Characterizations of *Colletotrichum* spp., Pathogens on Mango Fruits (*Mangifera indica* L. cv. 'Nam Dok Mai')

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ABSTRACT

Mango anthracnose disease is caused by several species of *Colletotrichum* including *C. gloeosporioides*, *C. acutatum*, *C. siamense* and *C. asianumand*. The disease is considered as one of the major constraints of Thai mango export. However, the classification of these pathogens was still unclear. Generally, *Colletotrichum* spp. are classied based on their morphology. The aims of this study were to characterize and confirm the species of *Colletotrichum* isolates collected from mango orchards for exporting located in 6 different provinces of Thailand. The characterization was based on pathogenicity test, morphological and molecular characteristics. Forty four isolates were obtained from the symptomatic fruits. Pathogenicity test of all isolates showed typical anthracnose symptoms on the tested mango fruits and Koch's postulates were fulfilled by re-isolation from the inoculated fruits. The morphological characterization identified all isolates as *C. gloeosporioides* with 6 morphotypes, as their conidia were hyaline, cylindrical with rounded-end and approximately 14.90x4.02 m in size. Setae on infected tissue were also observed is some isolates. Species specific primer analysis could confirm 39 isolates of *Colletotrichum* as *C. gloeosporioides*. Ribosomal DNA (rDNA) gene sequencing and homology analysis of other ve isolates revealed that these isolates were *C. gloeosporioides* except for the WH9 isolate which was identied as *C. acutatum*. By combining the results from molecular analysis with morphological characterization and pathogenicity test, we report that *C. gloeosporioides*is is the main causal pathogen of mango anthracnose in the mango orchards for exporting in Thailand.

Key words: *Colletotrichum gloeosporioide*, *C. acutatum*, anthracnose, mango

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Introduction

Thailand is among the world's five largest net exporters of fresh mango fruit (FAOSTAT, 2012) with the cultivated area of 1,967,904 rai giving the production approximately 1,462 kg/rai in 2017 (Office of Agricultural Economics, 2017). The cultivated areas of exported mango (cv.'Nam Dok Mai') in Thailand are Phitsanulok, Nakhon Ratchasima, Sakeao, PrachinBuri, Chiang Mai and Chchoengsao provinces. One of the major constraints of Thai mango export is anthracnose disease caused by *Colletotrichum* spp. Among them, *C. gloeosporioides* and *C. acutatum* were frequently reported to be the causal agents of the disease (Rivera-Vargas *et al*., 2006; Dhumtanom and Chantrasri, 2011). Traditionally, *Colletotrichum* spp. have been identified on the basis of morphological characteristics such as conidial size and shape, appressorium shape and pathogenicity test (Martnez-Culebras *et al*., 2000); however, these methods are often unreliable for species classication (Rivera-Vargas *et al*., 2006) due to the fact that morphological features expressing on the culture media may differ from those expressing on the host plant. As shown in the study of Smith (1990), *C. fragariae* associated with strawberry in the southeastern United States produced setae when they were observed on the plant tissue but not the media culture. Although wounded and unwounded inoculations of pathogenic *Colletotrichum* spp. led to the disease development, different disease levels were observed as shown in the study of Prihastuti *et al*., (2009). The application of molecular techniques has been advocated to characterize species of several plant pathogenic fungi including *Colletotrichum* spp. Random amplied polymorphic DNA (RAPD) marker had been used to separate *C. gloeosporioides* from *C. fragariae*, a causal agent of strawberry anthracnose (MacKenzie*et al*., 2008), and Internal Transcribed Spacer (ITS) of rRNA gene was widely applied to identify *Colletotrichum* spp. including *C. gloeosporioides*, *C. fragariae* and *C. kahawae* (Adaskaveg *et al*., 1997). In addition, ITS sequenceing analysis was able to separate *C. acutatum*, *C. simmondsii* and *C. fragariae* from the other strawberry anthracnose pathogens (Anderson *et al*., 2012). Although several species such as *C. gloeosporioides*, a main fungus and *C. acutatum*, a minor one have been reported to be associated with mango anthracnose disease in the study of Rivera-Vargas *et al*. (2006), the identification of these fungal pathogens is still ambiguous in Thailand. The present study aimed to characterize and confirm the main species of *Colletotrichum* associated with mango fruit anthracnose obtained from different cultivated areas of Thailand by 1) determining

their pathogenicity on unwounded fruits 2) evaluating their morphological characteristics both on the host and culture media 3) Molecular analyzing using species-specific primers for *C. gloeosporioides* (CgInt/ITS4) and *C. acutatum* (CaInt2/ITS4) and DNA sequencing analysis of rDNA gene.

Materials and Method

1. Sample collection and isolation

Ten mango fruits were obtained from each mango exported orchard in Phitsanulok, Nakhon Ratchasima, Sakeao, Prachinburi, Chiang Mai and Chachoengsao provinces, Thailand during April-July of 2013-2014. Locations were recorded. The samples were incubated at room temperature (25± 5°C) until ripening. Fruits showing anthracnose symptoms were isolated for the causal pathogens by tissue transplanting method. Advanced margins of infected tissues were cut about 0.5x0.5 cm in size and surface disinfected with 1% sodium hypochlorite solution for 3 min, then rinsed 2 times, with sterilized distilled water for transferred onto potato dextrose agar (PDA) plates and incubated for 7 days at 25± 2°C under alternate white and near ultraviolet light 12 h light/dark cycle. Pure culture of each *Colletotrichum* isolate was obtained by single conidia isolation and was kept either on potato carrot agar (PCA) at 15°C or in 15% glycerol solution at -70°C for further studies.

