in the combined tree (Fig. 2) and, thus, were identified as that species. A single isolate in group D resided in the *C. kyotensis* species complex. This isolate grouped with the ex-type isolate of *C. aconidialis* in the *tef1*, *cmdA*, and *his3* trees as well as in the combined *tef1*/*tub2*/*cmdA*/*his3* tree (Fig. 2; Supplementary Figs. S1, S3, and S4) and, thus, it was identified as that species. In the *tub2* tree, this isolate formed a separate branch (Supplementary Fig. S2) but this was due to the *tub2* sequences of some ex-type isolates of species in the *C. kyotensis* not being available. In total, 31 isolates were identified as *C. queenslandica*, 4 isolates as *C. pseudoreteaudii*, 2 isolates as *C. reteaudii*, and 1 isolate as *C. aconidialis* (Table 1).

MAT gene amplification and mating type assignment

Portions of the *MAT1-1-1* and *MAT1-2-1* genes representing *MAT1-1* and *MAT1-2* idiomorphs, respectively, were successfully amplified in all 38 isolates of the four species identified in this study. Only the *MAT1-2* idiomorph was detected in all 31 isolates of *C. queenslandica.* For isolates of *C. pseudoreteaudii* and *C. reteaudii*, either *MAT1-1* or *MAT1-2* was detected for a given isolate (Table 1). In the case of the single isolate of *C. aconidialis*, both the *MAT1-1* and *MAT1-2* idiomorphs were detected, confirming that it is a homothallic species (Table 1).

Pathogenicity tests

Eleven isolates representing four *Calonectria* spp. including *C. queenslandica* (CSF12031, CSF12034, CSF12037, CSF12057, and CSF12059), *C. pseudoreteaudii* (CSF12040, CSF12041, and CSF12061), *C. reteaudii* (CSF12051 and CSF12058), and *C. aconidialis*

