Three *Colletotrichum* Species, Including a New Species, are Associated to Leaf Anthracnose of Rubber Tree in Hainan, China

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Abstract

Colletotrichum gloeosporioides and *C. acutatum* have been reported to be causal agents of anthracnose disease of rubber tree. Recent investigations have shown that both *C. gloeosporioides* and *C. acutatum* are species complexes. The identities of *Colletotrichum* species causing anthracnose disease of rubber tree in Hainan, China, are unknown. In this study, 106 isolates obtained from rubber tree with symptoms of anthracnose were collected from 12 counties of Hainan and identified at the species complex level based on the ITS sequences and colony morphologies. Seventy-four isolates were identified as *C. gloeosporioides* species complex and the other 32 isolates as *C. acutatum* species complex. Forty-two isolates were selected for further multilocus phylogenetic analyses in order to identify the isolates

to the species level. Twenty-six isolates from the *C. gloeosporioides* species complex were characterized for partial sequences of seven gene regions (*ACT*, *TUB2*, *CHS-1*, *GAPDH*, ITS, *ApMat*, and *GS*), and the other 16 isolates from the *C. acutatum* species complex for five gene regions (*ACT*, *TUB2*, *CHS-1*, *GAPDH*, and ITS). Three species were identified: *C. siamense* and *C. fruticola* from the *C. gloeosporioides* species complex, and a new species *C. wanningense* from the *C. acutatum* species complex. Artificial inoculation of rubber tree leaves confirmed the pathogenicity of the three species. The present study improves the understanding of species causing anthracnose on rubber tree and provides useful information for the effective control of the disease.

Rubber tree (*Hevea brasiliensis*), a perennial tree of the Euphorbiaceae family, is economically important for production of natural rubber (*cis*-1,4-polyisoprene), an essential industrial raw material (Leitch et al. 1998; Zou et al. 2009). Although native to the Amazon basin, it is now mainly planted in Southeast Asia, accounting for 97% of the global supply of natural rubber (Li and Fox 2012). Since the early 1950s, China has been investing heavily on rubber tree plantation and successfully cultivating rubber trees in the environments perceived to be marginal for rubber trees, mainly in Hainan, Yunnan, and Guangdong provinces (Pu et al. 2007). In China, rubber plantation areas reached nearly one million ha in 2009 with approximately 47% of the total planting area on Hainan Island (Mo 2010).

Anthracnose of rubber tree leaves is considered as one major cause for the decline in rubber yield in the Asian continent and South American regions (Jayasinghe and Fernando 1998). The disease occurs in the spring and causes leaf necrosis and deformation, and even secondary fall of the youngest leaves when they are infected at the early development stage (Guyot et al. 2001). In China, anthracnose has become a limiting factor for rubber production and may result in significant yield losses (Z. Y. Cai et al. 2009).

Morphological, molecular, and fungicide sensitivity studies suggested that *C. gloeosporioides* and *C. acutatum* are causal agents of anthracnose on rubber trees (Brown and Soepena 1994; Saha et al. 2002). In China, *C. gloeosporioides* was used to be considered as the only anthracnose pathogen. However, *C. acutatum* has recently been recorded also as a causal agent of anthracnose on rubber trees in Yunnan in 2008 (Zhang et al. 2008) and in Hainan in 2010 (Li et al. 2010).

Both *C. gloeosporioides* and *C. acutatum* are species complexes. There are 22 species in addition to one subspecies within the *C. gloeosporioides* species complex (Weir et al. 2012) and 31 species within the *C. acutatum* species complex (Damm et al. 2012). Recently, several new species were added to the both species complexes. *C. acutatum* species complex is believed to consist of 34 species and *C. gloeosporioides* species complex of *C. gloeosporioides* and 37 other closely related species (Jayawardena et al. 2016b; Marin-Felix et al. 2017).