2. Pathogenicity testing

Before being inoculated with the tested isolates, mature green mango fruits were dipped in hot water at 55°C for 5 min followed by dipping in cold water for 5 min and left untill completely dried. The unwounded fruit was articially inoculated with 25 µl of conidial suspension ($3x10^6$ conidia/ml) of each individual *Colletotrichum* isolate and was subsequently covered with sterile Whatman No.1 filter paper disc (0.5x0.5 cm) maintain moisture of the inoculum. Each fruit was inoculated at 3 positions including 2 cm from stem end, in the middle and 2 cm from stylar end on one side of the fruit. For control treatment, each fruit was dropped with 25 µl of sterile distilled water. All inoculated fruits were maintained under high relative humidity by covering with moist plastic bag at 25±5°C for 24 h. The plastic bag was then removed and inoculted fruits were further incubated at 25-28°C for 10 days, totally 4 inoculated fruits per each isolate were determined for disease incidence (%).

3. Morphological characterization

The morphological characteristics were investigated on infected tissue and culture media. Conidial suspension of each *Colletotrichum* isolate was prepared from 7-day-old culture with distilled water at

$3x10^6$ conidia/ml then artificially inoculated by spraying on unwounded mango fruit surface and incubated at 25-28°C for 10 days. Conidial, mass color, acervulus and setae characteristics were observed on fresh and thin section of the diseased lesion which was mounted on the slide with lactoglycerol and investigated under compound microscope at 40x magnification. Conidial size was measured with Axio Vision Rel. 4.9.1 software at 100 conidia per replication. Totally, 4 independent replications were performed. Appressorium and colony characteristics were observed on PDA culture with mycelium newly isolated from the disease symptom area. Appressorium was produced using a slide culture technique (Weir *et al*., 2012). Mycelium plug of each *Colletotrichum* isolate was cultured on PDA culture. Sterile cover slips (0.5x0.5 cm) were plugged into the culture media half way of its height and subsequently incubated at 25±2°C for 2 weeks. Vegetative hyphae were grown on the surface of the cover slip to produce appressoria. The cover slip was removed and placed on a lactoglycerol droplet on the glass slide. Appressorial shape was characterized under compound microscope at 40x magnification. Colony characteristics including growth rate and colony color on the Difco® PDA media were observed. For each isolate, 5 mm diameter plug of mycelium from the colony margin of 5-day-old culture was transferred and placed at the center of a 90 mm Pyrex® petri dish containing 30 ml Difco® PDA media. The cultured plates were incubated at 25±2°C under alternate white and near ultraviolet light 12 h light/dark cycle with five replications for each isolate. After incubation for 7 days, the radial growth of mycelia (mm) was measured daily, and radial growth rate was then calculated. In addition, the colony color of each isolate was recorded.

4. Molecular characterization

4.1 Genomic DNA extractions

Each isolate of *Colletotrichum* spp. was cultured in 75 ml of MB [Malt broth: malt extract (*Merck*)] for 2-3 days at room temperature (25±2°C) on orbital shaker at 120 rpm. Young mycelia were harvested and transferred to 1.5 ml sterile eppendorf tubes. Mycelia were grounded in 200 µl solutionI (0.1 M NaCl, 0.2 M sucrose, 0.01 M EDTA and 0.03 M Tris HCl) containing 10 µl protease K, then added with 200 µl solution II (50 mM Tris HCl, 50 mM EDTA and 2.5% SDS) and incubated at 65°C for 15 min. Subsequently, each tube was added with 128 µl solution III (73.14 g potassium acetate and 28.75 ml acetic acid in total volume at 300 ml of sterile water) and 300 µl of chloroform then mixed gently and kept in ice for 10 min. The tube was centrifuged at 14,000 rpm for 10 min. The

supernatant was transferred to new 1.5 ml sterile eppendorf tube then added 1 volume of isopropanol into the supernatant, mixed gently and centrifuged at 14,000 rpm for 5 min. The supernatant was discarded and the pellet collected. Three hundred microliters of 70% ethanol was added in the tube to wash the DNA pellet and centrifuged at 14,000 rpm for 1 min. The supernatant was discarded and 50 µl of sterile water was added to dissolve DNA in the tube. The DNA sample then was stored at -20^{O}C for further studies.

