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RESEARCH ARTICLE

Plant Pathology

Molecular and phenotypic characterization of *Colletotrichum plurivorum* and *Colletotrichum musae* causing banana anthracnose disease in the Central Province of Sri Lanka

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Abstract: Most of the commercial banana cultivars in Sri Lanka are susceptible to anthracnose disease. *Colletotrichum musae* has been known as the causal agent of banana anthracnose for decades and the pathogen has been identified using morphological characteristics. Molecular analyses based on multigene phylogenetics are now standard protocols to identify *Colletotrichum* species. The present study was aimed at identifying *Colletotrichum* species causing banana anthracnose by molecular and phenotypic characterization. Thirty-seven isolates were obtained from ripened bananas showing anthracnose symptoms, collected from different locations in the Central Province of Sri Lanka. Of them, 36 were preliminarily identified as *Colletotrichum* based on conidial morphology. The remaining isolate did not sporulate during the entire study period. Ten isolates taken for molecular studies consisted of eight with orange/white arial mycelia and orange conidial masses, one with a white to greyish colony and blackish clusters of ascomata, and one with a white to faint pink colour colony. DNA extracted from each isolate was subjected to multi-gene DNA sequence analysis using ITS, TUB, GAPDH and GS loci. Based on phylogenetic analyses, eight isolates were identified as *Colletotrichum musae*, and the other two as *C. plurivorum* and *C. siamense*. The vegetative morphology of *C. plurivorum* differed considerably from *C. musae* and *C. siamense*. Slight differences in colony morphology were observed among the *C. musae* isolates. Freshly harvested healthy bananas were artificially inoculated with isolates of *C. musae* or *C. plurivorum* and produced typical anthracnose lesions within a week. The *Colletotrichum siamense* isolate failed to develop anthracnose symptoms. This is the first report of *C. plurivorum* causing banana anthracnose.

Keywords: *Colletotrichum gloeosporioides* species complex, *Colletotrichum orchidearum* species complex, *C. plurivorum*, molecular phylogeny, pathogenicity.

INTRODUCTION

Bananas are among the most produced, traded and consumed fruits globally. More than 1000 varieties of bananas exist in the world. The most traded variety is the Cavendish banana. In 2020, the amount of bananas produced worldwide reached approximately 119.83 million tonnes (FAO, 2022).

Colletotrichum species comprise important plant pathogens that cause anthracnose disease in many economically important crops worldwide. Anthracnose is by far the most destructive postharvest disease in all banana-producing and marketing countries of the world (Abayasekara *et al*., 2013), causing serious damage to fruit quality and drastically reducing shelf life and marketability.

Colletotrichum musae (Berkeley & M.A. Curtis) Arx was known as the causal agent of banana anthracnose disease for decades. The identification was mainly based on the host specificity and morphological characters. *Colletotrichum* infects immature banana fruits long before harvest in the field. The fungus is abundant in transition

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leaves and diseased crop debris, including flower parts, and the last bunch bract (de Lapeyre de Bellaire *et al*., 2008), serving as major sources of the primary inoculum. Conidia, disseminated by rain and wind, adhere to the surface of the developing fruit, germinate producing germ-tubes, and form appressoria on the tip of germ-tubes. Infection pegs that emerge from appressoria penetrate the cuticle and epidermal cell wall of the host tissue. The fungus remains quiescent for long periods (Swinburne, 1983) and progressive lesion development takes place only with the commencement of fruit ripening, during storage or marketing. The typical anthracnose lesions in ripe bananas are dark, circular, sunken and numerous with salmon-pink conidia masses (Adikaram *et al*., 2010). Quiescent infections are rather difficult to be controlled than wound infections (Abayasekara *et al.*, 2013; Wanigasekara *et al*., 2014). Young fruits are usually free from visible symptoms. *Colletotrichum musae* is also associated with other diseases of bananas such as crown rot (Indrakeerthi & Adikaram, 2011), blossom-end rot and tip rot (Meredith, 1965).

Colletotrichum siamense Prihast., L. Cai & K.D. Hyde was identified as a pathogen causing anthracnose in bananas in Turkey using partial sequences of GAPDH, ACT and CHS-1 (Uysal & Kurt, 2020), India (Kumar *et al*., 2017) and Brazil (Vieira *et al*., 2017). *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (Cannon *et al*., 2008) was reported as a causal agent of banana anthracnose in Ecuador (Riera *et al*., 2019). In Malaysia, while 92% of isolates were identified as *C. gloeosporioides* (Penz.) Penz. & Sacc, only 8% of the isolates were *C. musae* (Sakinah *et al*., 2014). *Colletotrichum scovillei* Damm, P.F. Cannon & Crous (Zhou *et al*., 2017), *C. fructicola* Prihast., L. Cai & K.D. Hyde, *C. cliviicola* Damm & Crous, *C. siamense*, *C. karstii* You L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai, and *C. musae* (Huang *et al*., 2021) were quite recently reported as causing banana anthracnose in China.

Most of the commercial banana cultivars in Sri Lanka are susceptible to the pathogens causing anthracnose disease. Popular banana cultivars of Sri Lanka include, 'Ambon' (*Musa acuminata*, AAA), 'Anamalu' (*Musa acuminata*, AAA), 'Embul' (*M. acuminata* x *M. balbisiana*, AAB), 'Kolikuttu' (*M. acuminata* x *M. balbisiana*, AAB), 'Puwalu' (*M. acuminata* x *M. balbisiana*, AAB) and, 'Suwandal' (*M. acuminata* x *M. balbisiana*, AAB) (Anthony *et al*., 2004; Nazriya *et al*., 2007; Abayasekara *et al*., 2013).

Due to the global importance of *Colletotrichum* as a plant pathogenic genus, accurate diagnosis is essential to improve biosecurity and disease management strategies (Cannon *et al*., 2012). However, cultural and morphological characteristics alone are insufficient to identify or differentiate *Colletotrichum* at the species level. Therefore, a polyphasic approach involving morphological and molecular methods and pathogenicity tests is needed for accurate species-level identification of *Colletotrichum* spp. Molecular analyses based on multigene phylogenetics, and pathogenicity assays are now the standard protocols to identify *Colletotrichum* species.

