Eight *Colletotrichum* Species, Including a Novel Species, Are Associated With Areca Palm Anthracnose in Hainan, China

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Abstract

Genus *Colletotrichum* is one of the most important genera of plant-pathogenic fungi affecting numerous species, particularly tropical and subtropical crops and fruit trees. In this study, 43 *Colletotrichum* strains were isolated from areca palm leaves with anthracnose symptoms in 11 areca palm plantations in eight counties of Hainan, China. Based on the morphology, phylogenetic analysis of six loci (internal transcribed spacer, actin, chitin synthase 1, glyceraldehyde-3-phosphate dehydrogenase, β -tubulin, and mating type locus MAT1-2), and pathogenicity tests, eight *Colletotrichum* species were distinguished, comprising five previously known species (*C. cordylinicola*, *C. fructicola*, *C. gloeosporioides*, *C. siamense*, and *C. tropicale*), one unidentified *Colletotrichum* species, a new species

(*C. arenicola*) in the gloeosporioides species complex, and *C. karstii* in the boninense species complex. *C. siamense* was the most common species found in areca palm in Hainan, followed by *C. arenicola*. Pathogenicity tests showed that all eight species could cause anthracnose symptoms on areca palm leaves using a wound inoculation method and that the isolates from the gloeosporioides species complex caused larger lesions than the isolates from the boninense species complex. Further research is needed to understand the epidemiology of these pathogenic species on areca palm in order to develop management strategies.

Keywords: etiology, fungi, tropical plants

Areca palm (*Areca catechu* L.; Arecaceae) originates from Malaysia and is widely distributed in the tropical regions of Asia, East Africa, and the Pacific (Srimany et al. 2016). Fruits of *A. catechu* (areca nut or betelnut) are popular chewable items and more than 400 million people chew betel quid daily in tropical and subtropical countries (Peng et al. 2015). *A. catechu* is also used in traditional herbal medicine. Investigations demonstrate that *A. catechu* has various pharmacological activities, including antiparasitic, antioxidant, antibacterial, antifungal, anti-inflammatory, analgesic, and antiallergic effects and regulatory effects on blood glucose and lipids (Peng et al. 2015). China is the second largest producer of areca nut in the world, with 99% of its production in Hainan (Huang et al. 2016); areca palm is the second largest commercial crop in Hainan, where it is mainly planted in the eastern, central, and southern parts of the province.

A number of pathogens cause diseases on *A. catechu* at different growth stages. Anthracnose, caused by *Colletotrichum* spp., is an important disease on areca palm and the fungus can survive in the debris of the previous year's infected bunches for up to 8 months (Yashoda et al. 1992). In India, approximately 60% of the areca palms were affected by *C. gloeosporioides* (Lokesh et al. 2013), whereas the incidence of trees with anthracnose can reach 90% in Hainan where the

disease is prevalent (Zhu et al. 2015). Morphology, pathogenicity, and rDNA-internal transcribed spacer (ITS) sequence analysis of a single isolate indicated that *C. gloeosporioides* was the pathogen causing areca anthracnose in Hainan (Zhu et al. 2015). However, the identification of *Colletotrichum* spp. based solely on morphology and ITS sequences has proven difficult (Ko Ko et al. 2011). With the use of multilocus phylogenetic analysis, 22 species and one subspecies were accepted within the *C. gloeosporioides* complex (Weir et al. 2012). Since then, several new species were reported as belonging to this species complex (Liu et al. 2015; Manamgoda et al. 2013). A recent review reported 38 closely related species in the gloeosporioides species complex (Marin-Felix et al. 2017).

The objectives of this study were as follows: (i) to characterize and identify the *Colletotrichum* species associated with anthracnose of areca palm in Hainan, China, using molecular and morphological analyses; and (ii) to assess the pathogenicity of these different *Colletotrichum* species on areca palm leaves.

Materials and Methods

The general experimental framework followed our previous work on characterization of *Colletotrichum* isolates from rubber trees and coffee in Hainan, China (Cao et al. 2019a, b).

