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Calonectria species associated with diseased leaves and soils in southern China *Eucalyptus* plantations

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Abstract

Calonectria leaf blight (CLB) is one of the most important diseases of *Eucalyptus* trees grown in plantations. This disease poses a serious threat to the sustainability of *Eucalyptus* plantations in southern China. To better understand the causal agents of CLB, we collected samples of diseased leaves and soil from *Eucalyptus* plantations from nine regions in Guangdong Province where the disease has become a serious problem. A total of 606 *Calonectria* isolates were purified from the samples, with 399 and 207 originating from diseased leaves and soils, respectively. Phylogenetic analyses utilizing six gene regions resolved 303 isolates in the *C. kyotensis* species complex and an equal number of isolates in the *C. reteaudii* species complex. These two complexes were represented by ten known *Calonectria* species, including *C. aconidialis* (12.0%), *C. curvispora* (0.3%), *C. hongkongensis* (24.8%), *C. ilicicola* (0.9%), and *C. kyotensis* (12.0%) in the *C. kyotensis* species complex, and *C. crousiana* (1.0%), *C. Guangdongensis* (0.3%), *C. pseudoreteaudii* (40.7%), *C. queenslandica* (7.3%), and *C. reteaudii* (0.7%) in the *C. reteaudii* species complex. Pathogenicity tests showed that all species were capable of causing disease on two tested *Eucalyptus* genotypes, albeit at varying degrees of aggressiveness. Most isolates (98.3%) in the *C. reteaudii* species complex were from the diseased leaves, indicating that species in this complex are the main causal agents of CLB outbreak. In addition, a significant number of the *C. kyotensis* species complex isolates (66.7%) from the soil samples could also cause the disease on *Eucalyptus* leaves.

Keywords Calonectria leaf blight, Pathogenicity, Phylogeny, Plant pathogen, Species diversity

Background

Eucalyptus plantations have expanded rapidly in China and serve as an important component of commercial forestry (Xie et al. 2017). By 2018, the plantations occupied approximately 5.5 million hectares in China (Xu et al. 2019). However, most of these plantations are commonly established based on single species or limited numbers of clones, making them vulnerable to pests and diseases (Zhou and Wingfield 2011).

In recent years, *Eucalyptus* plantations in China have been threatened by numerous emerging diseases, including but not limited to the following diseases: stem canker caused by species of Botryosphaeriaceae (Li et al. 2018), Cryphonectriaceae (Chen et al. 2010; Wang et al. 2020), *Ceratocystis* (Chen et al. 2013), and *Teratosphaeria* (Chen et al. 2011a); leaf blight/spot caused by species of Teratosphaeriaceae (Burgess et al. 2006), *Calonectria* (Wang and Chen 2020a; Wu and Chen 2021), and *Quambalaria* (Chen et al. 2017); and bacterial wilt associated with

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Ralstonia pseudosolanacearum (Carstensen et al. 2017). Of these diseases, leaf blight caused by *Calonectria* spp. is one of the most serious threats to the health of *Eucalyptus* plantations in southern China (Chen et al. 2011b; Lombard et al. 2015a).

The genus *Calonectria* (Hypocreales, Nectriaceae) includes many important plant pathogens that are widely distributed in tropical and subtropical regions of the world (Crous 2002; Li et al. 2022). These species are pathogenic on more than 335 plant species, many of which are important forestry, agricultural, and horticultural crop plants (Crous 2002; Lombard et al. 2010a). The disease symptoms caused by *Calonectria* include cutting rot, damping-off, leaf spot, root rot, shoot blight, and stem canker (Crous 2002). Many species of *Calonectria*, such as *C. pseudoreteaudii* (Wang and Chen 2020a), *C. pteridis* (Freitas et al. 2019), *C. reteaudii* (Old et al. 2003), and *C. spathulata* (Rodas et al. 2005) are significant threats to *Eucalyptus* trees in plantations.

A total of 130 *Calonectria* species have been described based on multi-gene phylogenetic analyses and morphological comparisons (Crous et al. 2018, 2019, 2021a, 2021b; Wang et al. 2019; Liu et al. 2020; Mohali and Stewart 2021; Pham et al. 2022). They include 27 species identified in China based on DNA sequence analysis. Of these, 11 species originated from diseased *Eucalyptus* leaves (Feng et al. 2007; Yang et al. 2014; Liu et al. 2020; Li et al. 2023) and 13 from soils in *Eucalyptus* plantations or nurseries (Li et al. 2017; Wang et al. 2019; Liu et al. 2020; Liu et al. 2021; Wu and Chen 2021). Five species were from both leaves and soils in *Eucalyptus* plantations, and eight were from plants and soils other than being associated with *Eucalyptus* (Liu et al. 2020; Wu and Chen 2021).

Recent surveys in *Eucalyptus* plantations in Guangdong Province of southern China revealed disease symptoms with characteristics of CLB. This study aimed to identify *Calonectri*a spp. associated with this disease and to test their pathogenicity on *Eucalyptus*.

Results

Fungal isolations

Soil samples were collected from nine regions of Guangdong Province, and diseased leaf samples were collected from six regions other than Dongguan, Shaoguan, and Yunfu, where no disease symptoms were observed (Figs. 1, 2 and Table 1). A total of 207 isolates were obtained from the soil samples, with one to three isolates