Table 2. Isolates from other studies used in the phylogenetic analyses

Code[a]	Species	Isolate[b,c]	Other collection[c]	Hosts	Area of occurrence	Collector	GenBank accession numbers[d]				Reference or source of data
							tef1	*tub2*	*cmdA*	*his3*	
B1	*Calonectria acaciicola*	CMW 47173^T	CBS 143557	Soil (*Acacia auriculiformis* plantation)	Do Luong, Nghe An, Vietnam	N. Q. Pham and T. Q. Pham	MT412690	MT412930	MT335160	MT335399	Pham et al. 2019; Liu et al. 2020
		CMW 47174	CBS 143558	Soil (*A. auriculiformis* plantation)	Do Luong, Nghe An, Vietnam	N. Q. Pham and T. Q. Pham	MT412691	MT412931	MT335161	MT335400	Pham et al. 2019; Liu et al. 2020
B2	*C. acicola*	CMW 30996^T	–	*Phoenix canariensis*	Northland, New Zealand	H. Pearson	MT412692	MT412932	MT335162	MT335401	Gadgil and Dick 2004; Lombard et al. 2010a; Liu et al. 2020
		CBS 114812	CMW 51216	*P. canariensis*	Northland, New Zealand	H. Pearson	MT412693	MT412933	MT335163	MT335402	Gadgil and Dick 2004; Lombard et al. 2010a; Liu et al. 2020
B4	*C. aconidialis*	CMW 35174^T	CBS 136086; CERC 1850	Soil (*Eucalyptus* plantation)	HaiNan, China	X. Mou and S. F. Chen	MT412695	NA	MT335165	MT335404	Lombard et al. 2015a; Liu et al. 2020
		CMW 35384	CBS 136091; CERC 1886	Soil (*Eucalyptus* plantation)	HaiNan, China	X. Mou and S. F. Chen	MT412696	NA	MT335166	MT335405	Lombard et al. 2015a; Liu et al. 2020
B5	*C. aeknauliensis*	CMW 48253^T	CBS 143559	Soil (*Eucalyptus* plantation)	Aek Nauli, North Sumatra, Indonesia	M. J. Wingfield	MT412710	NA	MT335180	MT335419	Pham et al. 2019; Liu et al. 2020
		CMW 48254	CBS 143560	Soil (*Eucalyptus* plantation)	Aek Nauli, North Sumatra, Indonesia	M. J. Wingfield	MT412711	NA	MT335181	MT335420	Pham et al. 2019; Liu et al. 2020
B8	*C. asiatica*	CBS 114073^T	CMW 23782; CPC 3900	Debris (leaf litter)	Prathet Thai, Thailand	N. L. Hywel-Jones	AY725705	AY725616	AY725741	AY725658	Crous et al. 2004; Lombard et al. 2010a
B10	*C. australiensis*	CMW 23669^T	CBS 112954; CPC 4714	*Ficus pleurocarpa*	Queensland, Australia	C. Pearce and B. Paulus	MT412723	MT412946	MT335192	MT335432	Crous et al. 2006; Lombard et al. 2010a; Liu et al. 2020
B17	*C. brassicicola*	CBS 112841^T	CMW 51206; CPC 4552	Soil (*Brassica* sp.)	Indonesia	M. J. Wingfield	KX784689	KX784619	KX784561	NA	Lombard et al. 2016
B19	*C. bumicola*	CMW 48257^T	CBS 143575	Soil (*Eucalyptus* plantation)	Aek Nauli, North Sumatra, Indonesia	M. J. Wingfield	MT412736	NA	MT335205	MT335445	Pham et al. 2019; Liu et al. 2020
B20	*C. canadiana*	CMW 23673^T	CBS 110817; STE-U 499	*Picea* sp.	Canada	S. Greifenhagen	MT412737	MT412958	MT335206	MT335446	Kang et al. 2001b; Crous 2002; Lechat et al. 2010; Liu et al. 2020
		CERC 8952	–	Soil	HeNan, China	S. F. Chen	MT412821	MT413035	MT335290	MT335530	Liu and Chen 2017; Liu et al. 2020
B23	*C. chinensis*	CMW 23674^T	CBS 114827; CPC 4101	Soil	Hong Kong, China	E. C. Y. Liew	MT412751	MT412972	MT335220	MT335460	Crous et al. 2004; Lombard et al. 2010a; Liu et al. 2020
		CMW 30986	CBS 112744; CPC 4104	Soil	Hong Kong, China	E. C. Y. Liew	MT412752	MT412973	MT335221	MT335461	Crous et al. 2004; Lombard et al. 2010a; Liu et al. 2020
	C. cochinchinensis	CMW 49915^T	CBS 143567	Soil (*Hevea brasiliensis* plantation)	Duong Minh Chau, Tay Ninh, Vietnam	N. Q. Pham, Q. N. Dang and T. Q. Pham	MT412756	MT412977	MT335225	MT335465	Pham et al. 2019; Liu et al. 2020
		CMW 47186	CBS 143568	Soil (*A. auriculiformis* plantation)	Song May, Dong Nai, Vietnam	N. Q. Pham and T. Q. Pham	MT412757	MT412978	MT335226	MT335466	Pham et al. 2019; Liu et al. 2020
B29	*C. colombiensis*	CMW 23676^T	CBS 112220; CPC 723	Soil (*E. grandis* trees)	La Selva, Colombia	M. J. Wingfield	MT412759	MT412980	MT335228	MT335468	Crous et al. 2004; Liu et al. 2020
		CMW 30985	CBS 112221; CPC 724	Soil (*E. grandis* trees)	La Selva, Colombia	M. J. Wingfield	MT412760	MT412981	MT335229	MT335469	Crous et al. 2004; Liu et al. 2020
B30	*C. crousiana*	CMW 27249^T	CBS 127198	*E. grandis*	FuJian, China	M. J. Wingfield	MT412761	MT412982	MT335230	MT335470	Chen et al. 2011b; Liu et al. 2020
		CMW 27253	CBS 127199	*E. grandis*	FuJian, China	M. J. Wingfield	MT412762	MT412983	MT335231	MT335471	Chen et al. 2011b; Liu et al. 2020
B31	*C. curvispora*	CMW 23693^T	CBS 116159; CPC 765	Soil	Tamatave, Madagascar	P. W. Crous	MT412763	NA	MT335232	MT335472	Victor et al. 1997; Crous 2002; Lombard et al. 2010b; Liu et al. 2020
		CMW 48245	CBS 143565	Soil (*Eucalyptus* plantation)	Aek Nauli, North Sumatra, Indonesia	M. J. Wingfield	MT412764	NA	MT335233	MT335473	Pham et al. 2019; Liu et al. 2020
B46	*C. heveicola*	CMW 49913^T	CBS 143570	Soil (*Hevea brasiliensis* plantation)	Bau Bang, Binh Duong, Vietnam	N. Q. Pham, Q. N. Dang, and T. Q. Pham	MT412786	MT413004	MT335255	MT335495	Pham et al. 2019; Liu et al. 2020
		CMW 49928	CBS 143571	Soil	Bu Gia Map National Park, Binh Phuoc, Vietnam	N. Q. Pham, Q. N. Dang, and T. Q. Pham	MT412811	MT413025	MT335280	MT335520	Pham et al. 2019; Liu et al. 2020
B48	*C. hongkongensis*	CBS 114828^T	CMW 51217; CPC 4670	Soil	Hong Kong, China	M. J. Wingfield	MT412789	MT413007	MT335258	MT335498	Crous et al. 2004; Liu et al. 2020
		CERC 3570	CMW 47271	Soil (*Eucalyptus* plantation)	BeiHai, GuangXi, China	S. F. Chen, J. Q. Li, and G. Q. Li	MT412791	MT413009	MT335260	MT335500	Li et al. 2017; Liu et al. 2020
B51	*C. ilicicola*	CMW 30998^T	CBS 190.50; IMI 299389; STE-U 2482	*Solanum tuberosum*	Bogor, Java, Indonesia	K. B. Boedijn and J. Reitsma	MT412797	NA	MT335266	MT335506	Crous 2002; Lombard et al. 2010a; Liu et al. 2020
B52	*C. indonesiae*	CMW 23683^T	CBS 112823; CPC 4508	*Syzygium aromaticum*	Warambunga, Indonesia	M. J. Wingfield	MT412798	MT413015	MT335267	MT335507	Crous et al. 2004; Liu et al. 2020
		CBS 112840	CMW 51205; CPC 4554	*S. aromaticum*	Warambunga, Indonesia	M. J. Wingfield	MT412799	MT413016	MT335268	MT335508	Crous et al. 2004; Liu et al. 2020