***Colletotrichum* isolates.** In 2018 to 2019, leaves with typical anthracnose symptoms were randomly collected from 11 areca plantations in eight counties in Hainan, China (Fig. 1); in total, 55 leaf samples were used for fungal isolation (four to seven samples for each plantation). Small pieces (5 × 5 mm) of leaf tissues from the lesion margins were disinfected in 70% ethanol for 30 s and followed by 1 min in 1% sodium hypochlorite. Then these leaf pieces were washed three times with sterile distilled water for 30 s and finally plated on potato dextrose agar (PDA). The plates were incubated at room temperature (28 to 30°C) under laboratory lighting conditions; after 2 to 3 days, actively growing hyphal tips were transferred to new plates with fresh PDA.

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

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Funding: This work was financially supported by grants from the Natural Science Foundation of Hainan Province, China (2019RC277), the Hainan Major Research Fund of Science and Technology (ZDKJ201817), and the Central Public-Interest Scientific Institution Basal Research Fund for Chinese Academy of Tropical Agricultural Sciences (1630042017023).

*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary figure and one supplementary table are published online.

The author(s) declare no conflict of interest.

Accepted for publication 20 December 2019.

Genomic DNA extraction, PCR, and sequencing. Mycelia from fresh colonies grown on PDA for 5 days at 25°C were harvested with a sterile 10-μl pipette tip. Total genomic DNA was extracted using the Tiangen Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. Six genomic loci, including ITS, actin (*ACT*), β-tubulin (*TUB2*), chitin synthase 1 (*CHS-1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and the mating type locus MAT1-2 (*ApMat*), were amplified and sequenced using the following primer pairs, respectively: ITS-1/ITS-4 (Gardes and Bruns 1993 and White et al. 1990, respectively), ACT-512F/ACT-783R (Carbone and Kohn 1999), T1/Bt2b (O'Donnell and Cigelnik 1997 and Glass and Donaldson 1995, respectively), CHS-79F/CHS-354R (Carbone and Kohn 1999), GDF1/GDR1 (Templeton et al. 1992), and AMF1/AMR1 (Silva et al. 2012).

PCR was performed in a thermal cycler (C1000; Bio-Rad Laboratories, Hercules, CA) in a 25-μl reaction volume, composed of 12.5 μl of Taq-Plus PCR Forest Mix (NOVA, Yugong Biolabs, Jiangsu, China), 1 μl of each primer (5 μM), 1 μl of DNA template, and 9.5 of μl ddH₂O. PCR conditions were performed as described by Cao et al. (2019a). PCR products were purified and sequenced by SinoGenoMax Co. Ltd. (Chinese National Human Genome Center, Beijing, China).

Phylogenetic analyses. The forward and reverse sequences were assembled to generate consistent sequences using MEGA version 6.0. All sequences were blasted in GenBank. Multiple sequence alignments of each gene were made with ClustalW (as implemented in MEGA 6.0) and edited manually as necessary. The sequences were deposited in GenBank (Supplementary Table S1). Bayesian inference (BI) was used to construct a phylogenetic tree with MrBayes version 3.2.6 (Ronquist et al. 2012). For the isolates from the gloeosporioides complex, two concatenated datasets (with or without *ApMat* gene sequences) were generated because some of the type strains in the gloeosporioides species complex did not have the *ApMat* gene region. MrModeltest version 2.3 (Nylander 2004), as implemented in MrMTgui, was used to determine the best-fit model of nucleotide substitution for each genomic locus using the corrected Akaike information criterion. For the gloeosporioides species complex, the following nucleotide substitution models were used: SYM+I+G for ITS, HKY+I for *GAPDH*, GTR+G for *ACT* and *TUB2*, K80+I+G for *CHS-1*, and HKY+G for *ApMat*. For the boninense species complex, the following models were used: SYM+I+G for ITS, HKY+G for *GAPDH*, GTR+G for *ACT*, GTR+I for *TUB2*, and K80+G for *CHS-1*. Eight simultaneous Markov Chain Monte Carlo chains were run from random trees with 6×10^6 generations for the gloeosporioides species complex and 3×10^6 for the boninense species complex with the trees were sampled every 1,000 generations. The first 25% of trees were discarded as burn-in and the remaining sampled trees were used to calculate posterior probabilities (PPs). In addition, maximum likelihood (ML) analyses

of the multilocus alignments were performed using RaxmlGUI version 1.3.1 (Silvestro and Michalak 2012). A GTRGAMMAI substitution model was applied with 1,000 bootstrap replicates.