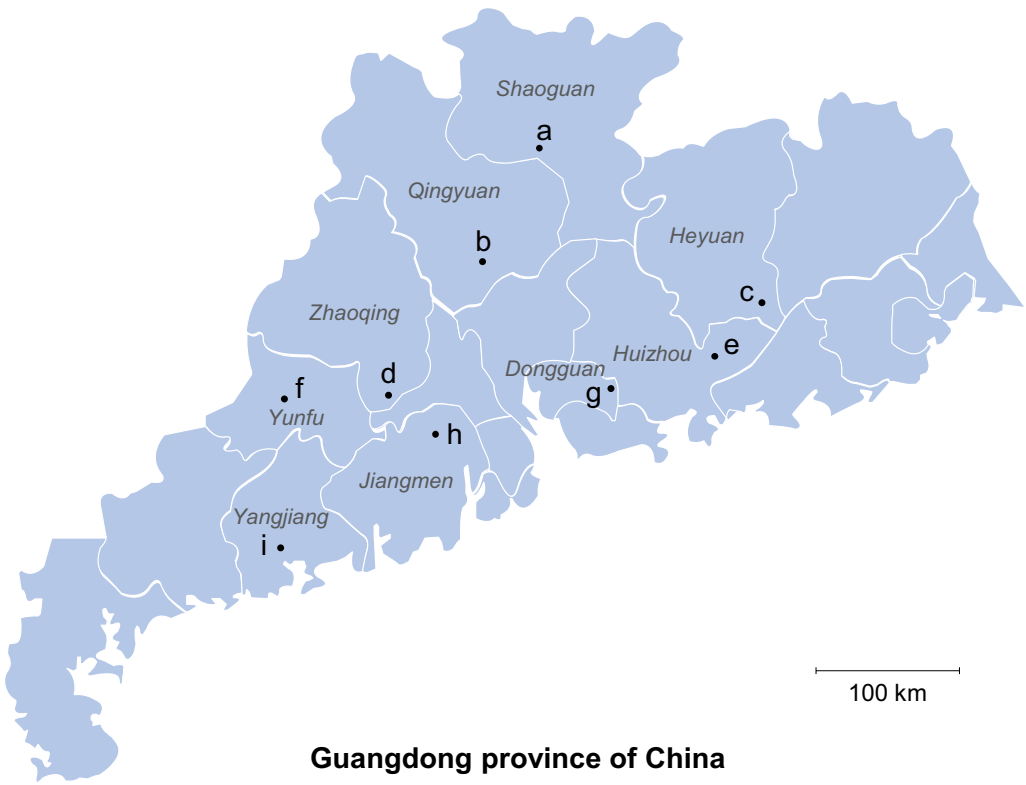

Fig. 1 Map showing the locations in Guangdong Province where the leaf and soil samples were collected. **a** Shaoguan. **b** Qingyuan. **c** Heyuan. **d** Zhaoqing. **e** Huizhou. **f** Yunfu. **g** Dongguan. **h** Jiangmen. **i** Yangjiang

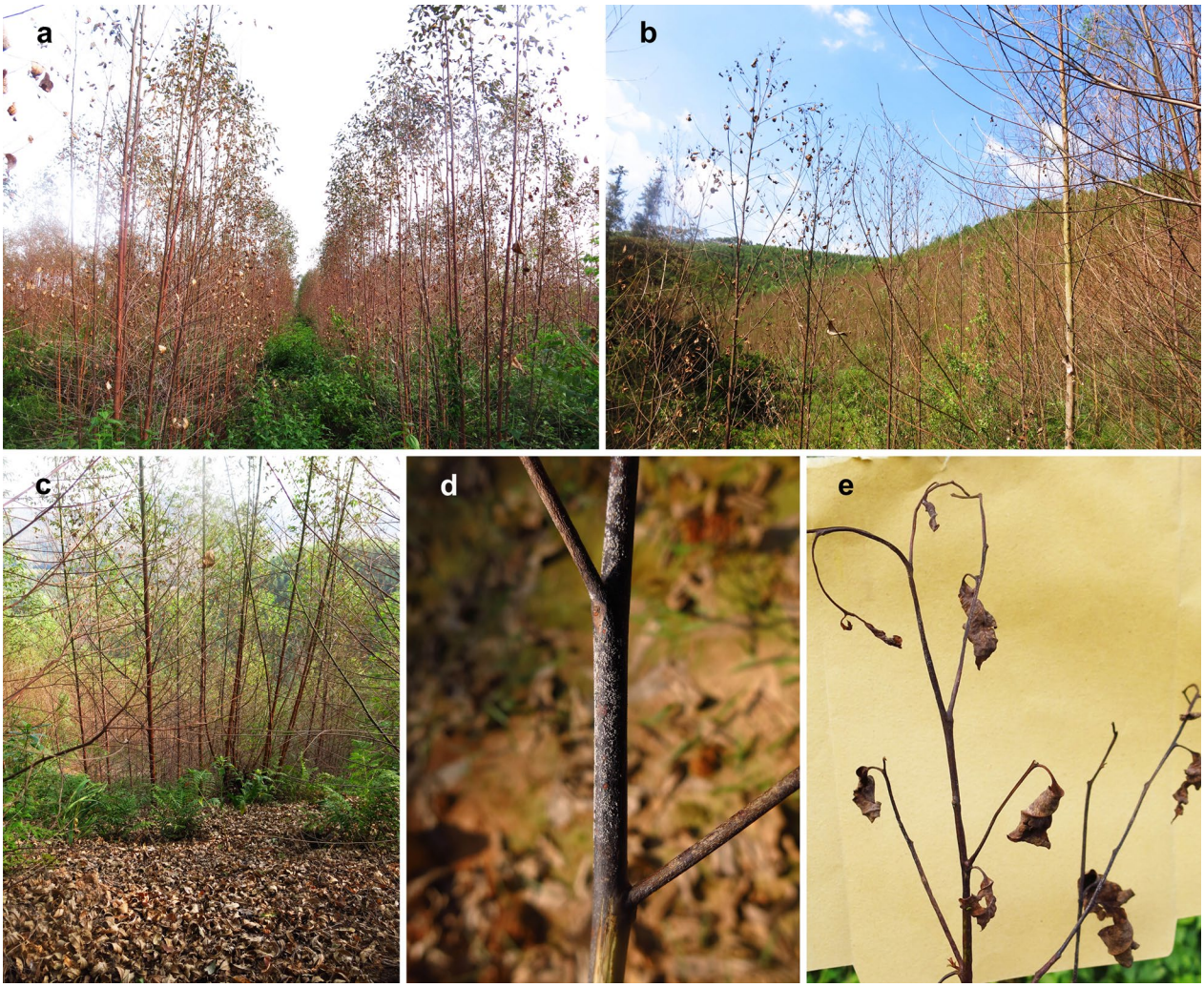

Fig. 2 Disease symptoms on *Eucalyptus* trees in plantations caused by *Calonectria* spp. **a**, **b** different *Eucalyptus* genotypes in the plantations from Yangjiang (**a**) and Zhaoqing (**b**) infected by *Calonectria* spp. **c** defoliation caused by *Calonectria*. **d** mass of conidiophores of *Calonectria* on the stem. **e** top dieback caused by *Calonectria*

retrieved per sample (Fig. 1 and Table 1). Between 28 and 51 diseased leaf samples were collected for each region other than Heyuan, where only one sample was collected, and the proportion of diseased leaf samples having *Calonectria* infection ranged from 96.1% to 100%. A total of 399 isolates were obtained from diseased leaves, with one to six isolates collected per sample (Fig. 1 and Table 1). In total, 606 isolates with typical morphological characteristics of *Calonectria* spp. were obtained from the nine regions considered (Table 1 and Additional file 1: Table S1).