In addition to *C. acutatum*, several other species (*C. citri*, *C. latiphilum*, *C. nymphaeae*, and *C. simmondsii*) were also isolated from rubber trees (Damm et al. 2012; Hunupolagama et al. 2017); all these species belong to the *C. acutatum* species complex. There have been no systematic studies conducted on species related to anthracnose caused by the *C. gloeosporioides* species complex, which is considered to be the dominant species complex in Hainan (Cao et al. 2017). Furthermore, the exact species identities from the *C. acutatum* species complex causing anthracnose of rubber tree in Hainan are unknown. Therefore, this study aimed to identify the *Colletotrichum* species associated with anthracnose disease of rubber tree in Hainan based on multigene phylogenetic analyses and morphology. The pathogenicity of different *Colletotrichum* species on rubber tree leaves was then assessed.

Materials and Methods

Fungal isolation. Rubber tree leaves with anthracnose symptoms were collected from different geographical locations in Hainan, China, from 2015–2017. Three pieces (5 × 5 mm) of tissue were taken from the margin of a single anthracnose lesion to isolate the fungus. The leaf pieces were surface sterilized with 70% ethanol

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for 30 s, 1% NaClO for 1 min, rinsed in sterile distilled water for 30 s, and finally plated onto potato dextrose agar (PDA). The plates were then incubated at room temperature (28 to 30°C) under continuous darkness until fungal colonies had grown. Growing edges of any fungal colony from the tissues were then transferred aseptically to new PDA.

The type specimen of a new species from this study was deposited in the Mycological Herbarium, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China (HMAS), and ex-type living culture deposited in the China General Microbiological Culture Collection Center (CGMCC).

Morphological analysis. Mycelial discs (9 mm diameter) were taken from 5-day-old cultures, plated on PDA, and incubated at 25°C in the dark. Daily growth rate was calculated as the 7-day average of mean daily growth (mm/day); colony characteristics were recorded. Characteristics of conidial, conidiophores, and appressoria were determined using methods described previously (L. Cai et al. 2009). Appressoria were induced on synthetic nutrient-poor agar (SNA) medium and measured using a slide culture technique (L. Cai et al. 2009). Characteristics of conidia, conidiophores, and appressoria were assessed under a digital microscope (Eclipse 80i, Nikon, Japan).

PCR amplification and DNA sequencing. Isolates were grown on PDA for 7 days at 25°C with a 12-h photoperiod. A small amount of aerial mycelium was scraped with a sterile 10 µl pipette tip from the colony surface; genomic DNA was extracted using the E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek, Germany) following the manufacturer's instructions. Isolates were identified to the species complex level based on cultural characteristics on PDA, spore morphology, growth rate, and internal transcribed spacer (ITS) sequence.

Forty-two isolates were then selected for taxonomical identification to the species level: 26 and 16 from the *C. gloeosporioides* and *C. acutatum* species complexes, respectively. Isolates of *C. gloeosporioides* species complex were analyzed for partial sequences of seven genomic loci: ITS, actin (*ACT*), β -tubulin (*TUB2*), chitin synthase 1 (*CHS-1*), an intron sequence of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), glutamine synthetase (*GS*), and the mating type locus MAT1-2 (*ApMat*). These were amplified and sequenced using the primer pairs ITS-1 (Gardes and Bruns 1993) + ITS-4 (White et al. 1990), ACT-512F + ACT-783R (Carbone and Kohn 1999), T1 (O'Donnell and Cigelnik 1997) + Bt2b (Glass and Donaldson 1995), CHS-79F + CHS-354R (Carbone and Kohn 1999), GDF1 + GDR1 (Templeton et al. 1992), GSF1 + GSR1 (Stephenson et al. 1997), and AMF1 + AMR1 (Silva et al. 2012), respectively. Isolates of the *C. acutatum* species complex were analyzed for partial sequences of five loci: ITS, *ACT*, *TUB2*, *CHS-1*, and *GAPDH*.

PCR amplifications were performed with a C1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA) in a 25 µl reaction volume. The PCR mixtures contained 1 µl of DNA template, 12.5 µl of TaKaRa Premix Taq (Ex Taq version, TaKaRa, Tokyo, Japan), 1 µl of 5 µM of each primer. PCR reactions for ITS were performed using the following conditions: initial denaturation at 95°C for 4 min, followed by 35 cycles each consisting of 30 s at 95°C, 30 s at 52°C plus extension for 45 s at 72°C, with a final extension step at 72°C for 7 min. PCR conditions for other loci were the same except the annealing temperatures: *ACT* at 58°C, *TUB2* at 55°C, *GAPDH* at 60°C, *CHS-1* at 58°C, *GS* at 60°C, and *ApMat* at 62°C.