4.2 Identification of *Colletotrichum* species with CgInt/ITS4, CaIn2/ITS4 and ITS1/ITS4 primers

Identification of *Colletotrichum* isolates was performed by polymerase chain reaction (PCR) with CgInt/ITS4, CaInt2/ITS4 and ITS1/ITS4 primers. The species-specific primer, CgInt/ITS4 was reported for *C. gloeosporioides* identification (Brown *et al*. 1996). The CgInt (GGACTCCCGCCTC-CGGGCGG) for forward primer and ITS4 (TCCTCCGCTTATTGATATGC) for reverse primer were employed for PCR amplication. The negative control was without DNA template, and the MN001 isolate of *C. gloeosporioides* was used as positive control (MN001 isolate was confirmed a species by DNA sequence analysis of the 18S rRNA ITS region and it shared 99% similarity with *C. gloeosporioides*). PCR reaction was performed in total volume of 50 µl containing 1 µl of 100 ng DNA, 5 µl of 10X DreamTaq buffer, 2.5 mM each of dNTP at 4 µl, 20 µM of each primer at 2 µl, 0.2 µl of DreamTaq DNA polymerase (5 U/µl) (Thermo Scientific; Watham, MA, USA) and 37.8 µl of distilled water. Reaction was carried out in DNA Thermal Cycler (MiniCycler^{TM}; Bio-Rad, Hercules, CA, USA) with the following condition: 95^{o}C for 5 min, 30 cycles of 95^{o}C for 30 s, annealing of 62^{o}C for 30 s and 72^{o}C for 30 s, with a final extension step of 72^{o}C for 5 min. The species-specific primer, CaInt2/ITS4 was reported for *C. acutatum* identification (Brown *et al*., 1996). The species-specific primer of *C. acutatum* was amplied using CaInt2 (GCCGCCGGCCCCGTCACGGGGG) for forward primer and ITS4 for reverse primer. The negative control was without DNA template and CaNR14 isolate of *C. acutatum* isolated from chili was used as positive control (CaNR 14 isolate was confirmed a species by DNA sequence analysis of the 18S rRNA ITS region and it shared 99% similarity with *C. acutatum*). Reaction was performed in total volume of 50 µl containing 1 µl of 100 ng DNA, 5 µl of 10X Dream Taq buffer, 2.5 mM each of dNTP at 4 µl, 10 M each of primers at 2 µl, 0.2 µl of Dreams Taq DNA polymerase (5 U/µl) (Thermo Scientic, Watham, MA, USA) and 37.8 µl of distilled water. Reaction was carried out in a DNA Thermal Cycler (MiniCycler^{TM}; Bio-Rad, Hercules, CA, USA)

with the following condition: 95°C for 5 min, 30 cycles of 95°C for 45 s, annealing of 60.5°C for 45 s and 72°C for 45 s with a final extension step of 72°C for 5 min. The internal transcribed spacer (ITS) of rDNA was amplified using ITS1 (GCCGTAGGT-GAACCTGCGG) for forward reaction and ITS4 for reverse reaction (Martnez-Culebras *et al*. 2000). Reaction was performed in total volume of 50 µl containing 1 µl of 100 ng DNA, 5 µl of 10X Dream Taq buffer, 2.5 mM each of dNTP at 4 µl, 20 µM each of primers at 2 µl and 0.2 µl of Dreams Taq DNA polymerase (5 U/µl) (Thermo Scientic; Watham, MA, USA) and 37.8 µl of distilled water. Reaction was carried out in a Biometra T Gradient Thermocycler (Biometra GmbH, Gttingen, Germany) with the following condition: 94°C for 5 min, 35 cycles of 94°C for 1 min, annealing of 60.5°C for 2 min and 72°C for 2 min with a final extension step of 72°C for 10 min. PCR products were stained by novel juice, a non-mutagenic DNA staining reagent (GeneDireX Inc., Taiwan) and were separated on a 1% w/v agarose gel. PCR product sizes were determined using HyperLadder™ 1kb marker (BIOLINE, London, UK) with 1X TBE at 100V for 30 min. PCR products were visualized under UV light and recorded with GelVue UV Transilluminator (Syngene, Cambridge, UK). For the *Colletotrichum* isolates which were unable to identify by species-specific primers, their genomic DNA were subjected to amplify the ITS-fragments with ITS1/ITS4 primer set. The DNA products were subsequently purified and sequenced at Macrogen (Seoul, Korea). DNA alignment was carried out with Vector NTI Suite 9. The ITS-sequences of unknown isolates were compared against the GenBank nucleotide database.

Results and Discussion

1. Sample collection and isolation

Mango anthracnose in Thailand was reported to be caused by *C. gloeosporioides* group. However, identification of these species particularly based on their morphology and disease symptoms cannot clearly seperate them. In this study, we characterized and confirmed the species of *C. gloeosporioides* group of mango anthracnose based on their pathogenicity test, morphological features mainly on the host tissue and molecular characteristics using PCR technique with two species-specific primers and ITS primers. Totally, forty-four isolates of *C. gloeosporioides* group were examined. Details of their origin were described in Table 1.

2. Pathogenicity test

Forty-four isolates of *Colletotrichum* spp. obtained in this study were pathogenic on the mango fruits under unwounded inoculation regime. The symptoms of anthracnose appeared as water soaked,

Table 1 Isolates of *C. gloeosporioides* group from mango (Nam Dok Mai), geographical origins