Phylogenetic analyses

The translation elongation factor 1-alpha (*tef1*) gene fragment was successfully amplified for all 606 isolates (Additional file 1: Table S1). Based on the genotypes determined based on *tef1* sequences as well as information on localities and isolation sources, 417 representative isolates were selected to sequence the β-tubulin (*tub2*) gene fragment (Additional file 1: Table S1). The combination of *tef1* and *tub2* sequence data was then used to select 131 isolates to sequence the calmodulin (*cmdA*), histone H3 (*his3*), the second largest subunit of RNA polymerase (*rpb2*), and actin (*act*) gene fragments (Additional file 1: Table S1).

The amplified sequences were approximately 525 bp for *tef1*, 565 bp for *tub2*, 685 bp for *cmdA*, 435 bp for *his3*, 860 bp for *rpb2*, and 270 bp for *act*. Based on the results of jModeltest, the TIM2+G model was selected for *tef1*, the TPM3uf+I+G model for *tub2*, the TIM1+G

Table 1 The number of *Calonectria* spp. detected in *Eucalyptus* plantations from nine regions of Guangdong Province

Region	Soil samples				Diseased leaf samples				Total number of *Calonectria* isolates
	No. of soil samples	No. of soil samples from which *Calonectria* were isolated	Percentage of soil samples from which *Calonectria* were isolated (%)	No. of *Calonectria* spp. isolated from soil samples	No. of diseased leaf samples	No. of diseased leaf samples from which *Calonectria* spp. were isolated	Percentage of diseased leaf samples from which *Calonectria* spp. were isolated	No. of *Calonectria* spp. isolated from diseased leaf samples	
Shaoguan (a)	44	1	2.27	2	0	0	0	0	2
Qingyuan (b)	50	24	48.0	40	51	49	96.1%	112	152
Heyuan (c)	55	10	18.2	19	1	1	100%	6	25
Zhaoqing (d)	31	20	64.5	32	32	31	96.9%	38	70
Huizhou (e)	57	15	26.3	28	28	27	96.4%	91	119
Yunfu (f)	20	2	10.0	2	0	0	0	0	2
Dongguan (g)	50	20	40.0	37	0	0	0	0	37
Jiangmen (h)	47	10	21.3	20	40	40	100%	108	128
Yangjiang (i)	30	15	50.0	27	32	32	100%	44	71
Total	384	117	30.5	207	184	180	97.8%	399	606

model for *cmdA*, the TIM2+I+G model for *his3*, the TIM1ef+I+G model for *rpb2*, and the TIM2ef+G model for *act*. A maximum likelihood (ML) tree constructed from the concatenated dataset with bootstrap values from ML and posterior probabilities from Bayesian inference (BI) are presented in Fig. 3. Individual gene trees were included in the Additional file 2: Figures S1–S6.

Based on phylogenetic analyses of combined sequence datasets, 104 isolates in the *C. kyotensis* species complex clustered in five lineages designated as Group 1 to Group 5 (Fig. 3). Of these, 23 isolates grouped with *C. aconidialis* (Group 1), 37 with *C. hongkongensis* (Group 3), and 40 with *C. kyotensis* (Group 5), three isolates (CSF12277, CSF12383, and CSF12618) grouped with *C. ilicicola* (Group 4), and a single isolate (CSF12265) with *C. curvispora* (Group 2). Twenty-seven isolates belonging to the *C. reteaudii* species complex clustered in five lineages together with known reference isolates. They were identified as five species (Fig. 3), of which two isolates (CSF12377 and CSF12379) clustered with *C. crousiana* (Group 6), two isolates (CSF12447 and CSF12448) in Group 7 were identified as *C. Guangdongensis*, 15 isolates grouped with *C. pseudoreteaudii* (Group 8), six isolates in Group 9 were identified as *C. queenslandica*, clustering with the ex-type isolate of that species and Group 10, most closely related to *C. reteaudii*, included two isolates (CSF12051 and CSF12385).

Distribution of *Calonectria* species

Of the 606 isolates collected, 207 were obtained from soils, and 399 were from diseased leaves. Three hundred and three isolates resided in the *C. kyotensis* species complex, and the same number were in the *C. reteaudii* species complex (Fig. 4a). *C. pseudoreteaudii* (*C. reteaudii* species complex) was the most dominant and accounted for 40.7% of all the isolates. This species was found in six regions, and the majority of the isolates were from five of those regions (Fig. 4b). *C. hongkongensis* in the *C. kyotensis* species complex represented 24.8% of the isolates and was found in eight of the nine sampled regions. *C. aconidialis* and *C. kyotensis* (*C. kyotensis* species complex), each accounted for 12% of the isolates, were distributed in six and seven regions, respectively. The remaining species were found in very small numbers and only detected in one or two regions (Fig. 4a).

The highest number of *Calonectria* spp. recovered (nine) was from Jiangmen (Region h), followed by Zhaoqing (Region d) and Huizhou (Region e) each with six species recovered. Between two and four species were recovered from Qingyuan (Region b), Heyuan (Region c), Yunfu (Region f), Dongguan (Region g), and Yangjiang (Region i), and only one species was found in Shaoguan (Region a) (Fig. 4b).