PCR products were examined by electrophoresis in 1.5% agarose gels stained with GoodView Nucleic Acid Stain (Beijing SBS Genetech, Beijing, China) in 1.0 × Tris-acetate acid EDTA (TAE) buffer and photographed under UV light. The PCR products were purified with the Qiagen purification kit (Qiagen, USA) according to the manufacturer's instructions. DNA sequencing was conducted by SinoGenoMax Co., Ltd. (Chinese National Human Genome Center, Beijing, China) with an ABI 3730XL automatic sequencer (Applied Biosystems, USA).

Phylogenetic analysis. The DNA sequences generated with forward and reverse primers were used to obtain consensus sequences using MEGA version 6.0 (Tamura et al. 2013). Multiple sequence alignments were generated using MEGA 6.0 and edited manually

if necessary. The consensus sequences were deposited in GenBank (Supplementary Table S1).

Bayesian inference (BI) was used to construct the phylogenetic tree using MrBayes version 3.2.6 (Ronquist et al. 2012). The best evolutionary model for each partition was determined separately according to the corrected Akaike information criterion (AIC) by ModelTest version 3.7 (Posada and Crandall 1998) as implemented in MrMTgui. Markov Chain Monte Carlo (MCMC) analysis with four chains was run twice for 3,000,000 generations, and trees were sampled every 1,000 generations. After omitting the first 25% of saved trees (burn-in), the remaining sampled trees were used to estimate a 50% majority rule consensus tree. Clades were regarded as significantly supported if they had a posterior probability ≥ 0.95 (Ronquist et al. 2012).

Maximum parsimony (MP) analyses based on the multilocus alignment were conducted with PAUP v. 4.0b10 (Swofford 2002). Phylogenetic trees were generated using the heuristic search option with tree bisection reconnection (TBR) branch swapping and 1,000 random sequence additions. Maxtrees were unlimited, with branches of zero length collapsed, and all multiple parsimonious trees were saved. Clade stability was assessed using a bootstrap analysis of 1,000 replicates. Tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were calculated.

An additional maximum likelihood (ML) analysis was carried out with RAxML v. 7.2.6 (Stamatakis 2006) using a GTRGAMMA substitution model with 1,000 bootstrap replicates.

Genealogical concordance phylogenetic species recognition analysis. New species and their most closely related neighbors were analyzed using the genealogical concordance phylogenetic species recognition (GCPSR) model by performing a pairwise homoplasy index (PHI) test (Quaedvlieg et al. 2014). The PHI test was performed in SplitsTree4 (Huson 1998; Huson and Bryant 2006) to determine the recombination level within phylogenetically closely related species using five- or seven-locus concatenated dataset. A PHI value below a 0.05 threshold ($\Phi_w < 0.05$) indicates the presence of a significant recombination in the dataset. The relationship between closely related species was shown in a split graph.

Pathogenicity assay. Detached rubber tree leaves (Reyan 7-33-97, one of the main clones in Hainan, China) were used to confirm pathogenicity of a number of identified *Colletotrichum* species. Four isolates from *C. wanningense* (Hainan 4, Hainan 5, Hainan 14, Hainan 34) – a new species identified in the present study (see the Results section), three isolates from *C. siamense* (Hainan 12, Hainan 20, Hainan 22), and one isolate from *C. fruticola* (Hainan 25) were selected for pathogenicity tests.

Leaves at the light green stage without disease symptoms were wounded by piercing with a sterilized needle. Four wounds on either side of the midrib of each leaf were made. A 10 µl droplet of conidial suspension (10^6 conidia ml⁻¹) was placed over every wound; wounds inoculated with sterile water were used as control. Inoculated leaves were placed on a piece of moist tissue paper, incubated in a moist chamber at 25°C, and assessed daily for lesion development. Lesion diameter at each inoculation site was measured 7 days after inoculation. The experiment followed a completely randomized design with three replicate leaves for each isolate. The experiment was performed twice.