To clearly identify the genes and/or loci in which variation is responsible for differentiating the new species, all six loci (ITS, *ACT*, *CHS-1*, *GAPDH*, *TUB2*, and *ApMat*) were analyzed individually for the isolates from the gloeosporioides species complex using BI and ML methods.

Alignments and tree files derived in this study were deposited in TreeBASE (<https://www.treebase.org/treebase-web/home.html>; accession number S25099).

Morphological analysis. Morphological and cultural characterizations were conducted based on published methods (Cai et al. 2009) and hence are only described briefly. Mycelial discs (5-mm diameter), taken from the edge of 5-day-old colonies in PDA, were transferred onto fresh PDA and incubated at 25°C with a 12/12-h fluorescent light/dark cycle. There were three replicate plates for each isolate. Colony diameters were measured daily for 5 days to calculate mean daily growth (in millimeters per day). The shape, color, and density of colonies were recorded after 5 days of incubation. Appressoria were induced using a published slide culture technique (Cai et al. 2009). The shapes and sizes of conidia and appressoria were assessed under a digital microscope (Eclipse 80i; Nikon, Tokyo, Japan) and 30 conidia and appressoria selected randomly were recorded for each strain. For the new species, morphological and cultural characteristics of the holotype on oatmeal agar (OA) and synthetic nutrient-poor agar medium (SNA) were recorded.

Prevalence of *Colletotrichum* species. The prevalence of each *Colletotrichum* species was calculated as $(Ns/Nt) \times 100\%$, where *Ns* is the number of leaf samples yielding at least one isolate of the species concerned and *Nt* is the number of leaf samples examined.

Pathogenicity tests. Fourteen *Colletotrichum* isolates, comprising one isolate from each of *C. tropicale* and *C. gloeosporioides* (as only one isolate was obtained for these two species) and two isolates from each of the other six species, were tested for their pathogenicity against detached tender healthy leaves (the youngest fully expanded leaves) of areca palm (cultivar Reyan 1) using both nonwound and wound inoculation methods. The tested leaves were washed three times with sterile water and air-dried on pieces of sterilized filter paper. For the wound inoculation method, the leaves were punctured with a sterilized needle (0.5-mm diameter) and hyphal plugs (5-mm diameter) from actively growing margins of PDA cultures were then placed onto each wound site. For the method of nonwound inoculation, hyphal plugs from actively growing margins of PDA cultures were placed on the leaves without wounding. Leaves, inoculated with sterile PDA plugs onto the wound or nonwound, were treated as the controls. After inoculation, the leaves were placed onto pieces of moist tissue papers in sterilized plastic containers. The containers were sealed in plastic and maintained in a chamber at 25°C with a 12/12-h light/dark regime. Lesion diameter was measured across two perpendicular directions 11 days after inoculation for the wound-inoculated leaves, whereas the leaves inoculated without wounding were assessed 30 days after inoculation. A completely randomized design was used with three replicates per isolate; there were three leaves in each replicate with two inoculation points per leaf.

Data analysis. One-way analysis of variance was used to compare the sizes of conidia and appressoria, growth rates among species, and lesion diameters (averaged over the two perpendicular directions) among isolates tested, and means were compared based on the Fisher's least significant difference test. SAS software (version 8.0; SAS Institute, Cary, NC) was used.

Results

Phylogenetic analyses and species identification. Forty-three *Colletotrichum* isolates (two to six isolates for each plantation sampled) were obtained from 55 leaf samples. Based on the morphology and BLAST search in GenBank, 41 isolates were identified as belonging to the gloeosporioides species complex and the other two

Fig. 1. Map of locations and compositions of *Colletotrichum* spp. isolates sampled in Hainan, China. Numerals in parentheses indicate the number of isolates obtained from that areca palm plantation.

to the boninense species complex. The six-gene (ITS, *ACT*, *CHS-1*, *GAPDH*, *TUB2*, and *ApMat*) and the five-gene (ITS, *ACT*, *CHS-1*, *GAPDH*, and *TUB2*) alignment of the gloeosporioides species complex contained 3,264 characters including gaps with the 1,539 unique site patterns and 2,264 characters including gaps with the 925 unique site patterns, respectively. The combined gene (ITS, *ACT*, *CHS-1*, *GAPDH*, and *TUB2*) alignment of the boninense species complex comprised 2,213 characters including gaps with the 576 unique site patterns. The ML tree confirmed the tree topology from the Bayesian analysis.