A total of 207 isolates were obtained from soils, of which 202 (97.6%) were in the *C. kyotensis* species complex and five (2.4%) in the *C. reteaudii* species complex. In contrast, 399 isolates were from diseased leaves, 298 (74.7%) of which belonged to the *C. reteaudii* species complex and 101 (25.3%) to the *C. kyotensis* species

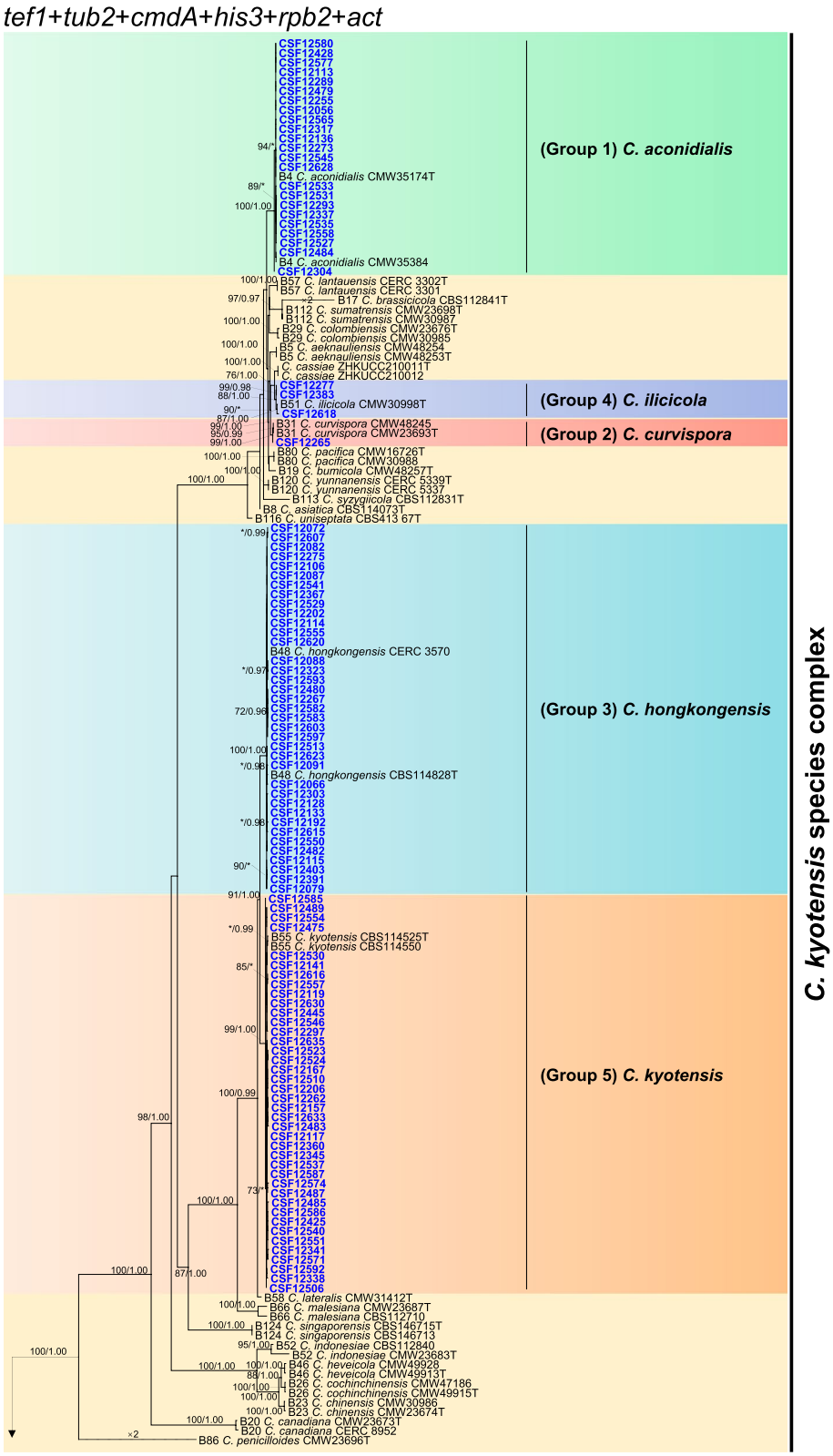

Fig. 3 Phylogenetic tree obtained from Maximum Likelihood (ML) analysis of the combined dataset of six (*tef1*, *tub2*, *cmdA*, *his3*, *rpb2*, and *act*) gene regions. Bootstrap values ≥ 70% from ML analysis and posterior probability values ≥ 0.95 obtained from Bayesian inference (BI) are indicated at nodes as ML/BI. Bootstrap values < 70% or posterior probability values < 0.95 are marked with '*'. Isolates reported in this study are highlighted in blue and in bold type; Ex-type isolates are indicated with 'T'. 'B-' species codes are consistent with those in Liu et al. (2020)

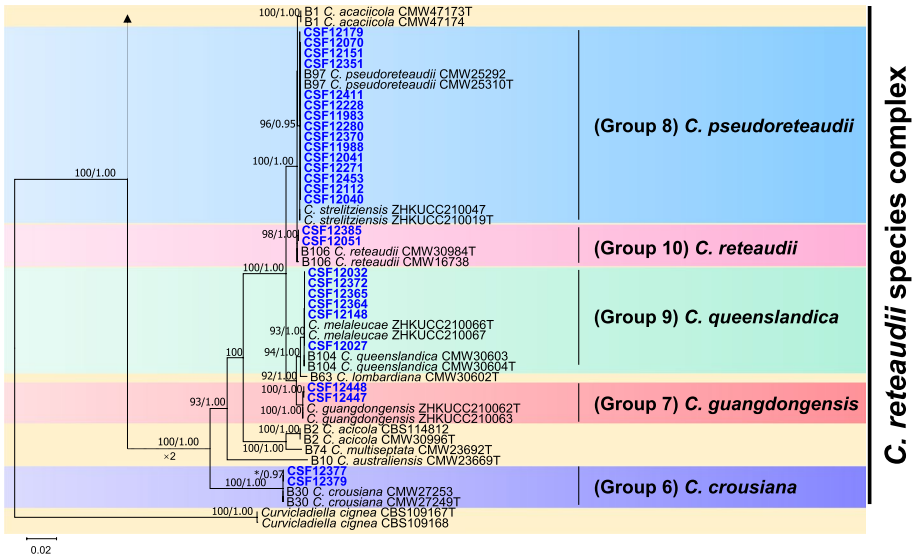

Fig. 3 continued

complex. Of the 606 isolates collected in total, 303 were in the *C. kyotensis* species complex, with 66.7% from soils and 33.3% from diseased leaves. The remaining 303 isolates belonged to the *C. reteaudii* species complex, of which 98.3% were from diseased leaves and 1.7% were from soils (Fig. 5a). In every sampled region, most isolates (88.9–100%) from soils were in the *C. kyotensis* species complex, and most (53.8–100%) from diseased leaves belonged to the *C. reteaudii* species complex. The only exception was *C. curvispora* (*C. kyotensis* species complex), which was found only on diseased leaves in Heyuan (Region c; Fig. 5b–j).