Results

Collection of *Colletotrichum* species. In total, 106 *Colletotrichum* strains were isolated from diseased leaves of rubber trees from 12 cities/counties of Hainan, China. Based on the BLAST search results of the NCBI database with the ITS sequences and the colony morphological characteristics on PDA, all these *Colletotrichum* strains were initially identified as either the *C. gloeosporioides* species complex (74 isolates) or the *C. acutatum* species complex (32 isolates).

Phylogenetic analyses. Twenty-six isolates in the *C. gloeosporioides* species complex and 16 isolates in the *C. acutatum* species complex were selected for multilocus phylogenetic analyses on the basis of their geographical location, morphology (colony shape and color and characteristics of aerial mycelia and conidia), and ITS sequences.

Colletotrichum gloeosporioides species complex. The seven-locus (*ACT*, *TUB2*, *CHS-1*, *GAPDH*, ITS, *ApMat*, and *GS*) phylogenetic analysis of the *C. gloeosporioides* species complex included 75 isolates with *C. boninense* (ICMP 17904) as the outgroup (Fig. 1). The concatenated dataset comprised 4,105 characters including the alignment gaps, of which 703 characters were parsimony informative, 808 parsimony-uninformative, and 2,594 constant. A maximum of 1,000 equally most parsimonious trees were saved (Tree length = 3,019, CI = 0.612, RI = 0.756, RC = 0.463, and HI = 0.389). For the Bayesian analyses, MrModeltest suggested that *ACT*, *TUB2*, *GAPDH*, ITS, *ApMat*, and *GS* partitions should be analyzed with dirichlet state frequency distributions, while the *CHS-1* and ITS partitions should be analyzed with fixed state frequency distributions. The following models recommended by MrModeltest were used: GTR+G for *ACT* and *GS*, GTR+I for *TUB2*, K80+G for *CHS-1*, HKY+I for *GAPDH*, SYM+G for ITS, and HKY+G for *ApMat*. Bayesian analysis of the combined alignment of the *C. gloeosporioides* species complex was done using 1,841 unique site patterns (*ACT*: 135, *TUB2*: 285, *CHS-1*: 93, *GAPDH*: 217, ITS: 144, *ApMat*: 546, and *GS*: 421). The trees generated from the PAUP and RaxML analyses were similar to that from the Bayesian analysis (Fig. 1). The 26 tested isolates from the *C. gloeosporioides* species complex clustered in two clades. Twenty-five isolates clustered with *C. siamense*, and one isolate with the *C. fructicola* clade.

Colletotrichum acutatum species complex. The five-gene (*ACT*, *TUB2*, *CHS-1*, *GAPDH*, and ITS) phylogenetic analysis of the

C. acutatum species complex included sequences of 52 isolates with *C. orchidophilum* (CBS 632.80) as the outgroup (Fig. 2). The concatenated dataset comprised 1,834 characters including the alignment gaps, of which 190 characters were parsimony informative, 211 parsimony-uninformative, and 1,433 constant. A maximum of 1,000 equally most parsimonious trees were saved (TL = 705, CI = 0.674, RI = 0.821, RC = 0.553, and HI = 0.326). For the Bayesian analyses, MrModeltest suggested that *ACT*, *TUB2* and *GAPDH*, and ITS partitions should be analyzed with dirichlet state frequency distributions, while the *CHS-1* partition should be analyzed with fixed state frequency distributions. The following models recommended by MrModeltest were used: GTR+G for *ACT*, HKY+G for *TUB2* and *GAPDH*, K80+I for *CHS-1*, and HKY+I for ITS. The Bayesian analysis of the combined alignment was based on 448 unique site patterns (*ACT*: 83, *TUB2*: 122, *CHS-1*: 43, *GAPDH*: 126, and ITS: 74). The trees generated from the PAUP and RaxML analyses were similar to that from the Bayesian analysis (Fig. 2). All of the 16 tested isolates from the *C. acutatum* species complex clustered in the same clade (closely related to *C. cairnsense*), which was distinct from all other known species, and is herein described as a new species in the present study, *C. wanningense*.