(*Continued on next page*)

[a] Species codes (B1 to B120) of the 120 accepted *Calonectria* spp. resulting from Liu et al. (2020).
[b] T = ex-type isolates of the species.
[c] ATCC = American Type Culture Collection, Virginia, U.S.A.; CBS = Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CERC = Research Institute of Fast-Growing Trees (RIFT)/China Eucalypt Research Centre, ZhanJiang, GuangDong Province, China; CMW = 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, U.K.; MUCL = Mycotheque, Laboratoire de Mycologie Systematique et Appliqee, I'Universite, Louvian-la-Neuve, Belgium; and STE-U = Department of Plant Pathology, University of Stellenbosch, South Africa.
[d] Abbreviations: *tef1* = translation elongation factor 1-α, *tub2* = β-tubulin, *cmdA* = calmodulin, *his3* = histone H3, and NA = information is not available.

Table 2. (*Continued from previous page*)

Code[a]	Species	Isolate[b,c]	Other collection[c]	Hosts	Area of occurrence	Collector	GenBank accession numbers[d]				Reference or source of data
							tef1	*tub2*	*cmdA*	*his3*	
B55	*C. kyotensis*	CBS 114525^T	ATCC 18834; CMW 51824; CPC 2367	*Robinia pseudoacacia*	Japan	T. Terashita	MT412802	MT413019	MT335271	MT335511	Crous 2002; Lombard et al. 2016; Liu et al. 2020
		CBS 114550	CMW 51825; CPC 2351	Soil	China	M. J. Wingfield	MT412777	MT412995	MT335246	MT335486	Lombard et al. 2016; Liu et al. 2020
B57	*C. lantauensis*	CERC 3302^T	CBS 142888; CMW 47252	Soil	LiDao, Hong Kong, China	M. J. Wingfield and S. F. Chen	MT412803	NA	MT335272	MT335512	Li et al. 2017; Liu et al. 2020
		CERC 3301	CBS 142887; CMW 47251	Soil	LiDao, Hong Kong, China	M. J. Wingfield and S. F. Chen	MT412804	NA	MT335273	MT335513	Li et al. 2017; Liu et al. 2020
B58	*C. lateralis*	CMW 31412^T	CBS 136629	Soil (*Eucalyptus* plantation)	GuangXi, China	X. Zhou, G. Zhao, and F. Han	MT412805	MT413020	MT335274	MT335514	Lombard et al. 2015a; Liu et al. 2020
B63	*C. lombardiana*	CMW 30602^T	CBS 112634; CPC 4233; Lynfield 417	*Xanthorrhoea australis*	Victoria, Australia	T. Baigent	MT412926	MT413133	MT335395	MT335635	Crous 2002; Crous et al. 2006; Lombard et al. 2010b
B66	*C. malesiana*	CMW 23687^T	CBS 112752; CPC 4223	Soil	Northern Sumatra, Indonesia	M. J. Wingfield	MT412817	MT413031	MT335286	MT335526	Crous et al. 2004; Liu et al. 2020
		CBS 112710	CMW 51199; CPC 3899	Leaf litter	Prathet, Thailand	N. L. Hywel-Jones	MT412818	MT413032	MT335287	MT335527	Crous et al. 2004; Liu et al. 2020
B74	*C. multiseptata*	CMW 23692^T	CBS 112682; CPC 1589	*E. grandis*	North Sumatra, Indonesia	M. J. Wingfield	MT412830	MT413044	MT335299	MT335539	Crous et al. 1998, 2006; Crous 2002; Liu et al. 2020
B80	*C. pacifica*	CMW 16726^T	A1568; CBS 109063; IMI 354528; STE-U 2534	*Araucaria heterophylla*	Hawaii, United States	M. Aragaki	MT412842	NA	MT335311	MT335551	Kang et al. 2001b; Crous 2002; Crous et al. 2004; Liu et al. 2020
		CMW 30988	CBS 114038	*Ipomoea aquatica*	Auckland, New Zealand	C. F. Hill	MT412843	NA	MT335312	MT335552	Crous 2002; Crous et al. 2004; Lombard et al. 2010a; Liu et al. 2020