In the six-gene tree, the 41 isolates in the gloeosporioides species complex clustered into seven clades as follows: two with *C. cordylinicola* (3.6% of the samples examined; Bayesian PP value, 0.97; RAxML bootstrap support ML value, 60), three with *C. fructicola* (5.5%; PP, 0.94; ML, 95), 16 with *C. siamense* (29.1%; PP, 1; ML, 90), one with *C. gloeosporioides* (1.8%; PP, 1; ML, 100), one with *C. tropicale* (1.8%; PP, 1; ML, 84), and three *Colletotrichum* sp. clustered with *C. ledongense* and *C. syzygicola* (5.5%). In addition, 15 isolates (27.3% of the samples examined; closely related to *C. cordylinicola*) were clustered distantly from any known species in the complex (PP, 1; ML, 93) and are herein described as a new species, *C. arenicola* (Fig. 2). Although the topology of the five-gene

tree of the gloeosporioides species complex was similar to that of the six-gene tree (Fig. 3), the five-gene trees failed to delimit *C. cordylinicola*, *C. arenicola*, and *Colletotrichum* sp. Furthermore, support for the *C. siamense* clade (PP < 0.9; ML < 50) and the *C. fructicola* clade (PP < 0.9; ML = 89) was low in the five-gene tree. The two isolates in the boninense species complex were clustered with *C. karstii* (3.6% of the samples examined; PP, 1; ML, 94) (Fig. 4).

Single-locus phylogenetic analyses of the isolates from the gloeosporioides species complex showed that all seven species in this study could be delimited from the other species using the *ApMat* gene sequences only, although the *C. arenicola* isolates were separated into two distinct clades (Supplementary Fig. S1). No more than three of the seven species could be differentiated when each of the other sequences (ITS, *ACT*, *CHS-1*, *GAPDH*, and *TUB2*) was used individually.

Taxonomy. Based on the morphology and phylogenetic analysis, the 43 *Colletotrichum* isolates from areca palm in Hainan were identified to be from eight species, including one new species that is described below.

Colletotrichum arenicola X.R. Cao, H.Y. Che & D.Q. Luo, sp. nov. - MycoBank MB 831251; Figure 5.

Fig. 2. A Bayesian inference phylogenetic tree based on concatenated sequences of internal transcribed spacer (ITS), actin (*ACT*), chitin synthase 1 (*CHS-1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β -tubulin (*TUB2*), and mating type locus MAT1-2 (*ApMat*) to illustrate the relationships of *Colletotrichum* spp. isolates within the gloeosporioides species complex. The species *C. boninense* (ICMP 17904) was used as an outgroup. The Markov chain was run for 6×10^6 generations, each locus with a separate model of DNA evolution. Bayesian posterior probability values ≥ 0.90 and RAxML bootstrap support values (maximum likelihood $\geq 50\%$) are shown at the nodes. Ex-type isolates are in bold. Shaded blocks indicate clades containing isolates obtained from areca palm in this study.

Etymology. Referring to the name of the host (*Areca catechu*) where the species was first collected.

Sexual morph was not observed. Asexual morph developed on PDA. Colonies on PDA 67 mm in diameter after 5 days at 25°C, with white to dark gray colonies with dense aerial mycelium; chlamydospores were not observed. Vegetative hyphae were hyaline, smooth-walled, septate, and branched. Conidiomata and setae were not observed. Conidiophores formed directly on aerial mycelium, hyaline, smooth-walled, septate, and branched. Conidiogenous cells were hyaline, smooth-walled, cylindrical to ampulliform, 9 to 22 × 2 to 4.5 µm, apex 1 to 3 µm in diameter. Conidia were hyaline, smooth-walled, aseptate, straight, cylindrical to clavate, with both ends round or one end round and one end slightly acute, 10.5 to 16.0 × 4 to 6 µm (mean dimensions ± SD = 13.9 ± 1.2 × 4.9 ± 0.4 µm), length/width (L/W) ratio = 2.8. Appressoria were solitary, brown to dark brown, variable in shape, irregular or knobbed, 12 to 20 × 5 to 8 µm (mean dimensions ± SD = 14.9 ± 2.3 × 6.2 ± 0.7 µm), L/W ratio = 2.4.