In spite of the establishment of molecular-based species-level identification of *Colletotrichum*, the identity of *Colletotrichum* species causing anthracnose disease in banana has not been performed. Therefore, the objectives of the present study were to, (i) collect banana fruits showing anthracnose disease symptoms from locations within the Central Province of Sri Lanka, irrespective of the cultivar, and to isolate *Colletotrichum* spp. from diseased fruits, (ii) characterize isolates morphologically and by performing a phylogenetic analysis of a sample of ten selected isolates, and complete species level identification, and (iii) confirm their pathogenicity. The present study identified the *Colletotrichum* species causing banana anthracnose in the Central Province (CP) of Sri Lanka using both morphological and molecular markers and pathogenicity tests.

MATERIALS AND METHODS

Collection of diseased fruits and isolation of fungi

Ripe fruits of banana cultivars, 'Anamalu' (*Musa acuminata*, AAA), 'Emban' (*M. acuminata*, AAA), 'Kolikuttu' (*M. acuminata* x *M. balbisiana*, AAB), 'Puwalu' (*M. acuminata* x *M. balbisiana*, AAB), and 'Seeni' (*M. acuminata* x *M. balbisiana*, ABB), showing characteristic symptoms of anthracnose disease were collected from fruit stalls and markets in different locations within the Central Province (CP), Sri Lanka in 2020. Diseased fruits were brought in sealed polythene bags to the laboratory at the National Institute of Fundamental Studies (NIFS), Kandy.

Peel segments (0.5 × 0.5 cm^2), cut from the advancing margins of anthracnose lesions, were surface sterilized in 1% NaOCl (Clorox®, USA) for 1–3 min and rinsed twice in sterile distilled water (SDW). After removing the excess liquid by placing on sterile filter paper, tissue segments were aseptically transferred onto PDA (Himedia Lab, India) plates (4 segments per plate) in replicates of four plates per specimen. The plates were incubated at room temperature (RT, 25 ºC). After 7 d, the mycelium that emerged from each of the tissue segment was sub-cultured on PDA.

Preparation of mono-conidial cultures

The mycelia scraped from 7 d old cultures, was suspended seperatly in 10 mL SDW. After shaking vigorously to release conidia, the suspensions were filtered through a muslin cloth. The concentration of conidia in the filtrate was adjusted to 5×10^6/mL. A loopful of each conidia suspension was streaked on Tap Water Agar (2 %, Himedia Lab, India). Following 12 to 18 h of incubation, a small piece of agar with a single germinated conidium located by moving the objective lens (× 25) of the light microscope (Euromax BB.1153 PLi model) along the streak line, was transferred onto fresh PDA. Single spore isolates were sub-cultured and used for molecular studies (Johnston & Booth, 1983).

DNA extraction

Ten isolates, labelled C-1 to C-10, were used for molecular studies (Table 1) of which 8 were selected randomly from among 35 cultures showing pink to orange conidial masses. The two remaining isolates had contrasting colony morphologies compared with the first 35 isolates. DNA extraction was performed using Promega Wizard® Genomic DNA Purification Kit (Promega Corporation, USA). Mycelia were scraped using a sterile inoculation loop, from fresh cultures grown on PDA at 25 °C for 7 to 10 d for DNA extraction, which was performed according to the manufacturer's protocol with modifications i.e., addition of 20 µL proteinase K, after cooling to RT, following the addition of cell lysis solution (20 µL), nuclei lysis solution (20 µL) and incubating for 1 h at 65 ºC. Finally, the DNA sample was stored at –20 °C in the freezer.

Table 1: The cultivars and locations from where the banana fruits were collected for obtaining the ten isolates used for DNA extraction.

Label	Collection location	Cultivar
C-1	Pilimatalawa	'Puwalu' (AAB)
C-2	Pilimatalawa	'Seeni' (ABB)
C-3	Kadugannawa	'Emban' (AAA)
C-4	Peradeniya	'Kolikuttu' (AAB)
C-5	Kandy	'Seeni' (ABB)
C-6	Kandy	'Puwalu' (AAB)
C-7	Pilimatalawa	'Seeni' (ABB)
C-8	Kadugannawa	'Anamalu' (AAA)
C-9	Kandy	'Seeni' (ABB)
C-10	Wattegama	'Kolikuttu' (AAB)

PCR amplification

Current taxonomic and phylogenetic studies on the genus *Colletotrichum* recommend the use of multiple gene regions for species-level identification (Damm *et al*., 2012). The present study used four loci, internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutamine synthetase (GS) and β-tubulin (TUB), and were amplified using primer pair ITS1 and ITS4, GD92F1 and GDR1, GSF1 and GSR1 and, BT2a and BT2b, respectively (White *et al*., 1990; Templeton *et al*., 1992; Gardes & Bruns,1993; Glass & Donaldson, 1995; Stephenson *et al*., 1997; Weir *et al*., 2012). All PCR amplifications were carried out using the Promega - 20 µl GoTaq® Green Master Mix, 0.2 µM each forward and reverse primers and 5 µL of unquantified DNA template. PCR reaction was performed using a thermal cycler (Applied Biosystems Veriti). The thermal cycler

was programmed to perform the PCR reactions using an initial denaturation at 95 ºC for 4 min, 35 cycles of denaturation at 95 ºC for 30 s and annealing at 52 ºC, 60 ºC, 54 ºC and, 55 ºC for ITS, GAPDH, GS and TUB2, respectively, for 30 s, extension at 72 ºC for 45 s and a final extension at 72 ºC for 7 min (Weir *et al*., 2012).

The amplified PCR products were separated by electrophoresis in 1% agarose gel, stained with ethidium bromide, and visualized with a UV transilluminator. The PCR products were sequenced with the same primers (Applied Biosystems, 3500 genetic analyzer).

Phylogenetic analyses

Sequenced data of the four gene regions of each strain were visualized, and ambiguous bases were edited manually using MEGA6 software v. 6.0 (Tamura *et al*., 2013). Sequences derived in this study were deposited in GenBank and the Accession numbers obtained are given in Supplementary Tables 1 and 2. These sequences were subjected to a similarity-based search using the NCBI, BLASTn programme. Initial blast results showed that the isolates belonged to two species complexes, the *C. gloeosporioides* and the *C. orchidearum* species complex. Hence, two different datasets were used to estimate two phylogenies, the *C. gloeosporioides* species complex tree based on combined ITS + GAPDH + TUB + GS regions, and the *C. orchidearum* species complex tree based on combined ITS + GAPDH. A Maximum Likelihood (ML) analysis was conducted using raxmlGUI v. 1.3 (Silvestro & Michalak, 2012). The optimal ML tree search was performed with 1000 separate runs, employing the default algorithm of the programme from a random starting tree for each run. The ultimate tree was chosen amongst suboptimal trees from each run by examining likelihood scores under the GTR + GAMMA substitution model. The resulting phylogenetic tree (Table 3) was visualized in FigTree v. 1.2.2 (Rambaut & Drummond, 2008).