B86	*C. penicilloides*	CMW 23696^T	CBS 174.55; STE-U 2388	*Prunus* sp.	Hatizyo Island, Japan	M. Ookubu	MT412869	MT413081	MT335338	MT335578	Crous 2002; Liu et al. 2020
B97	*C. pseudoreteaudii*	CMW 25310^T	CBS 123694	*E. urophylla* × *E. grandis*	GuangDong, China	M. J. Wingfield and X. D. Zhou	MT412885	MT413096	MT335354	MT335594	Lombard et al. 2010b; Liu et al. 2020
		CMW 25292	CBS 123696	*E. urophylla* × *E. grandis*	GuangDong, China	M. J. Wingfield and X. D. Zhou	MT412886	MT413097	MT335355	MT335595	Lombard et al. 2010b; Liu et al. 2020
	C. pentaseptata	CSF13036		*E. urophylla* × *E. tereticornis*	GuangDong, China	S. F. Chen, Q. C. Wang, and W. X. Wu	MN115915	MN115970	MN096291	MN115860	Wang and Chen 2020
		CSF13337		*E. urophylla* × *E. tereticornis*	GuangDong, China	S. F. Chen, Q. C. Wang, and W. X. Wu	MN115938	MN115993	MN096314	MN115881	Wang and Chen 2020
B104	*C. queenslandica*	CMW 30604^T	CBS 112146; CPC 3213	*E. urophylla*	Lannercost, Queensland, Australia	B. Brown	MT412898	MT413108	MT335367	MT335607	Kang et al. 2001a; Lombard et al. 2010b; Liu et al. 2020
		CMW 30603	CBS 112155; CPC 3210	*E. pellita*	Lannercost, Queensland, Australia	P. Q Thu and K. M. Old	MT412899	MT413109	MT335368	MT335608	Kang et al. 2001a; Lombard et al. 2010b; Liu et al. 2020
B106	*C. reteaudii*	CMW 30984^T	CBS 112144; CPC 3201	*E. camaldulensis*	Chon Thanh, Binh Phuoc, Vietnam	M. J. Dudzinski and P. Q. Thu	MT412901	MT413111	MT335370	MT335610	Kang et al. 2001a; Crous 2002; Crous et al. 2006; Liu et al. 2020
		CMW 16738	CBS 112143; CPC 3200	*Eucalyptus* leaves	Binh Phuoc, Vietnam	M. J. Dudzinski and P. Q. Thu	MT412902	MT413112	MT335371	MT335611	Kang et al. 2001a; Crous 2002; Crous et al. 2006; Liu et al. 2020
B112	*C. sumatrensis*	CMW 23698^T	CBS 112829; CPC 4518	Soil	Northern Sumatra, Indonesia	M. J. Wingfield	MT412913	NA	MT335382	MT335622	Crous et al. 2004; Liu et al. 2020
		CMW 30987	CBS 112934; CPC 4516	Soil	Northern Sumatra, Indonesia	M. J. Wingfield	MT412914	NA	MT335383	MT335623	Crous et al. 2004; Liu et al. 2020
B113	*C. syzygiicola*	CBS 112831^T	CMW 51204; CPC 4511	*Syzygium aromaticum*	Sumatra, Indonesia	M. J. Wingfield	KX784736	KX784663	NA	NA	Lombard et al. 2016
B116	*C. uniseptata*	CBS 413.67^T	CMW 23678; CPC 2391; IMI 299577	*Paphiopedilum callosum*	Celle, Germany	W. Gerlach	GQ267307	GQ267208	GQ267379	GQ267248	Lombard et al. 2016
B120	*C. yunnanensis*	CERC 5339^T	CBS 142897; CMW 47644	Soil (*Eucalyptus* plantation)	YunNan, China	S. F. Chen and J. Q. Li	MT412927	MT413134	MT335396	MT335636	Li et al. 2017; Liu et al. 2020
		CERC 5337	CBS 142895; CMW 47642	Soil (*Eucalyptus* plantation)	YunNan, China	S. F. Chen and J. Q. Li	MT412928	MT413135	MT335397	MT335637	Li et al. 2017; Liu et al. 2020
	Curvicladiella cignea	CBS 109167^T	CPC 1595; MUCL 40269	Decaying leaf	French Guiana	C. Decock	KM231867	KM232002	KM231287	KM231461	Decock and Crous 1998; Crous et al. 2006; Lombard et al. 2015b
		CBS 109168	CPC 1594; MUCL 40268	Decaying seed	French Guiana	C. Decock	KM231868	KM232003	KM231286	KM231460	Decock and Crous 1998; Crous et al. 2006; Lombard et al. 2015b