No.	Isolates	Geographic regions
1	PC1	Bang Khla, Chachoengsao province
2	PC5	Bang Khla, Chachoengsao province
3	PC7	Bang Khla, Chachoengsao province
4	PC12	Bang Khla, Chachoengsao province
5	PC23	Bang Khla, Chachoengsao province
6	PC28	Bang Khla, Chachoengsao province
7	PC40	Bang Khla, Chachoengsao province
8	PC51	Bang Khla, Chachoengsao province
9	Prachin1	Kabin Buri, Prachinburi province
10	Prachin5	Kabin Buri, Prachinburi province
11	Prachin7	Kabin Buri, Prachinburi province
12	Prachin8	Kabin Buri, Prachinburi province
13	Prachin10	Kabin Buri, Prachinburi province
14	Sakeao3	Amphoe Mueang Sa Kaeo, Sa Kaeo Sakeao province
15	Sakeao6	Amphoe Mueang Sa Kaeo, Sa Kaeo Sakeao province
16	pn1.5	Pak Chong, Nakhon Ratchasima Province
17	pn8.3	Pak Chong, Nakhon Ratchasima Province
18	pn8.5	Pak Chong, Nakhon Ratchasima Province
19	pnf15	Bang Khla, Chachoengsao province
20	225	Pak Chong, Nakhon Ratchasima Province
21	227	Pak Chong, Nakhon Ratchasima Province
22	233	Pak Chong, Nakhon Ratchasima Province
23	237	Pak Chong, Nakhon Ratchasima Province
24	240/2	Pak Chong, Nakhon Ratchasima Province
25	240/3	Pak Chong, Nakhon Ratchasima Province
26	npf5	Noen Maprang, Phitsanulok province
27	npf6	Noen Maprang, Phitsanulok province
28	npf8	Noen Maprang, Phitsanulok province
29	npf10	Noen Maprang, Phitsanulok province
30	npf13	Noen Maprang, Phitsanulok province
31	np5	Noen Maprang, Phitsanulok province
32	wp16	Noen Maprang, Phitsanulok province
33	Prao7	Phrao, Chiang Mai province
34	Prao10	Phrao, Chiang Mai province
35	Thaton8	Tha Ton, Amphoe Mae Ai, Chiang Mai province
36	Thaton10	Tha Ton, Amphoe Mae Ai, Chiang Mai province
37	Thaton11	Tha Ton, Amphoe Mae Ai, Chiang Mai province
38	Thaton12	Tha Ton, Amphoe Mae Ai, Chiang Mai province
39	Thaton16	Tha Ton, Amphoe Mae Ai, Chiang Mai province
40	WH1	Wiang Haeng, Chiang Mai province
41	WH5	Wiang Haeng, Chiang Mai province
42	WH9	Wiang Haeng, Chiang Mai province
43	WH14	Wiang Haeng, Chiang Mai province
44	WH25	Wiang Haeng, Chiang Mai province

circular, black lesions with gelatinous orange, yellow-orange and yellow colour conidial masses on the lesion (Figure 1 a, b). Pathogenicity of all tested isolates was confirmed on the mango fruits with unwounded inoculation method. Despite *Colletotrichum* is known as the genus containing serious pathogen species of plants, they may also have an endophytic, epiphytic and saprobic phase in their life cycle (Hyde *et al*., 2009). Furthermore, *Colletotrichum* spp. naturally infects on the fruits directly, but pathogenicity tests in the past were mostly examined using the wounded inoculation method. This approach may cause misinterpretation on their pathogenic capability, since symptoms appearing after inoculation may be caused by the fungal isolate undergoing either saprophytic or weak parasitic phase instead of pathogenic phase (Hyde *et al*., 2009). The unwounded inoculation technique that mimics the natural infection, could ensured that all isolates investigated in this study were the actual pathogens rather than endophytic *Colletotrichum* isolates acting as opportunistic pathogens. As no differences of symptom characteristic could be observed after inoculation with all isolates obtained in the study, it suggested that the symptom characteristics of mango anthracnose are not reliable criteria for *Colletotrichum* species differentiation. Unlike the study of Liu *et al*., (2016), a correlation between symptom types on the pepper fruits in the field and some *Colletotrichum* species such as *C. truncatum* and *C. scovillei* was observed but not in other species. Therefore, based merely on symptom characteristics it is still impractical to differentiate among *Colletotrichum* species in the pepper fields.

3. Morphological characterization

Traditionally morphological characteristics have been widely used for fungal classification. In *Colletotrichum*, conidial and appressorial size and shape, appressorial shape, the presence or absence of setae, colony color, conidial mass color and growth rate have been used for this purpose. Based on the morphological characteristic including conidial and appressorial size and shape, colony color and the presence or absence of setae were described by Sutton (1980). Unlike this study, the usefulness of morphotype grouping based on certain characteristics for *Colletotrichum* identification was noted. In this study the average conidial size of *C. gloeosporioides* was 14.86±0.09 (12.69-17.61) µm in length and 4.03±0.02 (3.60-5.17) min width with the length:width (L:W) ratio of 3.70±0.3 (3.20-4.52). Conidial shape was hyaline, one celled, cylindrical with rounded end.