Colony and reproductive morphology of isolates

Suspensions of conidia were prepared from 7–10 d old mono-conidial cultures of *Colletotrichum* isolates as described previously. Drops of conidia of each isolate were mounted on microscope slides and examined under a light microscope and photographed (Olympus BX53 with DP 74 Digital Camera & cellSens software ver. 2.1, Olympus, Japan). The dimensions of 50 randomly selected conidia from each drop were measured. The average width and length were calculated.

Appressoria were produced in fungal hyphae using a slide culture technique (Sutton, 1968). A few Petri plates were poured with a thin layer of PDA. Square pieces (10 mm^2) of PDA were cut and placed in the centre of empty sterile Petri plates. The four edges of each agar piece were inoculated with conidia taken from a sporulating culture and a sterile cover slip was placed over the inoculated agar. After 7 d, the appressoria formed on the underside of the cover slip were examined (Prihastuti *et al*., 2009) under a light microscope, and their morphology, dimensions and other features were recorded and photographed (Olympus BX53 with DP 74).

Pathogenicity test

Conidial suspensions of *C. musae* (C-1 to C-4 & C-7 to C-10) and *C. siamense* (C-6) were prepared by suspending the mycelium scraped from 14 d old cultures in SDW and filtering through a muslin cloth. The concentration of conidia was adjusted to approximately 1×10^6 /mL. Since isolate C-5 did not produce conidia, a suspension of ascospores was prepared. Clusters of perithecia, carefully picked from a 14 d old culture, were crushed and suspended in 10 mL SDW. The suspension was vigorously shaken on a Vortex mixer to force the release of ascospores and the suspension was filtered through a muslin cloth. The concentration of ascospores was adjusted to approximately 1×10^3 /mL. Seven drops (20 µL) of conidia or ascospores from each isolate were separately applied on to the surface of freshly harvested, mature fruits of bananas cv. 'Emban' (AAA) in triplicates, along the long axis, leaving a 2 cm space between each drop. A set of bananas, similarly treated with drops (20 µL) of SDW, was maintained as a control. Inoculated and control fruits were arranged in trays lined with moistened tissues and covered with glass plates, and incubated at 26–28 ºC. The fruits were observed daily for disease development. Once the symptoms appeared, the pathogen was re-isolated on PDA and compared with the original isolate used for inoculation.

RESULTS AND DISCUSSION

Collection of diseased bananas and isolation of fungi

A total of 37 isolates were collected from diseased banana fruits belonging to a range of cultivars. Thirty-six isolates (97.3%) produced oblong conidia and yellow/orange or pale orange conidiomata, which were tentatively identified as belonging to the genus *Colletotrichum* using colony and conidial morphology. The remaining isolate did not produce any conidia.

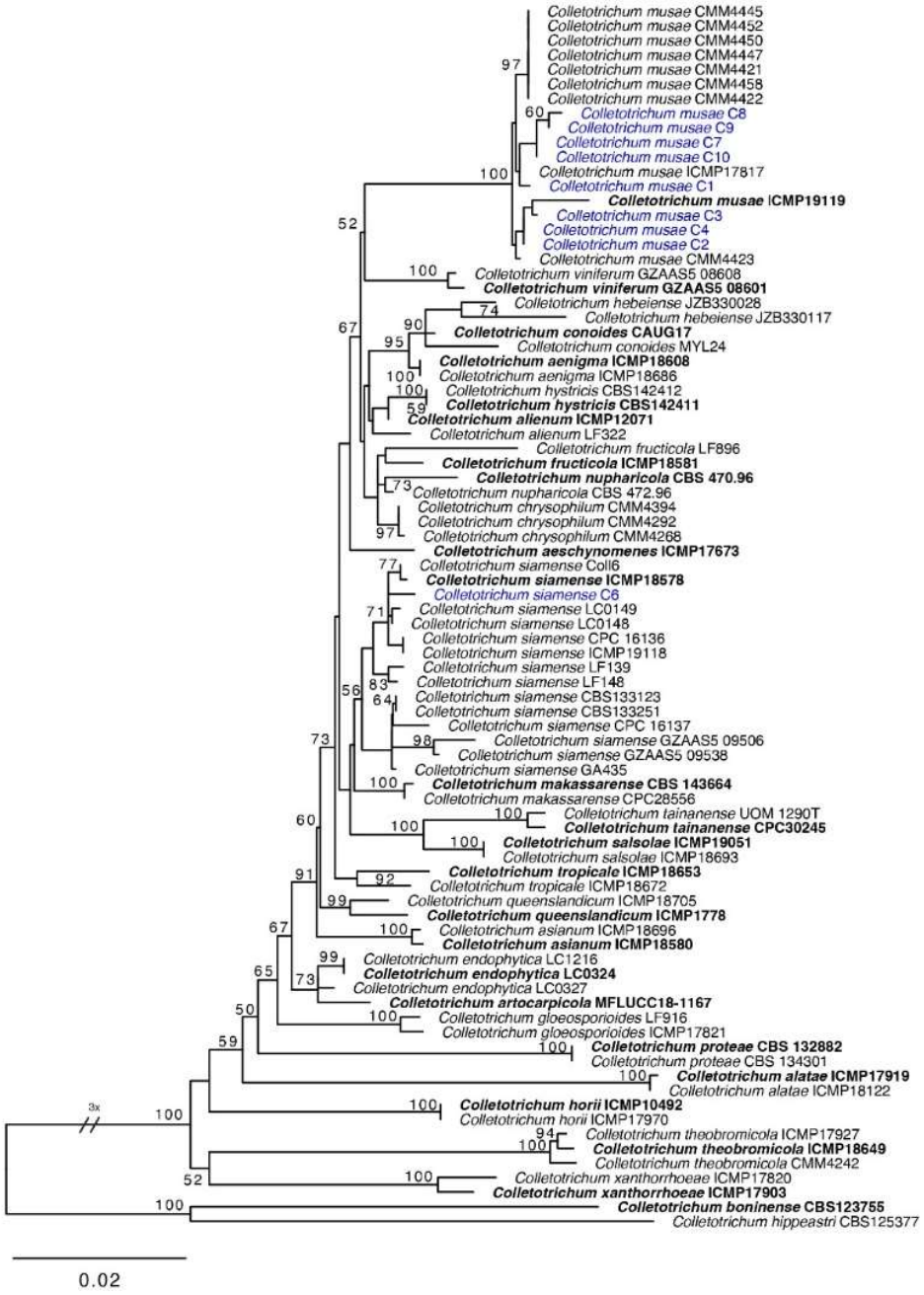

Figure 1: Phylogenetic relationships in the *Colletotrichum gloeosporioides* species complex based on combined ITS+GAPDH+GS+TUB loci. The isolates derived from the present study are in blue. Type strains are in bold.