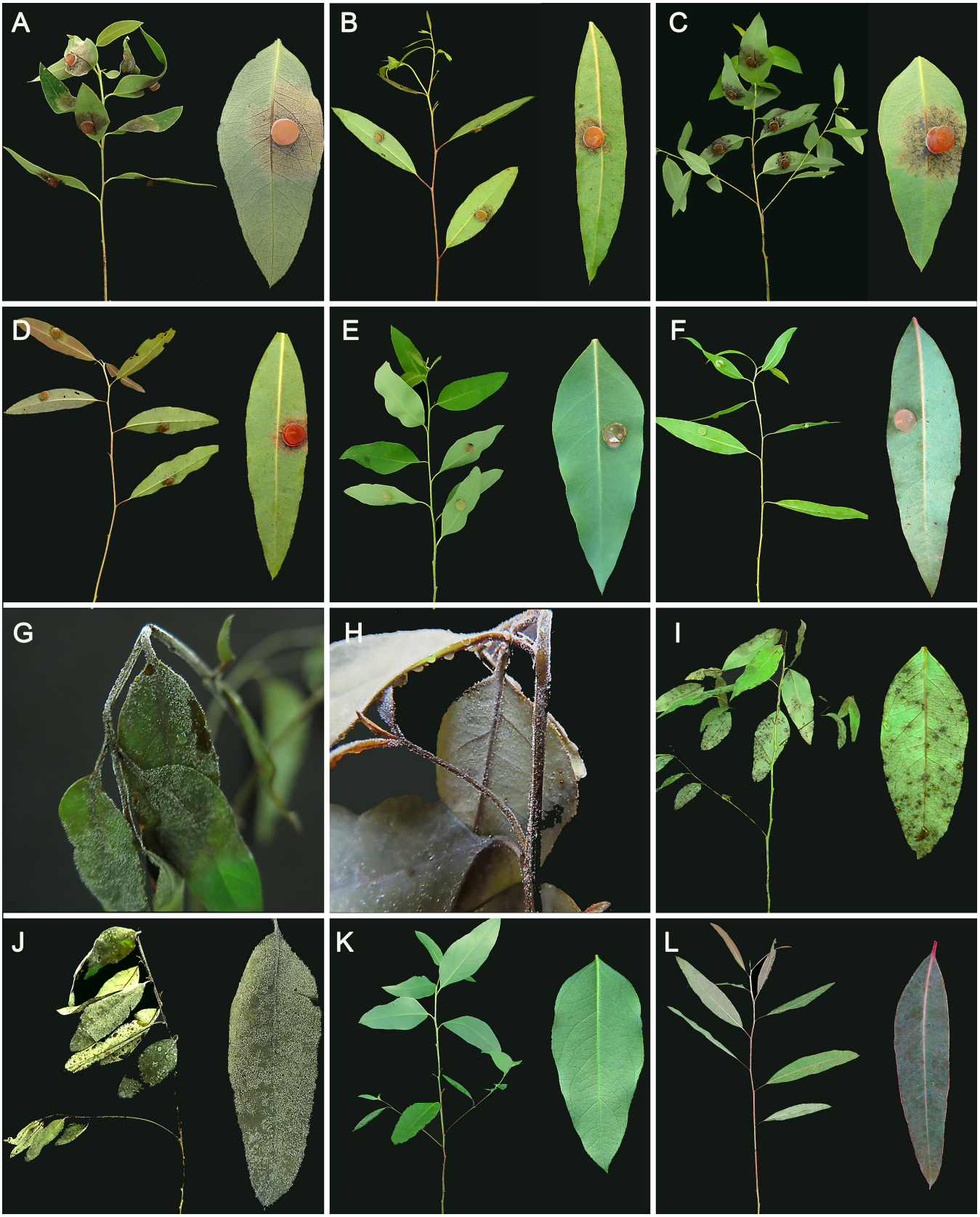

Fig. 3. Symptoms on seedlings of *Eucalyptus urophylla* × *E. tereticornis* hybrid genotype CEPT1876 and *E. urophylla* × *E. grandis* hybrid genotype CEPT1877 inoculated by **A to D,** *Calonectria* mycelial plugs; **E and F,** malt extract agar (MEA) plugs as controls; and **G to J,** conidial suspensions, with **K and L,** sterile water as controls of representative isolates of four *Calonectria* spp. A, CEPT1877 and B, CEPT1876 inoculated by isolate CSF12057 (*C. queenslandica*). C, CEPT1877 and D, CEPT1876 inoculated by isolate CSF12051 (*C. reteaudii*). Results indicated that CEPT1876 was more tolerant than CEPT1877. No symptoms were observed on leaves of E, CEPT1877 and F, CEPT1876 inoculated by MEA plugs (negative controls). Abundant white masses of conidiophores were observed on leaves and twigs of CEPT1877 inoculated with *C. queenslandica* isolates G, CSF12037 and H, CSF12057. I, More than 50% of leaf area of CEPT1877 was lesioned after inoculation by isolate CSF12037. J, More than 90% of leaf area of CEPT1877 was lesioned after inoculation by isolate CSF12057. No symptoms were observed on leaves of K, CEPT1877 and L, CEPT1876 inoculated by sterile water (negative controls). A to G, I, K, and L are in experiment one and H and J are in experiment two.

(CSF12056), were selected to inoculate seedlings of the two *Eucalyptus* genotypes (CEPT1876 and CEPT1877) (Table 1). The agar plug inoculations were carried out with all 11 isolates. Additionally, four isolates (CSF12034, CSF12037, CSF12057, and CSF12059) of *C. queenslandica* produced abundant masses of macroconidia in culture, making it possible to also inoculate plants with conidia of that species.

All 11 isolates produced distinct lesions on the leaves of the two *Eucalyptus* genotypes when inoculated with mycelial plugs (Fig. 3A to D). No disease symptoms were observed on the leaves inoculated with agar plugs as the negative controls (Fig. 3E and F). In the case of the inoculations with conidial suspensions from four *C. queenslandica* isolates, all seedlings of the two *Eucalyptus* genotypes developed leaf and shoot blight symptoms (Fig. 3G to J). No disease symptoms were observed for the negative controls (Fig. 3K and L). *Calonectria* isolates were successfully reisolated from trees inoculated with the mycelial plugs or conidial suspensions and morphological examination confirmed that isolates were the same as those used in the inoculations, thus fulfilling Koch's postulates.