Pathogenicity tests

Ten *Calonectria* species representing a range of genotypes and sources were selected for inoculations. Typical *Calonectria* symptoms were observed on all inoculated plants, and no symptoms appeared on the negative controls. *Calonectria* species were re-isolated from the lesions on inoculated plants but never from the negative controls.

The results of two inoculation experiments showed that all the *Calonectria* species found in this study were pathogenic to the two tested *Eucalyptus* genotypes (*E. urophylla*×*E. tereticornis* and *E. urophylla*×*E. grandis*). However, isolates of the same species displayed varying levels of aggressiveness with no apparent patterns associated with the isolation sources (Fig. 6). The overall data showed that both inoculated genotypes had similar levels of susceptibility to most of the tested isolates (Fig. 6).

Discussion

In this study, ten *Calonectria* species from diseased leaves or soils associated with infected *Eucalyptus* trees in 11 plantations across nine regions of Guangdong Province in southern China were identified using multi-gene phylogenetic analysis. *C. pseudoreteaudii* was the dominant species accounting for 40.7% of all the isolates. *C. crousiana*, *C. curvispora*, *C. Guangdongensis*, and *C. reteaudii* were isolated only from diseased *Eucalyptus* leaves, while the remaining six species were from both diseased leaves and soils. Two isolates of *C. curvispora* were isolated from the Heyuan region and accounted for 0.3% of the total isolates, and this is the first record of the species infecting *Eucalyptus* trees. Results of pathogenicity tests showed that all species identified in this study were pathogenic on two tested *Eucalyptus* genotypes.

Species in the *C. kyotensis* species complex, including *C. aconidialis*, *C. curvispora*, *C. hongkongensis*, *C. ilicicola*, and *C. kyotensis*, were isolated mainly from soils (66.7%), but a considerable number of the isolates were also obtained from diseased leaves. In previous studies, *C. aconidialis*, *C. hongkongensis*, and *C. kyotensis* were found widely distributed in Fujian, Guangdong, Guangxi, and Hainan of southern China, and they were all isolated from soils (Lombard et al. 2015a; Li et al. 2017; Liu et al. 2021; Wu and Chen 2021). In the present study, these species were isolated from both diseased *Eucalyptus* leaves and soils, although there were significantly more isolates from the soils than from the leaves. *C. curvispora* has only been isolated from soils in previous studies (Crous 2002; Pham et al. 2019; Liu and Chen 2023); this is the first record of it being found on infected plant tissues.