Molecular identification of isolates

Ten isolates, C-1 to C-10 (Table 1), were used for molecular studies. The combined GAPDH + GS + ITS + TUB dataset for the *C. gloeosporioides* species complex comprised 84 strains, including two out-group taxa from the *C. boninense* species complex (*Colletotrichum boninense* Moriwaki, Toy. Sato & Tsukib. strain CBS 123755 and *Colletotrichum hippeastri* Yan L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai strain ICMP 17920). The concatenated data matrixes comprised 2409 characters (GAPDH: 276, GS: 868, ITS: 564, and TUB: 701). The ML analysis for 1,000 bootstrap replicates yielded a tree with the likelihood value of ln: –18432.224367.

Based on the phylogenetic tree (Figure 1), the isolates C-1, C-2, C-3, C-4, C-7, C-8, C-9, and C-10 were identified as *C. musae* and one strain (C-6) clustered with the strains of *C. siamense*. Both *C. musae* and *C. siamense* belong to the *C. gloeosporioides* species complex. *Colletotrichum musae,* while showing its prominence as an anthracnose pathogen in bananas, is also involved in causing banana crown rot (Indrakeerthi & Adikaram, 2011).

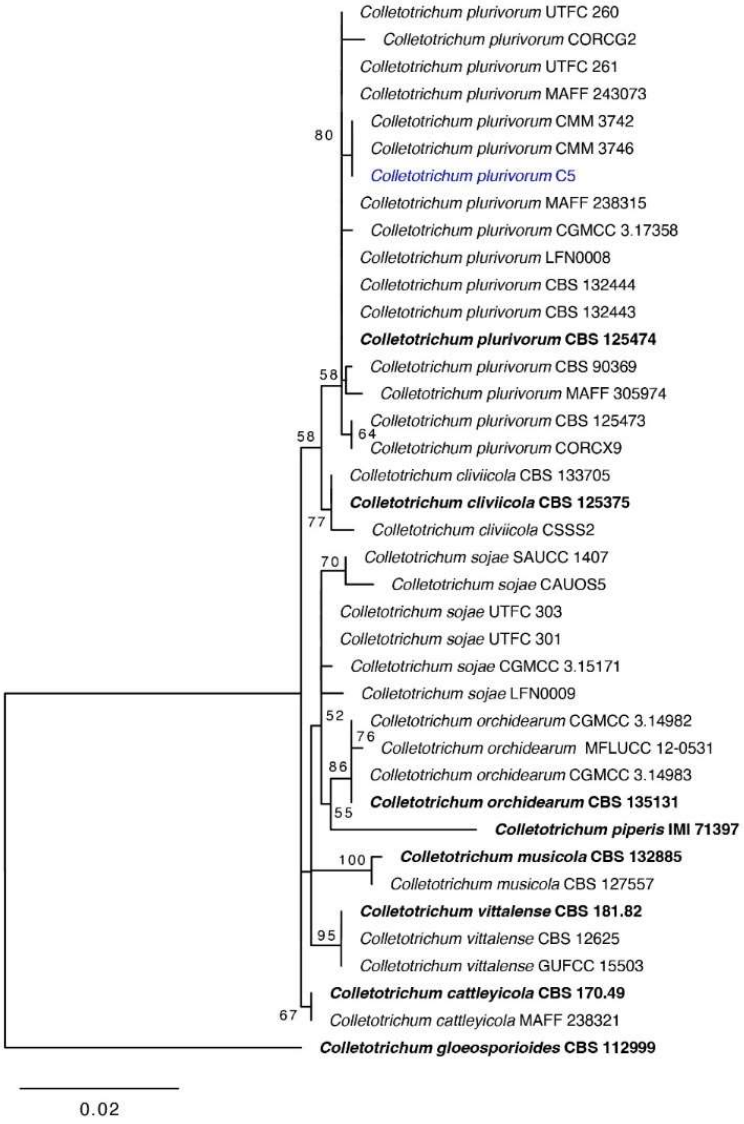

Figure 2: Phylogenetic relationships in the *Colletotrichum orchidearum* species complex based on combined ITS+GAPDH loci. The isolate derived from the present study is in blue.

The combined ITS + GAPDH dataset for the *C. orchidearum* species complex comprised 39 strains, including an out-group taxon from the *C. gloeosporioides* species complex (*C. gloeosporioides* isolate CBS 112999). The aligned data matrixes comprised 839 characters (ITS: 553 and GAPDH: 286). The ML analysis for 1,000 bootstrap replicates yielded a tree with the likelihood value of ln: –13321.232547. The isolate C-5 formed a clade with reference strains of *Colletotrichum plurivorum* Damm, Alizadeh & Toy. Sato (Figure 2).

Colletotrichum plurivorum, belonging to the *C. orchidearum* species complex, was previously isolated from leaves of *Musa* sp. in Japan (Damm *et al*., 2012), and known to be associated with the fruit rot of papaya in Taiwan (Sun *et al*., 2019), anthracnose disease in chilli pepper (*Capsicum annuum*) in Andaman and Nicobar Islands (Sakthivel *et al*., 2018), okra in Brazil (Batista *et al*., 2020), and cassava in China (Liu *et al*., 2019).

Colony and reproductive morphology

Colonies of *C. musae*, grown on PDA, produced orange to pinkish-white, cottony aerial mycelium and abundant bright orange conidial masses or conidiomata (Figure 3). The lower surface had white to greyish-orange pigmentation. The colony characteristics, texture, and pigmentation underneath, varied slightly within the eight *C. musae* isolates but pink/orange aerial mycelium was common to a majority (87.5%) of the identified isolates C1 to C-4, C-7, C-8, C-10. *Colletotrichum musae* was the fastest in colony growth on PDA among the three species, and *C. plurivorum* was the next (Table 2). The *Colletotrichum musae* isolate C-9, produced whitish to faint orange aerial mycelium and also tiny, faint orange coloured conidiomata over the centre of the colony (Figure 3).