The lesion sizes obtained with mycelial plug inoculations and disease indices obtained with conidial suspension inoculations did not conform to the normal distribution according to Kolmogorov-Smirnov normality test in SPSS v. 22.0 ($P < 0.05$). Therefore, all data were transformed (Kolmogorov-Smirnov normality test, $P = 0.2$) by conducting a Rank transformation using SPSS v. 22.0. The statistical analyses indicated that results of two experiments for mycelial plug inoculations were significantly different ($P < 0.05$), and significant differences were also found for the two repeat experiments where conidial suspensions were used ($P < 0.05$). Consequently, the data for each of the experiments were analyzed separately.

Mycelial plug inoculations. The results of both mycelial plug inoculation experiments showed that the lesions produced by all four *Calonectria* spp. were significantly larger than those from the controls ($P < 0.05$) (Figs. 3A to F and 4). The overall data showed that all four *Calonectria* spp. were pathogenic on both *Eucalyptus* genotypes. Isolates CSF12034 (*C. queenslandica*), CSF12041 (*C. pseudoreteaudii*), and CSF12051 (*C. reteaudii*) on *Eucalyptus* genotype CEPT1877 in experiment two, and isolate CSF12057 (*C. queenslandica*) on *Eucalyptus*

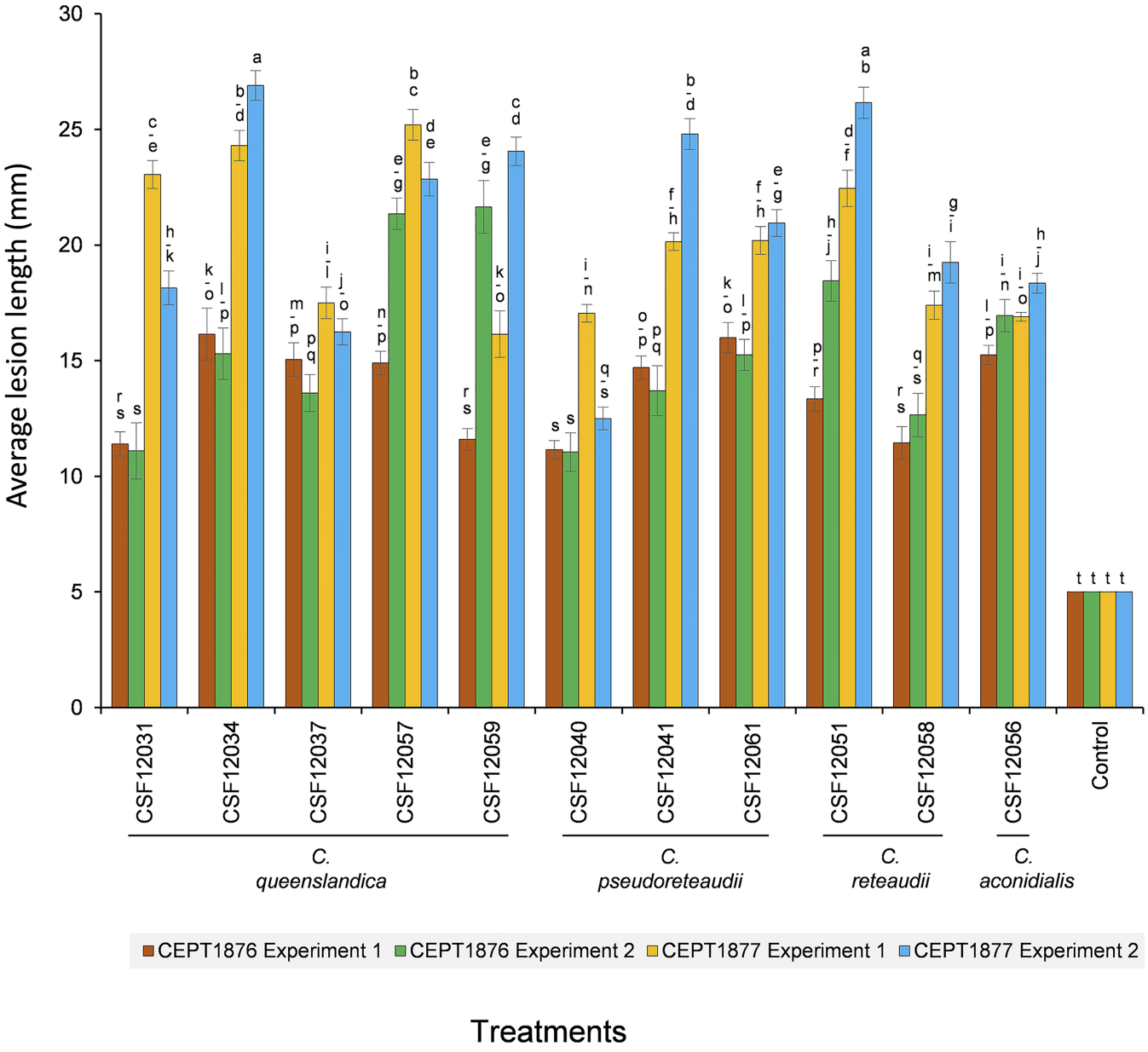

Fig. 4. Column chart indicating lesion length resulting from two mycelium plugs inoculation trials of two *Eucalyptus* hybrid genotypes inoculated with 11 isolates representing four *Calonectria* spp. and the controls. Vertical bars represent the standard error of the means. Bars with different letters indicate statistically significance at $P \leq 0.05$.