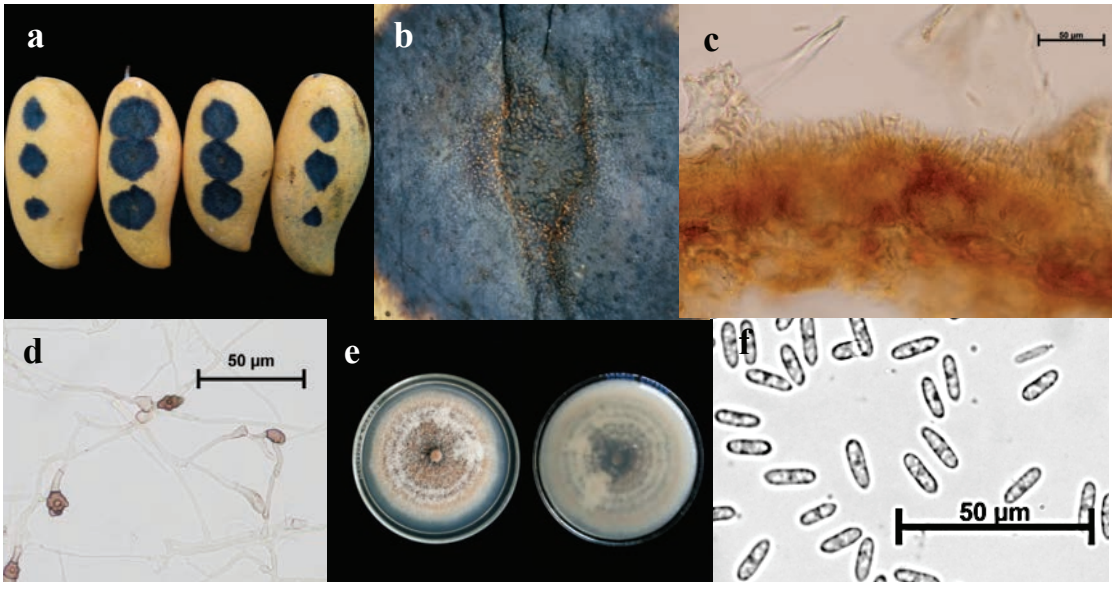

Figure 1 Morphological characteristics and pathogenicity test of *C. gloeosporioides* PC7 isolate. **a)** The symptom on unwounded fruits. **b)** Conidial mass on the diseased lesion. **c)** Acervuli. **d)** Appressoria. **e)** Colony appearances on PDA media. **f)** Conidia. Scale bar 50 µm.

While, the average conidial size of *C. acutatum* was 13.91 (10.83-17.55) µm in length and 3.85 (2.73-5.19) µm in width with the length:width (L:W) ratio of 4.03. Conidial shape was hyaline, one celled, cylindrical with rounded end and irregular shape appressorium. Mycelium growth rate on PDA medium was 3.4 mm/day and white with orange masses. The setae were not produced (Figure 2).

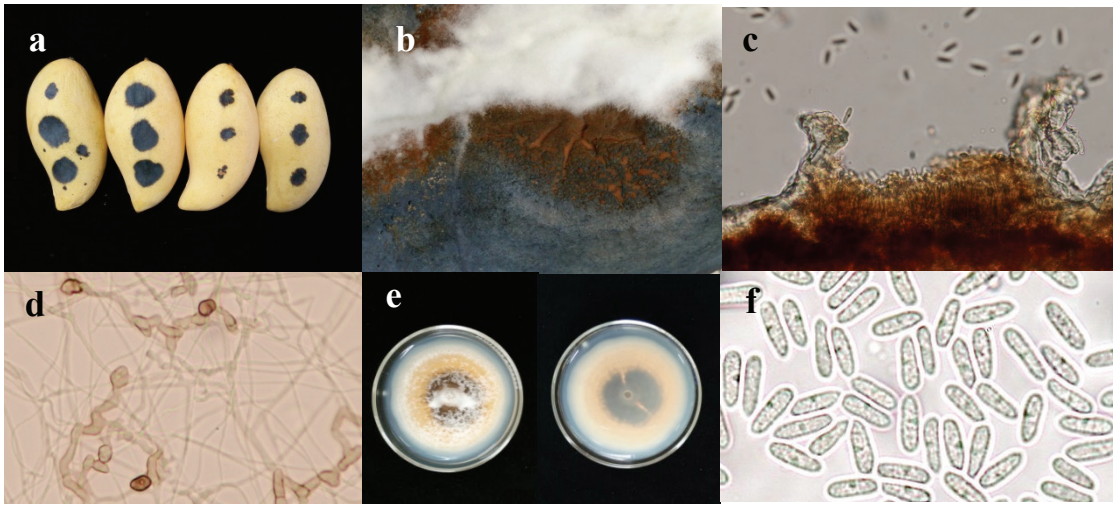

Figure 2 Morphological characteristics and pathogenicity of *C. acutatum* WH9 isolate. **a)** The symptom on unwounded fruits. **b)** Conidial mass on the diseased lesion. **c)** Acervuli. **d)** Appressoria. **e)** Colony appearances on PDA media. **f)** Conidia. Scale bar 50 µm.

Furthermore, appressorial characteristics were pale to dark brown, ovoid, clavate and irregular (Figure 1f). Most isolates were not able to produce setae on the infected tissues except three isolates which were 233, npf8 and Prao7. With the morphological characteristics described above, all isolates obtained in this study were identified as *C. gloeosporioides*, as they were tted into the current classification of *C. gloeosporioides* described by Sutton (1980). Furthermore, based on colony colour and conidial masscolour appearance on the infected tissues, all isolates were divided into six morphotypes (Table 2, Figure 3). The 1st morphotype comprised seventeen isolates having white colour colony with no appearance in their conidial mass. The average of mycelium growth rate of this group was 5.03±0.04(4.10-6.40) mm/day (Figure 3a). The 2nd morphotype consisted of eleven isolates having a gray colony with orange conidial mass and white color at the margin (Figure 3b) with 4.72±0.05 (3.40-5.60) mm/day of the mycelium growth rate. The 3rd morphotype included ten isolates having olive and white color colony with no conidial mass. The average of mycelium growth rate of this group was 4.78±0.12 (3.77-5.83) mm/day (Figure 3c). The 4th morphotype contained three isolates that were ochre in the origin with white at the margin and no conidial mass. The average of mycelium growth rate was 4.70±0.02 (4.50-5.00) mm/day (Figure 3d). The 5th morphotype had only one isolate with gray to white colony and no appearance of conidial mass. The average of mycelium growth rate was 5.90 (3.10-9.60) mm/day (Figure 3e). The 6th morphotype included three isolates having gray to white near to the margin of the colony with orange conidial mass. The average of mycelium growth rate was 4.30±0.03 (3.80-5.20) mm/day (Figure 3f).