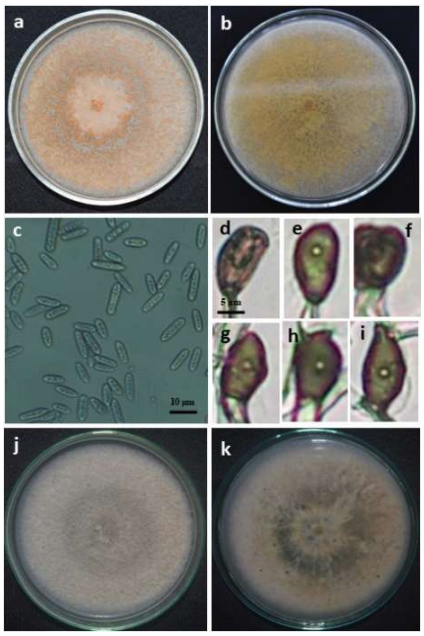

Figure 3: Colony morphology of *C. musae* isolate C-1, grown on PDA at 25 ℃ for 14 days, with orange aerial mycelium and conidiomata, common to seven *C. musae* isolates, C-1 to C-4, C-7, C-8 & C-10; (a) upper, and (b) lower surface, (c) conidia, and (d, e, f, g, h, i) appressoria. (j) *C. musae* isolate C-9 with slightly different colony morphology from the seven isolates, with whitish aerial mycelium and some conidiomata in the centre, upper, and (k) lower surface.

Conidia were abundant, hyaline, aseptate, guttulate, oval, elliptical or cylindrical, often with a flattened base and obtuse apex. Vegetative hyphae of all three species, grew on agar pieces in slide cultures and formed appressoria.

Each species produced appressoria of varied morphological forms. Appressoria of *C. musae* in slide cultures were medium to dark brown and irregular, the edges were entire or slightly lobed (Figure 3; Table 2).

The colony of *C. siamense* (C-6) on PDA was pale-yellow to pinkish white, circular with an irregular margin, white; aerial mycelium sparse to abundant and cottony, with pale orange conidial masses in the centre of colony. The lower surface was also pale-yellow to pinkish-white (Figure 5). Conidia hyaline, aseptate, smooth-walled, fusiform to cylindrical, both ends bluntly rounded. Appressoria single, medium to dark brown, smooth-walled, elliptical, navicular, bullet-shaped or irregular outline, with undulate or frequently lobate margin (Figure 5). These indicate that *C. siamense* shows slight differences in colony morphology compared to *C. musae*. The isolate was comparatively slow-growing (Table 2).

Colonies of *C. plurivorum* (C-5) on PDA were circular with dense, white to greyish aerial mycelium. The lower surface of the colony appeared yellowish green (Figure 4). Colony morphology was quite distinct from the other two species, *C. musae* and *C. siamense*, due to its white-to-greyish aerial mycelium containing dense, clusters of blackish ascomata spread over the colony (Figure 4). The eight *C. musae* isolates, and the single isolate of *C. siamense,* could be conveniently separated from *C. plurivorum* (Figure 4) using colony morphology (Figure 3). However, the morphological characteristics of singular isolates like *C. plurivorum* or *C. siamense* cannot be compared with species like *C. musae* with several isolates with confirmed identity in the present study.

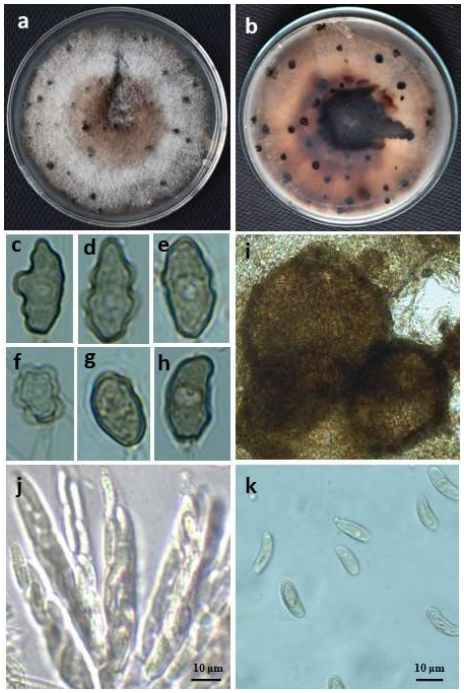

Figure 4: Colony morphology and microscopic characteristics of *Colletotrichum plurivorum.* A 14-day old culture (C-5) grown on PDA at 25 ℃ (a) upper surface, (b) lower surface, (c, d, e, f, g, h) appressoria produced in vegetative hyphae, (i) perithecia, (j) asci, and (k) ascospores.

Interspecific morphological variability could also be expected within individual species of *C. plurivorum* or *C. siamense*. Cultural characteristics are normally considered as less important criteria for distinguishing species within the genus *Colletotrichum* since they can be influenced by changing environmental factors and growth conditions (Cannon *et al*., 2012). Due to these inconsistencies, morphological characteristics alone are considered insufficient to identify or differentiate *Colletotrichum* at the species level.

Appressoria of *C. plurivorum* were single, pale to medium brown with a tint of light black background and smooth-walled with varied shapes, sub-globose to globose or irregular shaped, the edge undulated, crenate or slightly lobed (Figure 4; Table 2). Ascomata formed on PDA in clusters, covered by aerial mycelium, black, globose, solitary or gregarious and 140–290 µm diameter. Individual perithecia were ostiolate, the outer wall composed of greenish-grey angular cell. Asci were cylindrical, obclavate to clavate and 8-spored; ascospores were hyaline, smooth-walled, aseptate, fusoid, with rounded ends, straight or slightly curved (Figure 4; Table 2).