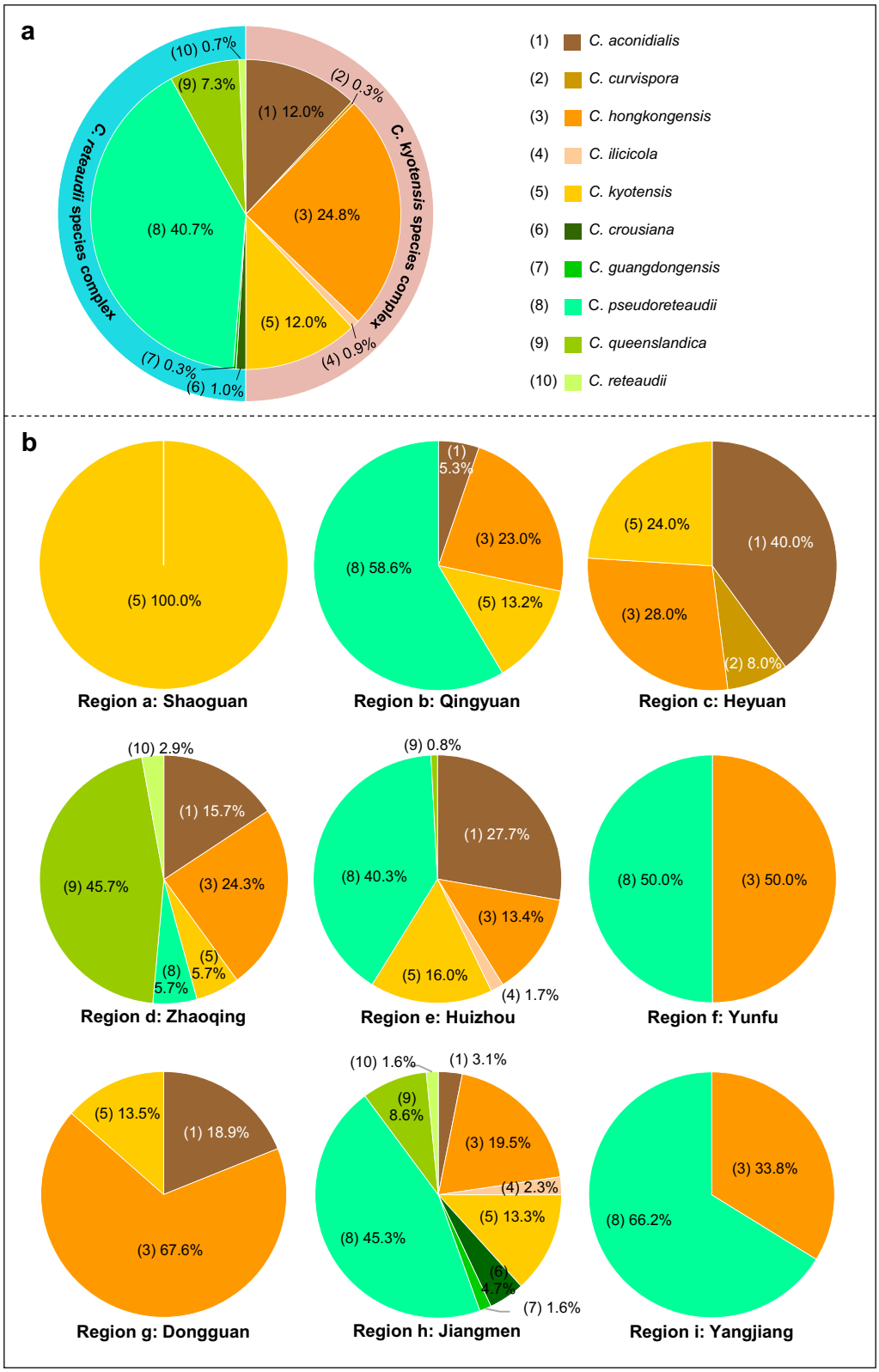

Fig. 4 Composition of *Calonectria* species from *Eucalyptus* plantations in nine regions in Guangdong Province. **a** In total, **b** per region

Fig. 5 Proportions of *Calonectria* species isolated from diseased leaves and soils. **a** In total, **b**–**j** per region. Species highlighted in orange reside in the *C. kyotensis* species complex. Species highlighted in blue reside in the *C. reteaudii* species complex

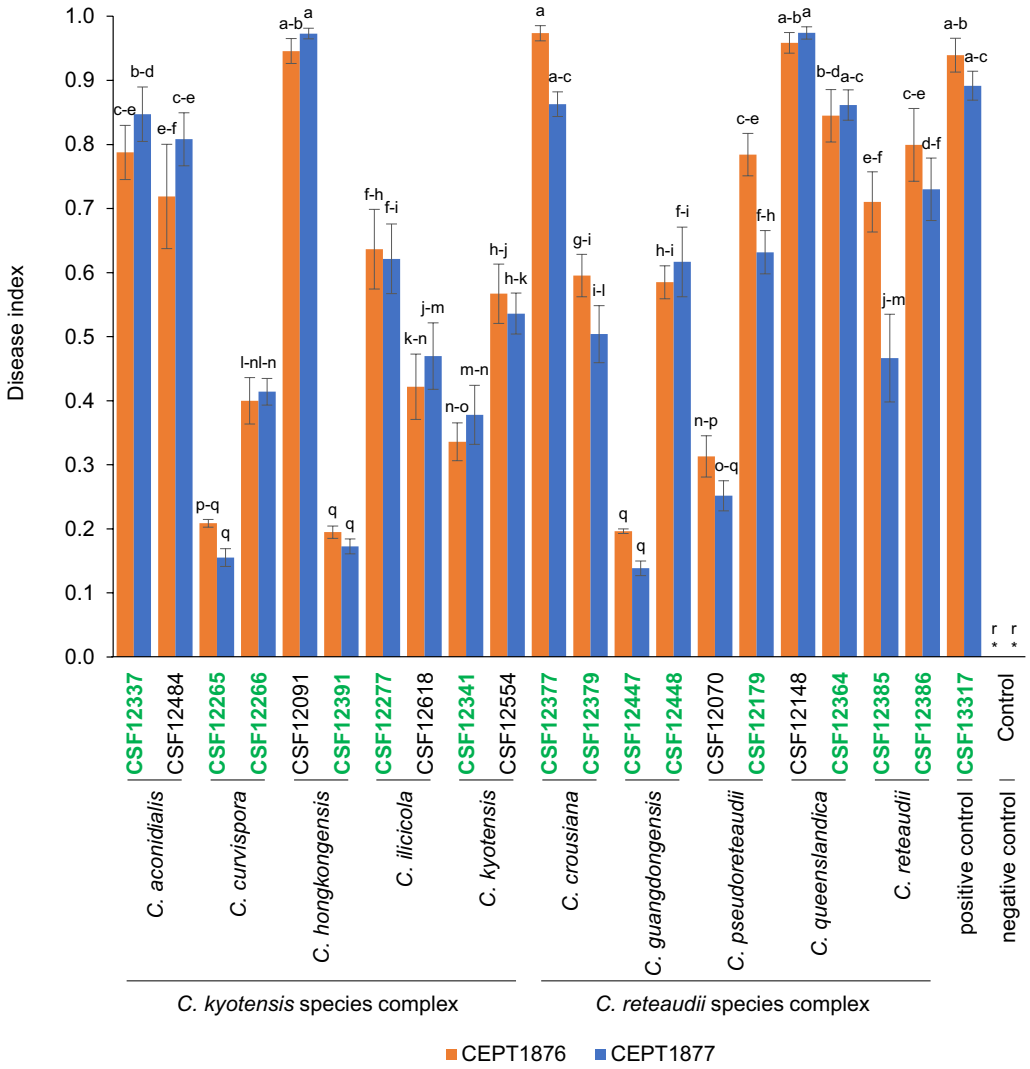

Fig. 6 Disease indices for 20 isolates representing ten *Calonectria* species inoculated on two *Eucalyptus* hybrid genotypes. The positive control was an isolate of *C. pseudoreteaudii* and the negative control was water. The numbers in green bold font represent the isolates from diseased leaves. Vertical bars represent the standard error of the means. Bars with different letters indicate treatment means that are significantly different based on Duncan's multiple range tests ($P = 0.05$)

The majority of the species (98.3%) residing in the *C. reteaudii* complex were from diseased leaves, with very few (1.7%) from soil samples. This is similar to the findings reported in previous studies (Lombard et al. 2010b; Chen et al. 2013; Lombard et al. 2015a; Li et al. 2017; Wang and Chen 2020a), where most species in this complex were from diseased leaves, and they seldom occurred in soil samples.

Little is known regarding the ability of *Calonectria* species in the *C. kyotensis* species complex to infect *Eucalyptus*. There are only two previous studies (Wu and Chen 2021; Liu and Chen 2022) where these species have been tested for pathogenicity, and the tested isolates were from soils. In the present study, representative isolates in the *C. kyotensis* species complex from both diseased leaves and soils were used in inoculation assays. The results, consistent with those of previous studies (Wu and Chen 2021; Liu and Chen 2022), showed that they were all pathogenic on the tested *Eucalyptus* genotypes.