genotype CEPT1877 in experiment one (Fig. 3A), produced larger lesions than other isolates on two *Eucalyptus* genotypes in the two experiments (Fig. 4). The results showed that the lesion sizes caused by isolates of the same species were significantly different. For example, in both experiments, the lesions produced by *C. pseudoreteaudii* isolates CSF12041 and CSF12061 were significantly larger than those for isolate CSF12040 ($P < 0.05$) on both *Eucalyptus* genotypes. With the exception of isolate CSF12056 (*C. aconidialis*) in two experiments and isolates CSF12057 (*C. queenslandica*) and CSF12040 (*C. pseudoreteaudii*) in experiment two, the average lesion size produced by all tested *Calonectria* isolates on *Eucalyptus* genotype CEPT1877 were significantly larger than those on CEPT1876 (Fig. 4). These results suggested that *Eucalyptus* genotype CEPT1876 is more tolerant than CEPT1877 to the four *Calonectria* spp. tested.

Conidial suspension inoculation. For the conidial suspension inoculations, the average DI showed that more than 50% of leaf area of two *Eucalyptus* genotypes was covered by lesions produced by each of the four tested *C. queenslandica* isolates (Figs. 3G to J and 5). In experiment one, there was no difference in pathogenicity obtained for isolates CSF12034, CSF12057, and CSF12059, whereas isolate CSF12037 yielded higher DI on *Eucalyptus* genotype CEPT1877 than on *Eucalyptus* genotype CEPT1876. In experiment two, isolates CSF12034 and CSF12037 yielded a higher DI on *Eucalyptus* genotype CEPT1876 than on *Eucalyptus* genotype CEPT1877, whereas isolates CSF12057 and CSF12059 yielded a higher DI on *Eucalyptus* genotype CEPT1877 than on *Eucalyptus* genotype CEPT1876 (Fig. 5).

Discussion

In this study, we determined the cause of a severe leaf blight disease in a 2-year-old *E. urophylla* × *E. grandis* plantation in the GuangDong Province in southern China. The disease showed typical symptoms of infection by species of *Calonectria*, and fruiting structures with morphological characteristics of these fungi were observed on diseased tissues. Fungal isolation followed by sequencing and multigene phylogenetic analyses showed that four *Calonectria* spp. were present, including