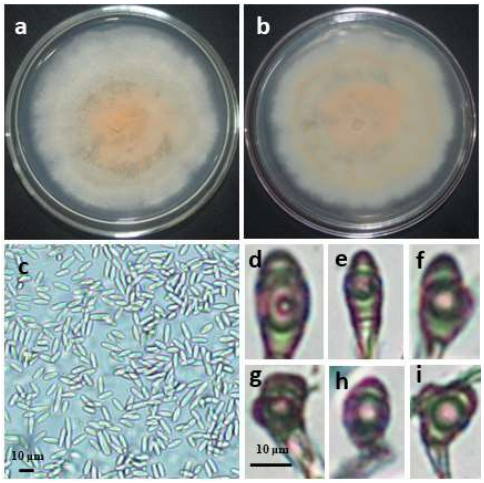

Figure 5: Colony and microscopic characteristics of *Colletotrichum siamense* (C-6) culture, grown on PDA for 14 d at 25 °C, (a) upper and (b) lower surface of the colony, (c) conidia, and (d, e, f, g, h, i) appressoria.

Figure 6: Anthracnose lesions developed in ripe banana cultivar 'Emban' (AAA), 7 d after inoculation with conidia of *Colletotrichum musae* (C-1 to C-4 & C-7 to C-10), *C. siamense* (C-6) and ascospores of *C. plurivorum* (C-5).

Pathogenicity test

The banana fruits, artificially inoculated with all nine *Colletotrichum* isolates separately, developed anthracnose lesions with the commencement of ripening. The lesions gradually became darker, slightly depressed, and expanded in size with time. Pink-coloured conidial masses appeared on the surface of colonies of *C. musae* or *C. siamense*. Symptoms developed by the isolates were typical of anthracnose disease, the size of lesions, however, varied slightly among the isolates (Figure 6). *Colletotrichum siamense* (C-6), on the other hand, produced only minute superficial specks none of which developed into progressive lesions.

The isolates of *C. musae* appeared highly pathogenic on the banana cultivar 'Emban'. The varied size of anthracnose lesions produced by *C. musae* isolates on inoculation indicates the existence of differences in virulence among isolates within the species. C-1 and C-9 appeared to be the most and the least virulent isolates of *C. musae* respectively. *Colletotrichum musae* isolate C-9 also displayed contrasting colony morphology from the rest of the *C. musae* isolates.

Table 2: Vegetative and reproductive morphology of *Colletotrichum* isolated and identified from banana fruits

Parameter	C-1 to 4 & C-7 to 10, *Colletotrichum musae*	C-5, *Colletotrichum plurivorum*	C-6, *Colletotrichum siamense*
Colony characteristics	Orange to pinkish-white, cottony mycelium with abundant yellow/ orange conidial masses, the lower surface was white to greyish orange colour (Figure 3).	White to greyish, dense aerial mycelium and, black, clusters of perithecia spread over the colony (Figure 4).	Colonies yellow to pinkish-white, sparse to abundant, aerial mycelium pale orange conidiomata (Figure 5).
Average colony growth mm d^{-1}	11.43	10.71	9.29
Conidial morphology	Hyaline, aseptate, oval to cylindrical	Isolate did not produce conidia.	Hyaline, aseptate, cylindrical to subcylindrical, spindle-shaped with obtuse ends.
Conidial dimensions (µm)	11.16 – 14.2 (12.5 ± 1.5) × 4.68 – 5.56 (5.0 ± 0.5)	---------	14.5 – 16.7 (15.8 ± 0.9) long × 4.2 – 4.9 (4.55 ± 0.35)
Perithecia, ascus, ascospore morphology & dimensions (µm)	(not produced)	Ascomata globose, irregular, solitary or gregarious, black, 150–300 µm diameter. Perithecia solitary, sub-globose, ostiolate outer wall composed of greenish grey angular cells, glabrous, 100 – 200 × 95 – 160. Asci cylindrical, obclavate to clavate 32–55 × 7.3–13, 8-spored. Ascospores hyaline, smooth-walled, aseptate, fusiform, straight or slightly curved, with rounded ends, straight or slightly curved. 14.5 – 16.7 (15.8 ± 0.9) × 4.2 – 4.9 (4.55 ± 0.35)	(not produced)
Appressoria morphology	Single, medium to dark brown, smooth-walled, oval, elliptical or heart-shaped, with undulate or slightly lobate margin.	Mostly single, pale to medium brown, smooth-walled, sub-globose to globose, the edge undulate, crenate or slightly lobed. Only a few appressoria were produced.	Single, medium to dark brown, smooth-walled, elliptical, navicular or bullet-shaped, with undulate or lobate margin.
Appressoria Dimensions (µm)	Mean ± SD = 11.5 ± 2.5 × 9 7.4 ± 1.5	Mean ± SD = 15.4 ± 3.0 × 8.8 ± 2.6	Mean ± SD = 12.5 ± 2.9 × 6.8 ± 1.3
Average lesion diameter of inoculated fruit (cm)	1.44 ± 0.54	1.1 ± 0.46	*C. siamense* did not develop lesions.

Banana fruits, inoculated with ascospores of the *C. plurivorum* isolate also developed fairly larger anthracnose lesions similar to suggesting a reasonably higher level of pathogenicity. The symptoms developed were quite similar to the progressive anthracnose lesions developed from natural infections. Although

C. plurivorum appeared to be moderately pathogenic to banana fruits, its association with the anthracnose disease in other fruit species is not very common.

The *C. siamense* isolate (C-6) failed to develop progressive lesions in bananas inoculated artificially. Instead, brown colored, superficial specks appeared only on the inoculation sites of the fruit peel, which did not expand into progressive lesions. The isolate (C-6) maybe a variant with an inability of forming appressoria on germ-tubes of germinating conidia, which could be a possible reason for its failure to develop anthracnose lesions. *Colletotrichum* species generally produce distinct appressoria, which facilitate the pathogen-penetration through intact fruit surface. Morphology of colonies, conidia or ascospores produced in cultures raised through re-isolation on PDA was similar to those of the *Colletotrichum* isolates used for fruit inoculation.

Two *Colletotrichum* species isolated from banana fruits in the present study were identified as belonging to the *C. gleoesporioides* species complex while *C. plurivorum* belongs to the *C. orchideorum* species complex based on molecular studies. Identification of new and unknown *Colletotrichum* species associated with banana anthracnose reflects the significance of continuous investigations into *Colletotrichum* systematics, which could help reduce the risk from unknown pathogens to the country's banana fruit industry.

Accurate identification is vital since the scientific name links the knowledge concerning a species including the biology, host range, distribution, and potential risk of the pathogen, which are necessary for planning effective control strategies, biosecurity and screening new banana cultivars against anthracnose (Bhunjun *et al*., 2021). Determination of the pathogenicity or the capability of the pathogen/s to cause host damage is also important, which usually relies upon the application of Koch's postulates for fungal plant pathogens. Similar trends have been found in the composition of *Colletotrichum* species causing anthracnose disease in other fruits, avocado (Sharma *et al*., 2017), chilli (Diao *et al*., 2017), and the ornamental plants, begonia (Wickramasinghe *et al*., 2020) and anthurium (Vithanage *et al*., 2021).