Pairwise homoplasy index (PHI) test. Application of the PHI test to the concatenated 5-locus sequences (ITS, *ACT*, *GAPDH*, *CHS-1*, and *TUB2*) revealed that no significant recombination event was observed between *C. wanningense* and *C. cairnsense* ($\Phi_W = 0.45$). Significant recombination ($\Phi_W < 0.0001$) was detected among the 26 *C. siamense*

Fig. 1. Phylogenetic consensus tree based on Bayesian inference, illustrating the relationships within the *Colletotrichum gloeosporioides* species complex of isolates obtained from rubber tree in Hainan, China. The tree was built with Bayesian analysis of concatenated sequences of *ACT*, *TUB2*, *CHS-1*, *GAPDH*, ITS, *ApMat*, and *GS*; the Markov chain was run for 3×10^6 generations, each locus with a separate model of DNA evolution. *C. boninense* (ICMP 17904) was used as the outgroup. Bayesian posterior probability values ≥ 0.95 , bootstrap support values $\geq 50\%$, and RaxML bootstrap support values (ML $\geq 50\%$) are shown at the nodes.

Taxonomy. Based on the morphology and the multilocus phylogeny analysis, 42 *Colletotrichum* isolates from rubber tree in Hainan are from three species, including one new species: *Colletotrichum wanningense* X.R. Cao, H.Y. Che & D.Q. Luo, sp. nov. - MycoBank MB824129 (Fig. 3). Etymology: Referring to the city of Hainan Province in China where the fungus was first collected. Sexual morph was not observed.

Asexual morph on SNA. Vegetative hyphae hyaline, smooth-walled, septate, branched. Chlamydospores not observed. Conidiomata not developed, conidiophores formed directly on hyphae. Setae not observed. Conidiophores hyaline, smooth-walled, septate, branched, 25 to 60 × 2 to 4 µm. Conidiogenous cells hyaline, smooth-walled, cylindrical to ampulliform, 5 to 15 × 2 to 4 µm, apex 1 to 2 µm diam. Conidia hyaline, smooth-walled, aseptate, straight, with both ends slightly acute or one end round and one end slightly

Fig. 2. Phylogenetic consensus tree based on Bayesian inference, illustrating the relationships within the *Colletotrichum acutatum* species complex of isolates obtained from rubber tree in Hainan, China. The tree was built with Bayesian analysis of concatenated sequences of *ACT*, *TUB2*, *CHS-1*, *GAPDH*, and *ITS*; the Markov chain was run for 3×10^6 generations, each locus with a separate model of DNA evolution. *C. orchidophilum* (CBS 632.80) was used as the outgroup. Bayesian posterior probability values ≥ 0.95 , bootstrap support values $\geq 50\%$, and RAxML bootstrap support values (ML $\geq 50\%$) are shown at the nodes.

acute, 7 to 15 × 2 to 4 μm, mean ± SD = 10.9 ± 1.7 × 3.0 ± 0.4 μm, L/W ratio = 3.6. Appressoria solitary, medium to dark brown, smooth-walled, ellipsoidal, clavate to irregular outline, entire edge or undulate, 6 to 15.5 × 4 to 8 μm, mean ± SD = 8.9 ± 1.6 × 6.2 ± 1.2 μm, L/W ratio = 1.4.

Asexual morph on PDA. Colonies on PDA 48 mm diam. after 7 days at 25°C in the dark, with white sparse aerial mycelium and orange conidial masses observed near the inoculum point at both above and reverse; strains Hainan 2, Hainan 9, Hainan 13, and Hainan 15 differ in forming white dense aerial mycelium and reverse white with

Fig. 3. Culture characteristics and microscopic features of the *Colletotrichum wanningsense* sp. nov. (A-B, E-F, I, K-M, Q-S from ex-holotype strain Hainan 14. C-D, G-H, J, N-P, T-V from strain Hainan 34). A-D, One-week-old colonies on PDA above and below. E-H, Conidiophores. I-J, Conidia. K-P, Appressoria on PDA. Q-V, Appressoria on SNA. Scale bars = 20 μm.