Asexual morph developed on OA. Setae were observed in the acervuli about 40 to 80 µm long. Conidiophores were hyaline to pale smooth-walled, septate, and branched. Conidiogenous cells were hyaline to pale brown, cylindrical to clavate, 12 to 30 × 2 to 5 µm, apex 1 to 3.5 µm in diameter. Conidia were hyaline, smooth-walled, aseptate, straight, cylindrical, both ends rounded, 11 to 17.5 × 3.5 to 6 µm (mean dimensions ± SD = 13.8 ± 1.4 × 4.9 ± 0.4 µm), L/W ratio = 2.8. Appressoria were solitary, brown to dark brown, cylindrical to elliptic in shape, 7 to 13 × 5 to 8 µm (mean dimensions ± SD = 9.0 ± 1.3 × 6.3 ± 0.6 µm), L/W ratio = 1.4.

Asexual morph developed on SNA. Setae were dark brown to black, opaque, tip acute, base cylindrical, and 35 to 90 µm long. Conidiophores were hyaline, smooth-walled, septate, and branched. Conidiogenous cells were hyaline, clavate, 8 to 23 × 2 to 4.5 µm, apex 1 to 2.5 µm in diameter. Conidia were hyaline, smooth-walled, aseptate, cylindrical, both ends rounded, 10.5 to 16.5 × 3.5 to 6 µm (mean ± SD = 14.3 ± 1.2 × 4.8 ± 0.5 µm), L/W ratio = 3.0.

Fig. 3. A Bayesian inference phylogenetic tree based on concatenated sequences of internal transcribed spacer (ITS), actin (*ACT*), chitin synthase 1 (*CHS-1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and β -tubulin (*TUB2*) to illustrate the relationships of *Colletotrichum* spp. isolates within the gloeosporioides species complex. The species *C. boninense* (ICMP 17904) was used as an outgroup. The Markov chain was run for 6×10^6 generations, each locus with a separate model of DNA evolution. Bayesian posterior probability values ≥ 0.90 and RAxML bootstrap support values (maximum likelihood $\geq 50\%$) are shown at the nodes. Ex-type isolates are in bold. Shaded blocks indicate clades containing isolates obtained from areca palm in this study.

Fig. 4. A Bayesian inference phylogenetic tree based on concatenated sequences of internal transcribed spacer (ITS), actin (ACT), chitin synthase 1 (*CHS-1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and β -tubulin (*TUB2*) to illustrate the relationships of *Colletotrichum* spp. isolates within the boninense species complex. The species *C. gloeosporioides* (ICMP 17821) was used as an outgroup. The Markov chain was run for 3×10^6 generations, each locus with a separate model of DNA evolution. Bayesian posterior probability values ≥ 0.90 and RAxML bootstrap support values (maximum likelihood $\geq 50\%$) are shown at the nodes. Ex-type isolates are in bold. Shaded blocks indicate clades containing isolates obtained from areca palm in this study.

Appressoria were dark brown, simple, irregular in shape, or bullet-shaped with an acute tip, sometimes irregularly lobed, 9 to 18×4 to $9.5 \mu\text{m}$ (mean $\pm$ SD = $13.1 \pm 2.1 \times 6.8 \pm 1.3 \mu\text{m}$), L/W ratio = 1.9.

Holotype (HMAS 248158): China, Hainan Province, Wenchang city, on a diseased areca palm leaf (*A. catechu*), March, 2019, X.R. Cao, ex-type living culture CGMCC 3.19667 = HNBLS.