In summary, the current study represents the most comprehensive investigation of *Colletotrichum* species on banana in the CP of Sri Lanka. The study revealed that *Colletotrichum plurivorum* and *C. musae* were the causal agents of banana anthracnose in the province. *Colletotrichum musae* was the most frequently isolated pathogen. *Colletotrichum siamense* was also isolated but the pathogenicity tests showed its inability to produce typical anthracnose symptoms from artificial inoculation.

CONCLUSIONS

The study identified *Colletotrichum musae* and *C. plurivorum* as the causal agents of banana anthracnose in the CP of Sri Lanka. *Colletotrichum musae* was the most frequently found species among the isolates subjected to molecular studies. *Colletotrichum siamense* isolated in the study was not found to be pathogenic to banana fruits (Emban, AAA) making its role in banana anthracnose quite uncertain.

This is the first report of the involvement of *C. plurivorum* causing banana anthracnose anywhere in the world.

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Competing Interests

All authors disclose that they have no competing interest.

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Molecular and phenotypic characterization of *Colletotrichum plurivorum* and *Colletotrichum musae* causing banana anthracnose disease in the Central Province of Sri Lanka

Supplementary Information

Supplementary Table 1: GenBank accession numbers of isolates included in *C. gloeosporioides* species complex tree. Sequences from the present study are in bold letters

Species	Culture	GenBank Accession number	
		ITS	GAPDH
Colletotrichum cattleyicola	MAFF 238321	MG600759	-
Colletotrichum cattleyicola	CBS 170.49	MG600758	MG600819
Colletotrichum cliviicola	CSSS2	GU109480	GU085868
Colletotrichum cliviicola	CBS 125375	MG600733	MG600795
Colletotrichum cliviicola	CBS 133705	MG600732	MG600794
Colletotrichum gloeosporioides	CBS 112999	JQ005152	JQ005239
Colletotrichum musicola	CBS 127557	MG600737	MG600799
Colletotrichum musicola	CBS 132885	MG600736	MG600798
Colletotrichum orchidearum	CGMCC 3.14982	KX853166	KX893585
Colletotrichum orchidearum	CGMCC 3.14983	KX853167	KX893586
Colletotrichum orchidearum	MFLUCC 12-0531	KT290264	KT290263
Colletotrichum orchidearum	CBS 135131	MG600738	MG600800
Colletotrichum piperis	IMI 71397	MG600760.	MG600820
Colletotrichum plurivorum	CBS 90369	MG600721	MG600784
Colletotrichum plurivorum	CBS 125474	MG600718	MG600781
Colletotrichum plurivorum	CORCG2	HM585397	HM585380
Colletotrichum plurivorum	CBS 132443	MG600719	MG600782
Colletotrichum plurivorum	UTFC 260	MG600723	MG600786
Colletotrichum plurivorum	MAFF 243073	MG600730	MG600793
Colletotrichum plurivorum	CBS 125473	MG600717	MG600780
Colletotrichum plurivorum	MAFF 305974	MG600731	-
Colletotrichum plurivorum	CORCX9	HM585398	HM585381
Colletotrichum plurivorum	CGMCC 3.17358	KJ955215	KJ954916
Colletotrichum plurivorum	UTFC 261	MG600722	MG600785
Colletotrichum plurivorum	LFN0008	KT696336	KT696289
Colletotrichum plurivorum	CMM 3746	KC702981	KC702942
Colletotrichum plurivorum	CBS 132444	MG600720	MG600783
Colletotrichum plurivorum	CMM 3742	KC702980.	KC702941
Colletotrichum plurivorum	MAFF 238315	MG600729	MG600792
Colletotrichum plurivorum	C5	**MT742141**	**MW192221**
Colletotrichum sojae	CGMCC 3.15171	-	KC843501
Colletotrichum sojae	UTFC 301	MG600756	MG600817
Colletotrichum sojae	LFN0009	KT696354	-
Colletotrichum sojae	CAUOS5	KP890107	KP890138
Colletotrichum sojae	UTFC 303	MG600757	MG600818
Colletotrichum sojae	SAUCC 1407	KT362184	KT362188
Colletotrichum vittalense	CBS 12625	MG600735	MG600797
Colletotrichum vittalense	GUFCC 15503	JN390935	-
Colletotrichum vittalense	CBS 18182	JN390935	MG600796

Supplementary Table 2: Collection details and GenBank accession numbers of isolates included in the *C. gloeosporioides* species complex tree. Sequences from present study are in bold letters