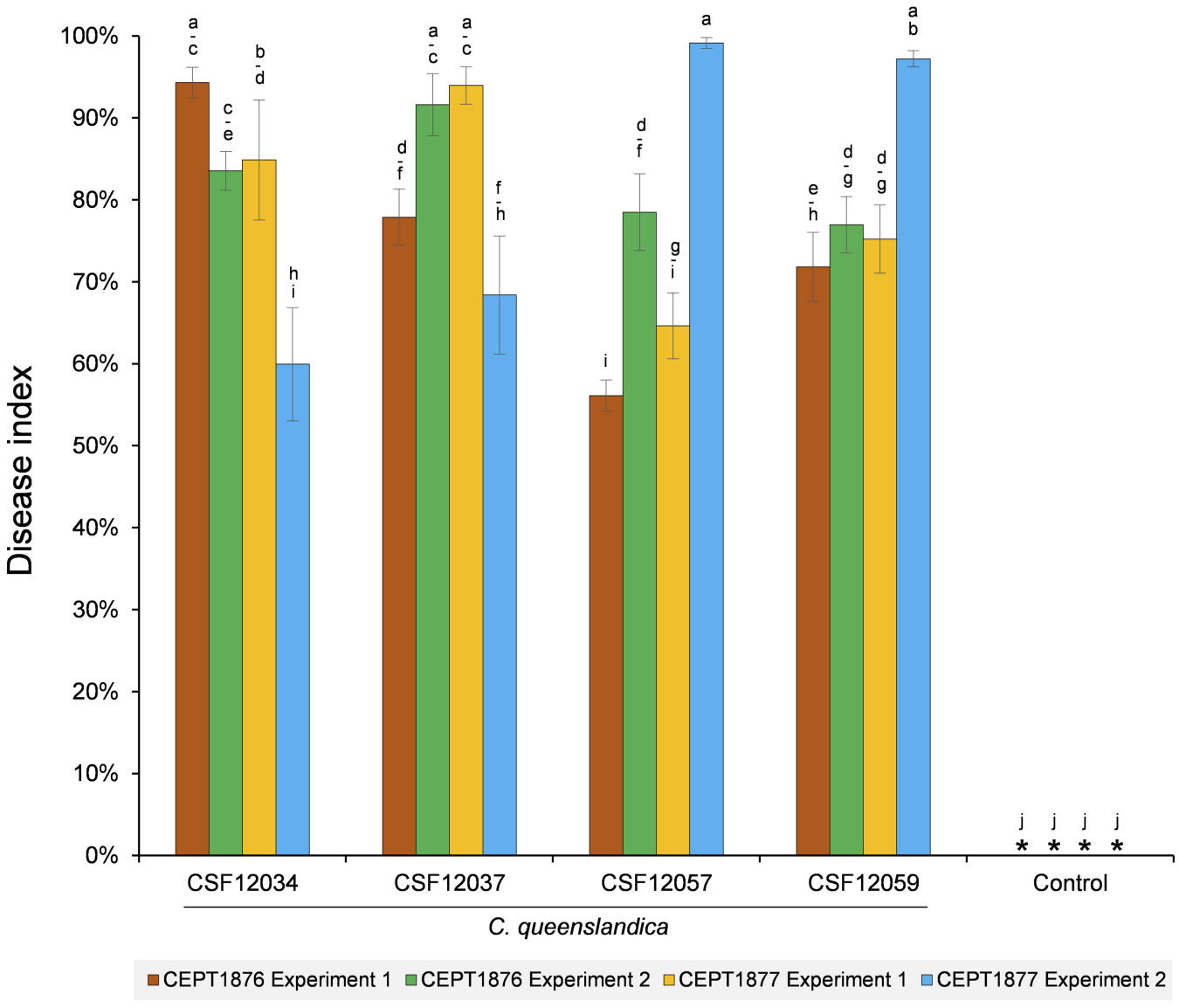

Fig. 5. Column chart indicating the disease index resulting from two conidial suspension inoculation trials of two *Eucalyptus* hybrid genotypes inoculated with four isolates of *Calonectria queenslandica* (CSF12034, CSF12037, CSF12057, and CSF12059) and the controls. Vertical bars represent the standard error of the means. Bars with different letters indicate statistically significance at $P \leq 0.05$ and the asterisk (*) indicates that the disease index of the negative control is zero.

C. queenslandica, *C. pseudoreteaudii*, *C. reteaudii*, and *C. aconidialis*. Of these, *C. queenslandica* was the dominant species, and this is the first report of the species outside Australia. Pathogenicity tests revealed that all four *Calonectria* spp. were equally pathogenic on two *Eucalyptus* genotypes that are widely planted in GuangDong Province. However, the high prevalence of *C. queenslandica* recovered from the infected tissues suggests that this is the main pathogen causing the leaf blight disease in this plantation.

C. queenslandica was first described causing a leaf blight disease on a *Eucalyptus* in Australia and its name reflects the geographic area where the species was described for the first time (Lombard et al. 2010b). This study represents not only the first report of *C. queenslandica* in China but also the first report of this species causing severe diseases in a *Eucalyptus* plantation. All 31 isolates of *C. queenslandica* recovered in this study had identical sequences at four different gene regions investigated and they were also of a single mating type (*MAT1-2*). This suggests that the fungus was introduced into the country with limited diversity, either from Australia or from some unknown source.

Prior to this study, *C. pseudoreteaudii* was considered to be the most widely distributed pathogen of *Eucalyptus* plantations in southern China, having been isolated in all studies on *Calonectria* spp. associated with diseased *Eucalyptus* in the past decade (Li et al. 2017; Lombard et al. 2015a; Wang and Chen 2020; Wu and Chen 2021). This species was also recovered in the current study but at a much lower frequency (10.5%) than *C. queenslandica* (81.6%). The result suggests that it should not be assumed that *C. pseudoreteaudii* is the main causal agent of every *Eucalyptus* leaf blight outbreak in China. Rather, rigorous surveys and diagnoses based on DNA -sequence comparisons should be undertaken to determine the cause of disease.

The results of pathogenicity tests showed that all four tested *Calonectria* spp. were pathogenic on *E. urophylla* × *E. grandis* and *E. urophylla* × *E. tereticornis* hybrids, which are widely planted in China. This result is not surprising given that similar results have been observed in previous studies showing that most *Calonectria* spp. are pathogenic (Alfenas et al. 2016; Wang and Chen 2020; Wu and Chen 2021). There were no clear differences in pathogenicity among the four species tested; isolates within and between different species differed in their relative aggressiveness. This emphasizes the need to include multiple isolates, preferably representing different genotypes, in disease resistance screening.

This study provides the first report of *C. queenslandica* as the main causal agent of a severe *Eucalyptus* leaf blight disease in southern China. It is also the first report of the species in China. It is unclear whether the investigated area (GuangDong Province) represents a point of introduction of the pathogen into the country or whether it now occurs in other regions of China. This question will need to be investigated, including extensive sampling and population genetic studies.

Acknowledgments

We thank G. Li for his assistance in collecting samples; Q. Wang, W. Wu, F. Liu, L. Sun, and J. Han for providing assistance with the pathogenicity trials; and Q. Liu for providing assistance with the phylogenetic analyses.

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