It is generally thought that species in the *C. kyotensis* species complex are mainly soil inhabitants (Lombard et al. 2015a; Li et al. 2017) and that they are unlikely to cause serious disease problems on *Eucalyptus* in plantations. In contrast, species in the *C. reteaudii* species complex are mainly isolated from diseased leaves and hence

considered as important plant pathogens (Wang and Chen 2020a, 2020b; Wu and Chen 2021; Li et al. 2023). The results of the present study show that species in the *C. kyotensis* species complex are able to infect *Eucalyptus* and that there were no significant differences in pathogenicity compared with species in the *C. reteaudii* species complex. However, it is relevant that relatively few isolates were utilized in our pathogenicity tests, and the results should not be over-interpreted relating to their relative importance as pathogens of *Eucalyptus* in China or elsewhere.

Conclusions

Results of this study showed that species of the *C. reteaudii* species complex, of which *C. pseudoreteaudii* was the dominant species, were the main causal agents of the disease outbreak investigated. This is based on the fact that species within the *C. reteaudii* species complex were isolated from diseased leaves most often (74.7%) than species in the *C. kyotensis* species complex (25.3%). While the larger number of isolates residing in the *C. kyotensis* species complex were isolated from soils, a considerable number of these were recovered from diseased *Eucalyptus* leaves. Inoculation test showed that species in both complexes are capable of causing infection on *Eucalyptus* hybrids. They should consequently all be considered as potential threats to *Eucalyptus* plantations.

Methods

Sample collection and fungal isolation

Disease surveys were conducted in *Eucalyptus* plantations in nine regions of Guangdong Province of southern China from September to October 2018. The surveyed regions included Dongguan, Heyuan, Huizhou, Jiangmen, Qingyuan, Shaoguan, Yangjiang, Yunfu, and Zhaoqing (Fig. 1). Typical symptoms caused by *Calonectria* spp. including leaf spots, shoot blight and defoliation, were observed on 3-year-old trees (Fig. 2). Both diseased leaves and soil samples were collected from plantations where Calonectria leaf blight was observed, and soil samples were also collected in plantations without the disease. Between 20 and 60 soil samples were collected at each site, and 30–50 samples of diseased tissue were collected in the infected *Eucalyptus* plantations other than in Heyuan, where only one diseased tree was found. At the time of sampling, plantations in Huizhou and Jiangmen had been damaged by a typhoon resulting in lodging and in these cases, leaves with typical symptoms of Calonectria leaf blight were collected from the upper sides of the fallen trees.

The symptomatic tissue samples were placed in Petri dishes (diameter 90 mm) containing two pieces of moist sterilized filter paper, and maintained at room temperature for 1–3 days to induce fungal sporulation. The soil samples were baited with *Medicago sativa* (alfalfa) seeds using the method described by Crous (2002). Conidial masses of *Calonectria* spp. on the symptomatic surfaces of germinating alfalfa seeds were lifted using sterile syringe needles under a dissection microscope (AxioCam Stemi 2000C, Carl Zeiss, Germany), transferred to 2% malt extract agar plates (MEA: 20 g malt extract and 20 g agar per liter of water), and incubated for 3–5 days at room temperature. To obtain pure cultures, a single hyphal tip was transferred to a new MEA medium plate and incubated at room temperature for one week.

All pure cultures were deposited in the culture collection (CSF) located 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 were used for DNA extraction, PCR sequencing, and sequence analysis. Mycelia were scraped from the surface of 7 to 10-day-old cultures grown on MEA plates using a sterile scalpel and transferred to 2 mL Eppendorf tubes. A CTAB extraction method described by Van Burik et al. (1998) was used to isolate total genomic DNA. The concentration and quality of extracted DNA were assessed using Nano-Drop 2000 Spectrometer (Thermo Fisher Scientific Inc. Waltham, MA, USA) and adjusted to about 100 ng/μL using ddH_2O.

Based on the study of Liu et al. (2020), six genes shown to be taxonomically informative for *Calonectria* species delimitation were amplified and sequenced, including *tef1*, *tub2*, *cmdA*, *his3*, *rpb2*, and *act* genes. The gene fragments were amplified using the primers EF1-728F/EF2 (*tef1*), T1/CYLTUB1R (*tub2*), CAL-228F/CAL-2Rd (*cmdA*), CYLH3F/CYLH3R (*his3*), fRpb2-5F/fRpb2-7cR (*rpb2*), and ACT-512F/ACT-783R (*act*). PCR reaction mixtures and cycling conditions were the same as those described by Liu et al. (2020). The *tef1* gene fragment was amplified and sequenced for all isolates to determine sequence genotypes. Based on the *tef1* sequence genotypes and considering the sampling site and source (leaves or soil), representative isolates were selected for the *tub2* gene fragment amplification and sequencing. Representative isolates of *tef1* and *tub2* genotypes and isolation sources were then selected to sequence the *cmdA*, *his3*, *rpb2*, and *act* gene fragments for final identification. The PCR products were examined using agarose gel electrophoresis and sequenced in both directions using the amplification primers mentioned above. Sequencing of the amplicons was conducted by Beijing

Genomics Institution, Guangzhou, Guangdong Province, China. Raw sequences were edited with Geneious v. 9.1.4 (Kearse et al. 2012), and consensus sequences were made from forward and reverse sequencing reads. All sequences generated in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov) (Additional file 1: Table S1).

Phylogenetic analyses

Preliminary identification of the isolates was achieved using a standard nucleotide BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The available sequences of all species residing in related species complexes, including the ex-type isolates, were used for sequence comparisons and phylogenetic analyses. Sequences for two isolates of *Curvicladiella cignea* (CBS 109167 and CBS 109168) were used as outgroup taxa in the analyses. Sequences obtained in the present study and reference sequences (Additional file 1: Table S2) from the datasets of Liu et al. (2020) were aligned using the online version of MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/) (Katoh and Standley 2013) with iterative refinement methods (FFT-NS-i). The resulting alignments were visualized and curated in MEGA v. 7.0 (Kumar et al. 2016).