Species	Culture	GenBank Accession numbers			
		ITS	GAPDH	TUB2	GS
Colletotrichum aenigma	ICMP18608	JX010244	JX010044	JX010389	JX010078
Colletotrichum aenigma	ICMP18686	JX010243	JX009913	JX010390	JX010079
Colletotrichum aeschynomenes	ICMP17673	JX010176	JX009930	JX010392	JX010081
Colletotrichum alatae	ICMP17919	JX010190	JX009990	JX010383	JX010065
Colletotrichum alatae	ICMP18122	JX010191	JX010011	JX010136	JX010449
Colletotrichum alienum	ICMP12071	JX010251	JX010028	JX010411	JX010101
Colletotrichum alienum	LF322	KJ955131	KJ954832	KJ955279	KJ954982
Colletotrichum artocarpicola	MFLUCC18-1167	MN415991	MN435568	MN435567	-
Colletotrichum asianum	ICMP18580	FJ972612	JX010053	JX010406	JX010096
Colletotrichum asianum	ICMP18696	JX010192	JX009915	JX010384	JX010073
Colletotrichum boninense	CBS123755	JQ005153	JQ005240	JQ005588	-
Colletotrichum chrysophilum	CMM4268	KX094252	KX094183	KX094285	KX094204
Colletotrichum chrysophilum	CMM4292	KX094248	KX094182	KX094284	KX094203
Colletotrichum chrysophilum	CMM4394	KX094239	KX094179	KX094282	KX094200
Colletotrichum conoides	CAUG17	KP890168	KP890162	KP890174	-
Colletotrichum conoides	MYL24	KY995389	KY995340	KY995473	-
Colletotrichum endophytica	LC0324	KC633854	KC832854	-	-
Colletotrichum endophytica	LC0327	KC633855	KC832846	-	-
Colletotrichum endophytica	LC1216	KC633853	KC832853	-	-
Colletotrichum fructicola	ICMP18581	JX010165	JX010033	JX010405	JX010095
Colletotrichum fructicola	LF896	J955221	KJ954922	KJ955366	KJ955071
Colletotrichum gloeosporioides	ICMP17821	JX010152	JX010056	JX010445	JX010085
Colletotrichum gloeosporioides	LF916	KJ955226	KJ954927	KJ955371	KJ955076
Colletotrichum hebeiense	JZB330117	MG763977	MG812555	MG812561	-
Colletotrichum hebeiense	JZB330028	KF156863	KF377495	KF288975	-
Colletotrichum hippeastri	CBS125377	JQ005230	JQ005317	JQ005664	-
Colletotrichum horii	ICMP10492	GQ329690	GQ329681	JX010450	JX010137
Colletotrichum horii	ICMP17970	JX010213	JX010000	-	-
Colletotrichum hystricis	CBS142411	KY856450	KY856274	KY856532	-
Colletotrichum hystricis	CBS142412	KY856451	KY856275	KY856533	-
Colletotrichum makassarense	CBS143664	MH728812	MH728820	MH846563	-
Colletotrichum makassarense	CPC28556	MH728815	MH728821	-	MH748262
Colletotrichum musae	C1	**MT742137**	**MW196689**	**OM274079**	**OM274088**
Colletotrichum musae	C10	**MT742138**	**MW196690**	**OM274082**	**OM274091**
Colletotrichum musae	C2	**MT742139**	**MW196691**	**OM274083**	**OM274092**
Colletotrichum musae	C3	**MT742140**	**MW196692**	**OM274084**	**OM274093**
Colletotrichum musae	C4	**MT742143**	**MW196693**	**OM274080**	**OM274089**
Colletotrichum musae	C7	**MT742144**	**MW196694**	**OM274086**	**-**
Colletotrichum musae	C8	**MT742145**	**MW196695**	**OM274081**	**OM274090**
Colletotrichum musae	C9	**MT742146**	**MW196696**	**OM274087**	**OM274094**
Colletotrichum musae	CMM4421	**KX094259**	**KX094194**	**KX094297**	KX094237
Colletotrichum musae	CMM4422	**KX094244**	**KX094189**	**KX094298**	KX094232
Colletotrichum musae	CMM4423	KX094243	KX094195	KX094294	KX094231
Colletotrichum musae	CMM4445	KX094241	KX094188	KX094293	KX094230
Colletotrichum musae	CMM4447	KX094251	KX094192	KX094296	KX094235
Colletotrichum musae	CMM4450	KX094245	KX094190	KX094295	KX094233
Colletotrichum musae	CMM4452	KX094253	KX094193	KX094291	KX094236
Colletotrichum musae	CMM4458	KX094249	KX094191	KX094292	KX094234
Colletotrichum musae	ICMP17817	JX010142	JX010015	JX01039	JX010084
Colletotrichum musae	ICMP19119	NG_06284	JX010050	JQ005861	JX010103
Colletotrichum nupharicola	CBS 470.96	JX010187	JX009972	JX010398	JX010088

Colletotrichum nupharicola	CBS 472.96	JX010188	JX010031	JX010399	JX010089
Colletotrichum proteae	CBS132882	KC297079	KC297009	KC297101	KC297032
Colletotrichum proteae	CBS134301	KC842385	KC842379	KC842387	KC842387
Colletotrichum queenslandicum	ICMP1778	JX010276	JX009934	JX010414	JX010104
Colletotrichum queenslandicum	ICMP18705	JX010185	JX010036	JX010412	JX010102
Colletotrichum salsolae	ICMP18693	JX010241	JX009917	-	-
Colletotrichum salsolae	ICMP19051	NR_120139	JX009916	-	-
Colletotrichum siamense	CPC 16136	KP703417	KP703347	KP703504	KP703758
Colletotrichum siamense	C6	**MT742142**	**MW192222**	**OM274085**	-
Colletotrichum siamense	CBS133123	JX145142	-	JX145193	-
Colletotrichum siamense	CBS133251	JX145144	-	JX145195	-
Colletotrichum siamense	CPC 16137	KP703418	KP703348	KP703505	KP703759
Colletotrichum siamense	Coll6	JX145153	-	JX145205	-
Colletotrichum siamense	GA435	KX620330	KX620264	KX620363	KX620295
Colletotrichum siamense	GZAAS5 09506	JQ247633	JQ247609	JQ247644	JQ247621
Colletotrichum siamense	GZAAS5 09538	JQ247632	JQ247608	JQ247645	JQ247620
Colletotrichum siamense	ICMP18578	JX010171	JX009924	JX010404	JX010094
Colletotrichum siamense	ICMP19118	JX010259	JX009974	JX010415	JX010105
Colletotrichum siamense	LC0148	KJ955078	KJ954779	KJ955227	KJ954929
Colletotrichum siamense	LC0149	KJ955079	KJ954780	KJ955228	KJ954930
Colletotrichum siamense	LF139	KJ955087	KJ954788	KJ955236	KJ954938
Colletotrichum siamense	LF148	KJ955088	KJ954789	KJ955237	KJ954939
Colletotrichum tainanense	UOM 1290T	MH728805	MH728819	-	MH748271
Colletotrichum tainanense	CPC30245	MH728805	MH728823	MH846558	MH748259
Colletotrichum theobromicola	CMM4242	KX094238	KX094173	KX094278	KX094197
Colletotrichum theobromicola	ICMP17927	JX010286	JX010024	JX010373	JX010064
Colletotrichum theobromicola	ICMP18649	JX010294	JX010006	JX010447	JX010139
Colletotrichum tropicale	ICMP18653	JX010264	JX010007	JX010407	JX010097
Colletotrichum tropicale	ICMP18672	JX010275	JX010020	JX010396	JX010086
Colletotrichum viniferum	GZAAS5 08601	JN412804	JN412798	-	JN412787
Colletotrichum viniferum	GZAAS5 08608	JN412802	JN412800	-	JN412784
Colletotrichum xanthorrhoeae	ICMP17820	JX010260	JX010008	-	-
Colletotrichum xanthorrhoeae	ICMP17903	JX010261	JX009927	JX010448	JX010138