Table 2 Summary of the six distinct morphotypes based on colony color of *Colletotrichum* spp. isolated from mango fruits 'Nam Dok Mai'

Mor-photype	characteristics of colony on PDA media	Mycelium growth rate on PDA medium (mm/day)	Conidia characteristics on mango fruit lesion				Disease incidence (%)[a]	Isolate
			Shape	Width (m)	Length (m)	L:W ratio		
1st	White colony color, with no conidial mass	5.03±0.04 (4.10-6.40)	Cylindrical with rounded end	3.99 (3.62-4.58)	14.80 (12.69-17.43)	3.72	90.20 (66.67-100.00)	PC5, PC40, 240/3, npf5, Prachin 1, Prachin 10, pnf 15, pn 1.5, pn 8.3, 237, Prao 7, WH14, npf10,WH1, WH5, npf8, Thaton 16
2nd	White at the margin and gray of the origin of colony, with orange conidial mass	4.72±0.05 (3.40-5.60)	Cylindrical with rounded end	3.92 (3.71-4.28)	14.66 (12.71-16.27)	3.75	94.70 (75.00-100.00)	WH 9,WH 25,Thaton 8, Thaton11, PC 7, PC 12, 225, 233, Prachin 8, np2, np5
3rd	Olive and white colony colour, with no conidial mass	4.75±0.03 (3.80-5.80)	Cylindrical with rounded end	4.02 (3.60-4.24)	15.02 (13.01-16.32)	3.75	94.17 (83.33-100.00)	PC1, Prachin7, PC23, PC28, PC51, sakeao 3, sakeao 6, np9, pn 8.5, Thaton 10
4th	Ochre at the origin and white at the margin of colony, with no conidial mass	4.70±0.02 (4.50-5.00)	Cylindrical with rounded end	4.27 (4.08-4.45)	14.52 (13.62-15.09)	3.40	91.67 (75.00-100.00)	Prachin 5, Thaton 12, Prao 10
5th	Gray to white colony color, with no conidial mass	5.90 (3.10-9.60)	Cylindrical with rounded end	4.12 (2.93-5.48)	14.53 (11.07-19.55)	3.53	100 (100.00-100.00)	wp 16
6th	Gray to white colony color at the margin, with orange conidial mass	4.30±0.03 (3.80-5.20)	Cylindrical with rounded end	4.41 (4.01-5.17)	15.81 (14.33-17.61)	3.60	100 (100.00-100.00)	240/2, npf 6, npf 13

[a] Lesions were measured at 10 days after inoculation. Data were mean ± standard error.