Notes: This new species was isolated from infected leaves of areca palm collected from four counties in Hainan, China; it was phylogenetically most closely related to *C. cordylinicola* (Fig. 2). Phylogenetic analyses showed that isolates from *C. arecicola* could be delimited from other species in the gloeosporioides species complex using the *ApMat* locus although the *C. arecicola* isolates were separated into two distinct clades (Supplementary Fig. S1). In morphology, *C. arecicola* differs from *C. cordylinicola* by having longer appressoria (12 to 20×5 to $8 \mu\text{m}$ versus 13 to 13.4×7.2 to $7.3 \mu\text{m}$) and knobbed appressoria on PDA.

Morphological characterization. All seven species from the gloeosporioides species complex developed white to gray or black colonies with dense aerial mycelia, whereas isolates of *C. karstii* from the boninense species complex developed white colonies with sparse aerial mycelia (Table 1). The width of *C. karstii* conidia was significantly ($P < 0.01$) larger than the other species, whereas the growth rate of *C. karstii* was slower ($P < 0.01$) than the other species (Table 1). There were also significant differences ($P < 0.01$) in the size of both conidia and appressoria and in growth rates among species within the gloeosporioides species complex.

Pathogenicity tests. Inoculation of wounded areca palm leaves (cultivar Reyan 1) resulted in typical symptoms of anthracnose for all tested isolates; the average lesion diameter ranged from 1.8 to

4.0 mm (Fig. 6). There were significant differences ($P < 0.01$) in the lesion diameter among species. Isolates of *C. arecicola* and *C. cordylinicola* resulted in larger lesions (diameter > 3 mm), whereas isolates of *C. karstii* and *C. tropicale* produced smaller lesions (diameter < 2 mm). There was also a significant difference between the lesion diameter two *C. siamense* isolates (2.3 and 3.2 mm). However, no symptoms were observed on those leaves inoculated without wounding 30 days after inoculation.

Discussion

The *Colletotrichum* species associated with anthracnose of areca palm in Hainan, China, were identified based on morphological characteristics and multilocus phylogenetic analyses. Eight *Colletotrichum* species (*C. cordylinicola*, *C. fruticola*, *C. gloeosporioides*, *C. siamense*, *C. tropicale*, an unknown species *Colletotrichum* sp., a new species *C. arecicola* from the gloeosporioides species complex, and *C. karstii* from the boninense species complex) were shown to be associated with anthracnose of areca palm in Hainan.

Species in the gloeosporioides species complex were previously reported to cause anthracnose on numerous plants worldwide, particularly in the tropical region (Freeman et al. 1998; Udayanga et al. 2013). More than 74.5% of the isolates obtained in this study were from the gloeosporioides species complex. This was in agreement with previous reports that *C. gloeosporioides* was the causal agent of anthracnose of areca palm in India and Hainan (Yashoda et al. 1992; Zhu et al. 2015). However, this study also indicated that a species belonging to the boninense species complex can cause anthracnose on areca palm.

C. siamense, *C. fruticola*, *C. tropicale*, and *C. karstii* were reported on several host plants in Hainan. For example, both *C. siamense* and

Fig. 5. Culture characteristics and microscopic features of the *Colletotrichum arecicola* sp. nov. **a and b**, Colonies on potato dextrose agar (PDA) above and below. **c, d, and e**, Conidiophores. **f**, Conidia. **g and h**, Setae. **i, j, k, l, m, n, o, p, q, and r**, Appressoria. **c, f, and i to k** were produced on PDA; **g, l, and m** were produced on oatmeal agar; and **h and o to r** were produced on synthetic nutrient-poor agar medium. Scale bars = $10 \mu\text{m}$.

C. fructicola were reported on tea-oil trees, rubber trees, and coffee (Cao et al. 2019a, b). In addition, *C. siamense* was reported on mango, partridge tea, and red gragon (Liu et al. 2018a; Qin et al. 2017; Zhao et al. 2018); *C. fructicola* on strawberry (Jayawardena et al. 2016); *C. tropicale* on *Sauropus androgynus* and coffee (Cao et al. 2019b; Liu et al. 2018b); and *C. karstii* on chili, stylo, and coffee (Cao et al. 2019b; Diao et al. 2017; Jia et al. 2017). In this study, these four species were isolated from areca palm in Hainan in 8, 2, 1, and 2 plantations of the 11 plantations sampled in Hainan, respectively (Fig. 1). In addition, *C. siamense* was the most common species associated with areca palm in Hainan. Similar results were obtained on rubber tree and coffee in Hainan (Cao et al. 2019a, b). Further research is needed to study whether *C. siamense* from areca palm, rubber tree, and coffee can cross-infect each other, as they are usually planted adjacent to or intercropped with each other in Hainan.