brown center surrounded with black. Chlamydospores not observed. Vegetative hyphae hyaline, smooth-walled, septate, branched. Conidiomata and setae not observed. Conidiophores formed directly on aerial mycelium, hyaline, smooth-walled, septate, branched, 20 to 60 × 2 to 4 µm. Conidiogenous cells hyaline, smooth-walled, cylindrical to ampulliform, 8 to 20 × 1.5 to 3.5 µm, apex 1 to 2 µm diam. Conidia hyaline, smooth-walled, aseptate, straight, with both ends slightly acute or one end round and one end slightly acute, 7.5 to 12.5 × 2 to 4 µm, mean ± SD = 9.8 ± 1.2 × 2.9 ± 0.4 µm, L/W ratio = 3.4. Appressoria solitary, medium to dark brown, smooth-walled, ellipsoidal, clavate to irregular outline, entire edge or undulate, 5 to 10.5 × 4 to 9 µm, mean ± SD = 7.2 ± 1.4 × 5.7 ± 1.2 µm, L/W ratio = 1.3.

Holotype (HMAS 247814): China, Hainan Province, Wanning city, on a living leaf of rubber tree, 1 April 2017, X.R. Cao, ex-type living culture CGMCC 3.18936 = Hainan14.

Notes: This new species was isolated from infected leaves of rubber tree collected from many counties in Hainan, China; it is phylogenetically most closely related to *C. cairnsense*. Although there is only 1 bp difference between the two species for *ACT*, *GAPDH*, and *ITS*, there were 8 bp and 3 bp differences for *TUB2* and *CHS-1*, respectively. *C. wanningense* differs in the colony growth rates and spore sizes from *C. cairnsense*: *C. wanningense* has faster growth rates than *C. cairnsense* (48 mm diam. after 7 days vs. 24 mm diam. after 7 days) and smaller spores than *C. cairnsense* (7.5 to 12.5 × 2 to 4 µm versus 13 to 15 × 4 µm). Colony color differs between the two species: white sparse aerial mycelium (*C. wanningense*) vs. pale white-gray to olivaceous gray aerial mycelium (*C. cairnsense*).

Pathogenicity assay. Inoculation of detached wounded leaves at the green stage of rubber trees resulted in typical anthracnose symptoms for all tested *Colletotrichum* isolates (Fig. 4). Necrotic lesions and typical orange conidial masses were often observed on leaves 5 to 7 days after inoculation. However, there were significant differences

in the average lesion diameter between three *Colletotrichum* species ($P < 0.01$). Isolates of *C. siamense* and *C. fruticola* resulted in larger necrotic lesions than those caused by *C. wanningense* (Table 1).

Discussion

Identities of *Colletotrichum* species associated with anthracnose of rubber tree leaves in Hainan, China, were determined using multilocus phylogenetic analyses, and morphological characters; their pathogenicity was confirmed in a detached leaf test. Both *TUB2* and *GAPDH* should be used to differentiate species within the *C. acutatum* species complex (Damm et al. 2012). Liu et al. (2015, 2016) used different sets of loci and different phylogenetic analytical methods on a large number of closely related *Colletotrichum* strains/species from the *C. gloeosporioides* species complex. They showed that *ApMat* should be combined with other loci such as *GS* to achieve satisfactory species delimitation in the *C. gloeosporioides* species complex. *ACT*, *CHS-1*, and *ITS* gene regions have been accepted for differentiating species in the *C. acutatum* and *C. gloeosporioides* species complexes (Alvarez et al. 2014; Damm et al. 2012; Jayawardena et al. 2016a; Marin-Felix et al. 2017; Weir et al. 2012). Therefore, five gene regions (*ACT*, *TUB2*, *CHS-1*, *GAPDH*, and *ITS*) and seven gene regions (*ACT*, *TUB2*, *CHS-1*, *GAPDH*, *ITS*, *ApMat*, and *GS*) were selected in this study for classifying isolates into species for the *C. acutatum* and the *C. gloeosporioides* species complexes, respectively.

C. laticophilum in the *C. acutatum* species complex was reported as a causal agent of rubber anthracnose disease in India and Colombia (Damm et al. 2012), while five species (*C. acutatum*, *C. citri*, *C. laticophilum*, *C. nymphaeae*, and *C. simmondsii*) in the *C. acutatum* species complex were associated with rubber anthracnose disease in Sri Lanka (Hunupolagama et al. 2017). The present study suggested that all the 16 tested isolates from the *C. acutatum* species complex, isolated from rubber tree leaf lesions in Hainan, China, belong to a

Fig. 4. Pathogenicity assay conducted on detached rubber tree leaves (Reyan 7-33-97) and the symptoms caused by *C. wanningense* (a, Hainan 14; b, Hainan 34), *C. fruticola* (c, Hainan 25), *C. siamense* (d, Hainan 20), and the control inoculated with water (e), 7 days after inoculation at 25°C.