ML and BI analyses were conducted for individual gene sequences as well as for the concatenated dataset of all six genes. Best-fit nucleotide substitution models were determined with jModelTest v.2.1.5 (Darriba et al. 2012). ML analyses were performed with RaxML v. 8.2.4 on the CIPRES Science Gateway v. 3.3 (Miller et al. 2010) with 1000 bootstrap replicates (Stamatakis 2014). BI analyses were conducted using MrBayes v. 3.2.6 (Ronquist et al. 2012), where four MCMC chains were run for five million generations, and trees were sampled every 100th generation. The first 25% of the trees sampled were discarded as burn-in, and those remaining were used to calculate the posterior probabilities. Phylogenetic trees were viewed using MEGA v. 7.0 (Kumar et al. 2016).

Pathogenicity tests

To evaluate the pathogenicity of the identified *Calonectria* species, two isolates per species were selected for inoculation trials. One isolate of *C. pseudoreteaudii* from a previous study (Wang et al. 2022) was included as a positive control. Three-month-old trees of two *Eucalyptus* genotypes (*E. urophylla*×*E. tereticornis* CEPT1876 and *E. urophylla*×*E. grandis* CEPT1877) were used in the inoculations.

Mycelial suspensions were used for inoculations using the method described by Wang et al. (2022). The concentrations of the mycelial fragments were determined using a spectrophotometer and adjusted to $ABS_{600}=1.0$. Before inoculation, seedlings of two *Eucalyptus* genotypes were placed in plastic chambers in a greenhouse for 24 h, where the temperature was maintained at 25–27°C and the humidity at 70–80%. Leaves of eight plants of each *Eucalyptus* genotype were sprayed with mycelial suspensions of each isolate until run-off, and an equal number of plants were treated with sterile water as negative controls.

After three days, disease indices (DI) were determined following the approach described by Mishra et al. (2009). The percentage of infected leaf area was determined using the 'Leaf Doctor' software (Pethybridge and Nelson 2015), and then assigned to a 0 to 5 scale, where 0 represented no lesion and DI 1–5, respectively, indicated 1–10%, 11–25%, 26–50%, 51–75%, 76–100% of the leaf area infected. The resulting data were analyzed using SPSS Statistics 22 software (IBM Corp., Armonk, NY, USA). Re-isolations were carried out to fulfill the Koch's postulates, and the entire experiment was repeated once under the same conditions.

Abbreviations

act	Actin
BI	Bayesian inference
CLB	Calonectria Leaf Blight
cmdA	Calmodulin
DI	Disease index
his3	Histone H3
ML	Maximum likelihood
rpb2	The second largest subunit of RNA polymerase
tef1	Translation elongation factor 1-alpha
tub2	β-Tubulin

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-023-00183-z.

Additional file 1. **Table S1**. Information of all isolates obtained in this study. **Table S2**. Isolates from other studies and used in the phylogenetic analyses.

Additional file 2. **Figure S1**. Phylogenetic tree obtained from Maximum Likelihoodanalysis of the tef1 gene sequences. Bootstrap values ≥70% from ML analysis and posterior probability values ≥0.95 obtained from Bayesian inferenceare indicated at nodes as ML/BI. Bootstrap values <70% or posterior probability values <0.95 are marked with '*'. Isolates reported in this study are highlighted in blue and in bold type; Ex-type isolates are indicated with 'T'. 'B-' species codes are consistent with those in Liu et al. (2020). **Figure S2**. Phylogenetic tree obtained from Maximum Likelihoodanalysis of the tub2 gene sequences. Bootstrap values ≥70% from ML analysis and posterior probability values ≥0.95 obtained from Bayesian inferenceare indicated at nodes as ML/BI. Bootstrap values <70% or posterior probability values <0.95 are marked with '*'. Isolates reported in this study are highlighted in blue and in bold type; Ex-type isolates are indicated with 'T'. 'B-' species codes are consistent with those in Liu et al. (2020). **Figure S3**. Phylogenetic tree obtained from Maximum Likelihoodanalysis of the cmdA gene sequences. Bootstrap values ≥70% from ML analysis and posterior probability values ≥0.95 obtained from Bayesian inferenceare indicated at nodes as ML/BI. Bootstrap values <70% or posterior probability values <0.95 are marked with '*'. Isolates reported in this study are highlighted in blue and in bold type; Ex-type isolates are indicated with 'T'. 'B-' species codes are consistent with those

in Liu et al. (2020). **Figure S4**. Phylogenetic tree obtained from Maximum Likelihoodanalysis of his3 gene sequences. Bootstrap values ≥70% from ML analysis and posterior probability values ≥ 0.95 obtained from Bayesian inferenceare indicated at nodes as ML/BI. Bootstrap values <70% or posterior probability values <0.95 are marked with '*'. Isolates reported in this study are highlighted in blue and in bold type; Ex-type isolates are indicated with 'T'. 'B-' species codes are consistent with those in Liu et al. (2020). **Figure S5**. Phylogenetic tree obtained from Maximum Likelihoodanalysis of the rpb2 gene sequences. Bootstrap values ≥70% from ML analysis and posterior probability values ≥0.95 obtained from Bayesian inferenceare indicated at nodes as ML/BI. Bootstrap values <70% or posterior probability values <0.95 are marked with '*'. Isolates reported in this study are highlighted in blue and in bold type; Ex-type isolates are indicated with 'T'. 'B-' species codes are consistent with those in Liu et al. (2020). **Figure S6**. Phylogenetic tree obtained from Maximum Likelihoodanalysis of act gene sequences. Bootstrap values ≥70% from ML analysis and posterior probability values ≥0.95 obtained from Bayesian inferenceare indicated at nodes as ML/BI. Bootstrap values <70% or posterior probability values <0.95 are marked with '*'. Isolates reported in this study are highlighted in blue and in bold type; Ex-type isolates are indicated with 'T'. 'B-' species codes are consistent with those in Liu et al. (2020).

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Authors' contributions
SC conceived and designed the experiments. SC and WL collected the samples. WL performed the laboratory work. WL drafted the manuscript. SC, MW, and TD revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

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Competing interests
The authors declare that they have no competing interests.

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