C. cordylinicola was isolated from leaves of *Cordyline fruticosa* and *Eugenia javanica* in Thailand and Laos (Phoulivong et al. 2010). It is herein reported in Hainan for the first time, although with low frequencies (3.6%) on areca palm. The new species, *C. arecicola*, was a common pathogen on areca palm in Hainan, present in 7 of the 11 areca plantations sampled. The unknown *Colletotrichum* sp. was clustered with *C. ledongense* and *C. syzygicola*; further research is needed to determine its taxonomical identity.

The resolution of *Colletotrichum* species differs depending on both the locus used and the species concerned; thus, there is no general agreement on the locus or loci to use for species identification (Marin-Felix et al. 2017). The *ApMat* locus was proven to be better in delimiting the gloeosporioides species complex than *ACT*, *CAL*, *GAPDH*, *GS*, *ITS*, and *TUB2* (Liu et al. 2015; Sharma et al. 2013; Silva et al. 2012). All seven species in the gloeosporioides species complex obtained in this study can be differentiated from the other species when using *ApMat* sequences only. However, Liu et al. (2015) reported that *ApMat* should be combined with other loci for species delimitation in the gloeosporioides complex. In this study, the *C. arecicola* isolates were separated into two distinct clades when using the *ApMat* locus only. The isolates identified as *C. cordylinicola*, *C. arecicola*, and *Colletotrichum* sp. in the six-gene tree could not be

delimited in the five-gene tree without the *ApMat* gene. Furthermore, both the *C. siamense* clade and the *C. fructicola* clade did not receive high support values in the five-gene tree without the *ApMat* gene, consistent with a previous report (de Silva et al. 2019).

Wounding is known to enhance infection by *Colletotrichum* species and subsequent disease development; thus, many studies of *Colletotrichum* spp. have been carried out in the laboratory using wound-based inoculation methods (Peres et al. 2005). In this study, all eight species were able to infect wounded detached areca palm leaves.

Fig. 6. Lesion diameters of *Colletotrichum* isolates on wounded leaves of areca palm (cultivar Reyan 1) 11 days after inoculation. The bars represent the standard error, and columns with the same letter do not differ significantly according to the Fisher's least significant difference test ($P = 0.05$).

Table 1. Morphological and cultural characteristics of *Colletotrichum* spp. causing anthracnose of areca palm in Hainan, China^z