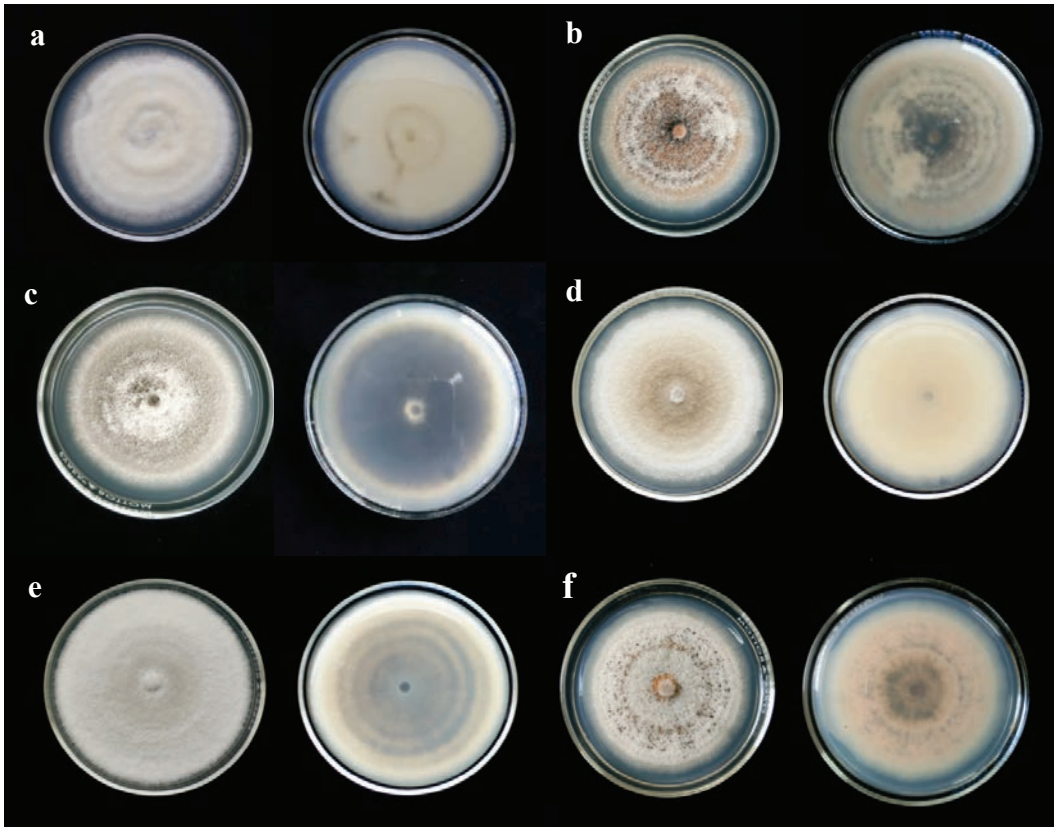

Figure 3 Six distinct morphotypes of *C. gloeosporioides* isolated from mango fruits 'Nam Dok Mai' on PDA media after incubated at 25±2^{O}C for 7 days. **a)** 1st morphotype. **b)** 2nd morphotype. **c)** 3rd morphotype. **d)** 4th morphotype. **e)** 5th morphotype. **f)** 6th morphotype

Than *et al*., (2008) reported a correlation between colony characteristics of *C. gloeosporiodes* from chili (*Capsicum* spp.) anthracnose in Thailand and phylogenetic groups differentiated by using the information on ITS and β-tubulin regions. Likewise, Nguyen *et al*., (2009) and Prihastuti *et al*., (2009) showed the usefulness of different characteristic for *C. gloeosporioides* identification including colony color, conidial shapes, conidial length and width, mycelial growth rate, pathogenicity test and substrate-utilization on ammonium tartrate medium. Previous study reported that *C. gloeosporioides* was not able to produce satae on the culture media (Damm *et al*., 2012), however, contradictory result was observed in this study. We found that some isolates of *C. gloeosporioides* can produce setae in acervuli of infected mango tissue. Therefore, these criteria should be taken into account for identification of *C. gloeosporioides*. Some studies reported the disadvantage of morphological characterization as a mean for *Colletotrichum* identification (Freeman

et al., 1998). This approach is still uncertain due to the fact that morphological features may express differently on the media and the host plant which may lead to the confusion on the identification at species level.

4. Molecular characteristics

Species specific primers for *C. gloeosporioides* (CgInt/ITS4) and for *C. acutatum* (CaInt2/ITS4) were used to confirm the species of fourty-four *Colletotrichum* isolates. Thirty-nine out of 44 isolates were identified as *C. gloeosporioides*, since 450 bp fragments were amplified with the *C. gloeosporioides* specific primers. No amplified PCR product was obtained from seven isolates including PC51, pn1.5, npf13, Prao7, WH1, WH9 and WH25 (Table 3), when the same species-specific primers were used. In the case of PCR amplification with *C. acutatum* specific primers, none of isolates produced the PCR product except the positive control, *C. acutatum* isolate CaNR14 (Table 3). Sequencing and homology analysis of rDNA gene using ITS1/ITS4 primers in the seven unidentified isolates revealed that the PC51, pn1.5, npf13, Prao7, WH1 and WH25 isolates showed 97-99% sequence similarity with *C. gloeosporioides*, whereas the WH9 isolate showed 98% sequence similarity with *C. acutatum*de posited in the Genbank (Table 4). Therefore, the application of molecular techniques has been advocated to characterize species of several plant pathogenic fungi including *Colletotrichum* spp. In our study, species of *C. gloeosporioides* group on mango fruits can be resolved into two species: *C. gloeosporioides* and *C. acutatum*, by combining PCR based identification with species-specific primers and DNA sequencing of ITS region. In fact, we found that there may be some nucleotide variations on Internal Transcribed Spacer (ITS) of rRNA gene, since the failures of PCR amplification with those primers in some isolates did exist. Higher resolution of the genetic relationships of species within a genus can be achieved by using gene multilocus analysis (Martens *et al*., 2008). Lima *et al*., (2013) classified the causal agent of mango anthracnose in northeastern Brazil by nucleotide sequencing of these following locus: glyceraldehyde-3-phosphate dehydrogenase, actin, β-tubulin, calmodulin, glutamine synthetase and rDNA-ITS. Therefore, further analysis on such multilocus of all isolates of *C. gloeosporioides* group obtained from mango in this study should be carried out in the future.