Table 1. Lesion diameter of *Colletotrichum* isolates on rubber tree leaves and colony growth rates

Species	Isolates	Lesion diameter (cm)		Growth rate (mm/day)	
		Mean	Standard deviation	Mean	Standard deviation
<i>C. siamense</i>	Hainan 12	1.92	0.12	11.33	0.23
	Hainan 20	1.89	0.13	10.93	0.31
	Hainan 22	1.10	0.12	11.20	0.20
<i>C. fruticola</i>	Hainan 25	1.83	0.32	10.80	0.35
<i>C. wanningense</i>	Hainan 4	1.23	0.14	7.33	0.31
	Hainan 5	1.33	0.18	6.67	0.12
	Hainan 14	1.17	0.12	7.20	0.20
	Hainan 34	1.19	0.18	7.40	0.40

new species, here named as *C. wanningense*. *C. acutatum* species complex was identified as the main cause associated with rubber tree in Sri Lanka (Jayasinghe et al. 1997), whereas it was first reported in Hainan in 2010 (Li et al. 2010).

A polyphasic approach, including geographical, ecological, morphological, and genetic data, is currently recommended to establish a natural classification system for the genus *Colletotrichum* (L. Cai et al. 2009). For species delimitation within this genus, phylogenetic analysis based on concatenated loci and genealogical concordance phylogenetic species recognition (GCPSR) has proven to be powerful tools (Cannon et al. 2012; Liu et al. 2016). GCPSR suggested that the absence of recombination between *C. wanningense* and its most closely related species *C. cairnsense*.

Both *C. siamense* and *C. fruticola* were originally described as causal agents of anthracnose on coffee berries in northern Thailand (Prihastuti et al. 2009). These two species are cosmopolitan and have diverse host species (Doyle et al. 2013; Liu et al. 2016; Schena et al. 2014; Sharma et al. 2015; Udayanga et al. 2013; Vieira et al. 2014; Weir et al. 2012). In Hainan, both *C. siamense* and *C. fruticola* were reported on *Camellia oleifera*, *Santalum album*, *Pueraria lobata*, *Alchornea davidii*, *Symplocos sumuntia*, *Maesa perularius*, and *Livistona chinensis* (Jiang et al. 2016; Li et al. 2016). Additionally, *C. siamense* was reported on *Khaya senegalensis*, *Mangifera indica*, and *Bauhinia purpurea* whereas *C. fruticola* was reported on *Aporosa chinensis*, *Alchornea davidii*, and *Dalbergia odorifera* (Jiang et al. 2016; Li et al. 2016). This report demonstrates that both species can infect leaves of rubber trees in Hainan and that *C. siamense* is the most prevalent species in the *C. gloeosporioides* species complex that causes leaf anthracnose of rubber tree in Hainan. Generally, isolates of *C. siamense* and *C. fruticola* caused larger necrotic lesions than those caused by *C. wanningense*, consistent with a previous report that *C. acutatum* has a lower level of pathogenicity than *C. gloeosporioides* (Jayasinghe et al. 2009).

C. siamense was considered as a species complex (Sharma et al. 2015; Udayanga et al. 2013; Vieira et al. 2014), but this has been rejected recently (Liu et al. 2016). The present study also indicated significant recombination among the 35 *C. siamense* isolates, suggesting that *C. siamense* is unlikely to be a species complex.

In summary, we have identified three *Colletotrichum* species, *C. siamense* and *C. fruticola* from the *C. gloeosporioides* species complex and a new species (*C. wanningense*) from the *C. acutatum* species complex, as causal agents of anthracnose on rubber tree leaves in Hainan, China. For effective disease management, further research is necessary to understand whether these three species differ in their response to climatic conditions, common fungicides, and popular rubber tree genotypes. Such knowledge will help development of more targeted disease management strategies.

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