Species	Colony characteristics	Conidia			Appressoria			Growth rates (mm/day)
		Shape	Length (μm)	Width (μm)	Shape	Length (μm)	Width (μm)	
<i>C. cordylinicola</i>	White with gray in center colony, dense mycelium	Cylindrical	14.0 ± 0.17 (10.5–16.5) c	4.7 ± 0.03 (4.0–5.5) d	Ovoid, clavate, or slightly irregular	12.6 ± 0.3 (8.5–17.5) bc	6.4 ± 0.1 (5.0–9.0) cd	12.7 ± 0.5 b
<i>C. fructicola</i>	White with gray to black in center, dense mycelium, with orange conidial masses	Cylindrical	14.2 ± 0.2 (12.0–16.5) bc	5.0 ± 0.06 (3.5–6.0) b	Ovoid, clavate, or slightly irregular	11.2 ± 0.5 (7.5–16.0) de	6.7 ± 0.2 (5.0–9.0) bc	12.8 ± 0.2 b
<i>C. gloeosporioides</i>	White to gray colony, dense mycelium, with orange conidial masses	Cylindrical	15.4 ± 0.2 (13.5–18.0) a	4.8 ± 0.05 (4.0–5.5) cd	Clavate or slightly irregular	12.0 ± 0.5 (8.5–16.5) cd	6.4 ± 0.2 (5.0–8.0) cd	14.6 ± 0.2 a
<i>C. karstii</i>	White colony, sparse mycelium	Oblong	14.7 ± 0.2 (12.0–18.5) b	7.0 ± 0.05 (6.0–8.5) a	Globose or ovoid	9.8 ± 0.2 (7.0–14.5) f	7.6 ± 0.2 (5.5–11.0) a	9.6 ± 0.1 c
<i>C. siamense</i>	White with gray in center colony, dense mycelium, with orange conidial masses	Cylindrical to fusiform	14.3 ± 0.1 (12.0–16.5) bc	4.9 ± 0.06 (3.5–6.0) bc	Ovoid or clavate	10.7 ± 0.2 (7.0–16.5) ef	7.0 ± 0.1 (5.5–9.5) b	12.6 ± 0.3 b
<i>C. tropicale</i>	White to gray colony, dense, with orange conidial masses	Cylindrical	14.4 ± 0.2 (12.0–16.5) bc	4.9 ± 0.04 (4.0–5.5) bc	Subglobose or clavate	10.4 ± 0.4 (7.5–14.5) ef	6.7 ± 0.2 (5.0–9.0) bc	14.8 ± 0.1 a
<i>C. arecicola</i>	White with gray to black in center colony, dense mycelium	Cylindrical to clavate	14.3 ± 0.2 (10.5–16.5) bc	4.9 ± 0.05 (4.0–6.0) bc	Irregular or knobbed	15.5 ± 0.3 (12.0–21.0) a	6.1 ± 0.1 (5.0–8.0) de	13.4 ± 0.1 b
<i>Colletotrichum</i> sp.	White to gray colony, dense mycelium, with orange conidial masses	Cylindrical to fusiform	14.7 ± 0.2 (12.5–17.5) b	4.8 ± 0.04 (4.0–6.0) cd	Irregular or knobbed	13.4 ± 0.2 (10.0–17.5) b	5.9 ± 0.1 (4.5–9.0) e	13.0 ± 0.2 b

^z Values within the same column followed by the same letters are not significantly different based on the one-way analysis of variance with Fisher's least significant difference test at $P = 0.05$.

Overall, the isolates from the gloeosporioides species complex caused larger lesions than those of *C. karstii*. There were also significant differences in the pathogenicity among species within the gloeosporioides species complex. Generally, isolates from *C. arenicola*, *C. siamense*, and *C. cordylincola* were found to cause larger lesions than other species. The former two species accounted for 27.3 and 29.1% of the isolates obtained from the 11 plantations in this study, respectively. The two *C. siamense* isolates also differed in lesion diameter, indicating the presence of intraspecies variability in the aggressiveness on areca palm. Similarly, such intraspecies variability in *C. siamense* was also observed on mango leaves and fruits (Mo et al. 2018). In three *Colletotrichum* spp., different pathotypes were identified on mature green and ripe chili fruit (Mongkolporn et al. 2010).

No visual symptoms were observed on those leaves when inoculated without wounding. Previous studies suggested that wounding is necessary for *Colletotrichum* species to infect various hosts, such as mango fruit (Freeman and Shabi 1996; Lima et al. 2013) and grape leaf (Hong et al. 2008). When 12 *Colletotrichum* species from cultivated pear were inoculated onto detached pear leaves, only two species were pathogenic to those leaves without wounding (Fu et al. 2019). This phenomenon may be related to the quiescent infection, which is an important feature of *Colletotrichum* spp. (Peres et al. 2005). The cuticle and epidermis may also act as a barrier to infection by *Colletotrichum* spp. (Auyong et al. 2015). Factors such as inoculum density, temperature, wetness duration, and plant age could influence the infection by *Colletotrichum* spp. and subsequent symptom development (Freeman et al. 1998; Hong and Hwang 1998; Uysal and Kurt 2017).

In 10 of the 11 areca palm plantations sampled, at least two *Colletotrichum* species were present, and the composition of *Colletotrichum* species varied greatly among the sampled plantations. Fungicide is one of the main strategies for areca palm anthracnose management (Li 2011; Nidhina and Hegde, 2014); however, different *Colletotrichum* species display different sensitivities to individual fungicides (Cao et al. 2019b; Chen et al. 2016). Therefore, it is essential to determine the species in a given plantation in order to maximize fungicide control efficacies.

Acknowledgments

We thank Dr. Fang Liu for help with the phylogenetic analyses.

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