Table 3 Species identification of *Colletotrichum* spp. isolates from mango fruit 'Nam Dok Mai' anthracnose by using species-specific primer CgInt/ITS4 (Cg), species-specific primer CaInt2/ITS4 (Ca) and ITS1/ITS4 primer (ITS)

Isolate	Primer reaction[a]			species	Isolate	Primer reaction[a]			species
	Cg	Ca	ITS			Cg	Ca	ITS	
Negative control of Cg	-	nd	nd		Prachin7	+	-	+	*C. gloeosporioides*
Positive control of Cg	+	nd	nd	*C. gloeosporioides*	Prachin8	+	-	+	*C. gloeosporioides*
Negative control of Ca	nd	-	nd		Prachin10	+	-	+	*C. gloeosporioides*
Positive control of Ca	nd	+	nd	*C. acutatum*	Sakeao3	+	-	+	*C. gloeosporioides*
Negative control of ITS	nd	nd	-		Sakeao6	+	-	+	*C. gloeosporioides*
Positive control of ITS	nd	nd	+	*C. gloeosporioides*	pn1.5	-	-	+	Unidentified
PC1	+	-	+	*C. gloeosporioides*	pn8.3	+	-	+	*C. gloeosporioides*
PC5	+	-	+	*C. gloeosporioides*	pn8.5	+	-	+	*C. gloeosporioides*
PC7	+	-	+	*C. gloeosporioides*	pnf15	+	-	+	*C. gloeosporioides*
PC12	+	-	+	*C. gloeosporioides*	225	+	-	+	*C. gloeosporioides*
PC23	+	-	+	*C. gloeosporioides*	233	+	-	+	*C. gloeosporioides*
PC28	+	-	+	*C. gloeosporioides*	237	+	-	+	*C. gloeosporioides*
PC40	+	-	+	*C. gloeosporioides*	240/2	+	-	+	*C. gloeosporioides*
PC51	-	-	+	Unidentified	240/3	+	-	+	*C. gloeosporioides*
Prachin1	+	-	+	*C. gloeosporioides*	npf5	+	-	+	*C. gloeosporioides*
Prachin5	+	-	+	*C. gloeosporioides*	npf6	+	-	+	*C. gloeosporioides*
npf13	-	-	+	Unidentified	npf8	+	-	+	*C. gloeosporioides*
np5	+	-	+	*C. gloeosporioides*	npf10	+	-	+	*C. gloeosporioides*
np9	+	-	+	*C. gloeosporioides*	np2	+	-	+	*C. gloeosporioides*
wp16	+	-	+	*C. gloeosporioides*	Thaton12	+	-	+	*C. gloeosporioides*
Prao7	-	-	+	Unidentified	Thaton16	+	-	+	*C. gloeosporioides*
Prao10	+	-	+	*C. gloeosporioides*	WH1	-	-	+	Unidentified
Thaton8	+	-	+	*C. gloeosporioides*	WH5	+	-	+	*C. gloeosporioides*
Thaton10	+	-	+	*C. gloeosporioides*	WH9	-	-	+	Unidentified
Thaton11	+	-	+	*C. gloeosporioides*	WH14	+	-	+	*C. gloeosporioides*
					WH25	-	-	+	Unidentified

All PCR amplifications were repeated at least twice[a] The genomics DNA were amplified with CgInt/ITS4 (Cg), CaInt2/ITS4 (Ca) and ITS1/ITS4 (ITS) primers; + = PCR product present, - = PCR product absent. nd = not determined. The negative control was without DNA template. MN001 was used as positive control for *C. gloeosporioides* whereas CaNR14 was used as positive control for of *C. acutatum*.

Table 4 BLAST result of ITS sequence identity between seven isolates of *Colletotrichum* spp. and some GenBank sequences

Isolates	Geographic location in Thailand	% identity	GenBank accession no.
PC51	Bang Khla, Chachoengsao province	97%	KM111484.1 *C. gloeosporioides*
pn1.5	Pak Chong, Nakhon Ratchasima province	99%	KF177685.1 *C. gloeosporioides*
npf13	Noen Maprang, Phitsanulok province	98%	KC816034.1 *C. gloeosporioides*
Prao7	Phrao, Chiang Mai province	99%	KT582187.1 *C. gloeosporioides*
WH1	Wiang Haeng, Chiang Mai province	99%	KF053199.1 *C. gloeosporioides*
WH9	Wiang Haeng, Chiang Mai province	98%	AF272782.1 *C. acutatum*
WH25	Wiang Haeng, Chiang Mai province	97%	AJ311883.1 *C. gloeosporioides*

Conclusions

This study provides the evidence to confirm the identity of causal agents of anthracnose disease on mango fruits in Thailand. By reclassification of *C. gloeosporioides* group with pathogenicity test, morphological and molecular characterizations, *C. gloeosporioides* is reported to be the main cause of fruit anthracnose while *C. acutatum is minor cause*. A number of techniques facilitating reclassification of *C. gloeosporioides* group were reaffirmed here in this study and will be useful for plant pathologists to confirm these species identity. For examples, wounded-inoculation method on mango fruit may not be required for pathogenicity test since high disease incidences can still be obtained with unwounded inoculation. We also suggested that only some morphological characteristics, i.e., conidial and appressorial shape and mycelium growth rate could be adopted for differentiating between *C. gloeosporioides* and *C. acutatum*. Furthermore, molecular technique by using PCR amplification with species-specific primers, i.e., CgInt/ITS4 for *C. gloeosporioides* and CaInt2/ITS4 for *C. acutatum* and DNA sequencing of Internal Transcribed Spacer (ITS) of rRNA gene are useful for identifying the species of *C. gloeosporioides* group. As two species of *Colletotrichum*; *C. gloeosporioides* and *C. acutatum*, are reported here, we cannot rule out other species within *C. gloeosporioides* group. Therefore, more isolates of the anthracnose pathogen from high number of sample fruits and various sampling locations are needed for further investigation. Furthermore, molecular characterization by analyzing sequencing data from other locus rather than ITS of rRNA gene is suggested for in-depth species confirmation.

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