RESEARCH ARTICLE Indian Journal of Agricultural Research, Volume 56 Issue 1: 81-90 (February 2022)

Diversity of *Colletotrichum* Species causing Anthracnose Disease from Mango cv. Nam Dork Mai See Tong based on ISSR-PCR

S. Bincader[1,2], R. Pongpisutta[1,2], C. Rattanakreetakul[1,2]

10.18805/IJARe.AF-691

ABSTRACT

Background: Anthracnose disease caused by the genus *Colletotrichum* is one of the crucial problems occurring in the field, along with postharvest diseases and affects mango quality in Thailand. In particular, the Nam Dork Mai See Tong cultivar, which is highly susceptible to the disease, is an important product for exportation.

Methods: In this research, thirty-seven *Colletotrichum* species isolate were obtained from anthracnose disease in mango cv. Nam Dork Mai See Tong in three provinces in Thailand. Morphological studies and molecular techniques using species-specific primers were investigated; moreover, the diversity of pathogens was analyzed using PCR amplification of inter simple sequence repeats (ISSRs) with 6 primers, including pathogenicity tests.

Result: Morphological studies and molecular detection with species-specific primers revealed that 32 isolates belonged to the *C. gloeosporioides* species complex and 5 isolates to the *C. acutatum* species complex. The genetic diversity of pathogens was analyzed. PCR amplification using 6 ISSR primers produced 35 polymorphic bands. These bands were used to construct UPGMA, in which cluster analysis divided the 37 isolates into 3 main groups and 8 subgroups at 61-73% Jaccard similarity coefficient with cophenetic correlation (*r*) = 0.6781. The ISSR technique showed the greatest genetic variation among isolates collected from different locations. Hence, a study based on ISSR markers was profitable to investigate the phylogenetic relationship of the genus *Colletotrichum*. Pathogenicity tests revealed that PC006 (*Ca*) and CS005 (*Cg*) showed the highest aggressiveness, with disease incidences of 84.74 and 80.90%, respectively. This study indicates that the diversity of pathogenic *Colletotrichum* species related to mango plantations in Thailand is increasing.

Key words: Anthracnose, *Colletotrichum*, Diversity, ISSR-PCR, *Mangifera indica* L.

INTRODUCTION

Colletotrichum is the most important genus of pathogenic plant fungi worldwide, especially in subtropical and tropical regions (Bailey and Jeger, 1992). This disease affects various parts of the mango tree. The symptom of this disease is commonly appears as a depressed black lesion that is subcircular, in which a spore mass develops, there are necrotic lesions on the young leaves and panicles and damage occurs in the mango fruit (Walker *et al*., 1991; Cannon *et al*., 2012; Lima *et al*., 2015). In particular, conidia of the fungus can germinate on most plant surfaces and produce infectious appressoria (Deising *et al*., 2008).

Cannon *et al*. (2012) studied the current status of genus *Colletotrichum* species based on morphology and DNA sequence analyses and the research indicated that it was difficult to visualize the interspecific genetic distance between the recognized species. Apart from this, morphological descriptions are mostly from agar cultures, conidia size and shape, including appressorium formation, using a slide culture technique. Schena *et al*. (2013) reported that the characteristics of *Colletotrichum* species can change under different growth conditions and can be lost with repeated subculturing.

In many instances, genetic recombination is constrained, such as mycelia or vegetative incompatibility in asexual pathogen populations producing clonal population structures. Molecular analyses, such as RAPD (random amplification of polymorphic DNA), RFLP (restriction fragment length polymorphism) and ISSR (inter simple sequence repeat), have demonstrated occasional transfer of specific chromosomes between apparently genetically distinct isolated clonal lines and the production of new

[1]Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Nakhon Pathom 73140, Thailand.
[2]Postharvest Technology Innovation Center, Ministry of High Education, Science, Research and Innovation, Bangkok 10400, Thailand.

Corresponding Author: R. Pongpisutta, Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Nakhon Pathom 73140, Thailand.
Email: agrryp@ku.ac.th

How to cite this article: Bincader, S., Pongpisutta, R. and Rattanakreetakul, C. (2022). Diversity of *Colletotrichum* Species causing Anthracnose Disease from Mango cv. Nam Dork Mai See Tong based on ISSR-PCR. Indian Journal of Agricultural Research. 56(1): 81-90. DOI: 10.18805/IJARe.AF-691.

Submitted: 08-09-2021 **Accepted:** 27-10-2021 **Online:** 27-11-2021

pathotypes on different host plants (Masel *et al*., 1996). ISSR markers are a simple rapid technique using a single primer for detection and random amplification, such as RAPD. Primers are designed to amplify the regions between microsatellite loci even using a single primer and thus generate highly polymorphic patterns between different individuals (Mort *et al*., 2003; Hettwer and Gerowitt, 2004). The objectives of this study were to reveal the relationship of *Colletotrichum* species causing mango anthracnose disease in the Nam Dork Mai See Tong cultivar using ISSR-PCR.

MATERIALS AND METHODS

Fungal isolation

The fungal pathogen was isolated from mango anthracnose symptoms using a tissue transplanting technique. A small piece (5×5 mm in size) of infected tissue was cut from the edge of the anthracnose lesion. The surface disinfected with 1.2% sodium hypochloride for 3 min and was then rinsed 3 times using sterile distilled water. The tissue was wiped and allowed to air dry, placed on the surface of potato dextrose agar (PDA) and later incubated at 25°C under a 12 hr light/12 hr dark photoperiod for 5 days to induce mycelial development. The fungus was purified using a single spore isolation technique on water agar (WA) and mycelia were transferred onto potato carrot agar (PCA) for further study.

Morphological identification

Morphological characteristics were described by Sutton (1980). Each petri dish containing 15 mL of PDA was inoculated with one mycelial disc 5 mm in diameter from the margins of the 5-day-old colonies growing on PDA and then incubated at 25°C under a 12 hr light/12 hr dark photoperiod. Colony type and spore mass color were evaluated at day 5 and the length and width of thirty conidia were measured. An appressorium study was induced by a modified slide culture technique (Johnston and Jones, 1997). Each isolate with a 5-day-old culture on PDA was transferred onto a 25.4×76.2 mm sterile microscope slide, covered with a 22×22 mm cover slide and incubated at 25°C for 4 days in a petri dish as a moisture chamber. The length and width of 30 appressoria per isolate were measured under a compound microscope with Olympus CellSens Standard software version 1.6.

DNA extraction

The method of total genomic DNA extraction was performed according to the modified protocol of Pongpisutta *et al*. (2013). *Colletotrichum* species were grown in spezieller nährstoffarmer broth (SNB) at room temperature for 48-72 hr. Mycelia were harvested by filter paper (Whatman No. 1) and crushed in liquid nitrogen. Then, 50 mg of mycelium powder was suspended in a 1.5-mL microcentrifuge tube containing 500 µl extraction buffer and 2.8 µL proteinase K. The mixture was incubated at 65°C for 1 hr then 250 µL phenol chloroform isoamyl alcohol (25:24:1) was added and mixed with vortex and centrifuge for 10 min at 13,000 rpm. A 400 µL aliquot of the supernatant was transferred to a new sterile microcentrifuge tube. Sedimentation of genomic DNA was performed using 800 µL chilled absolute alcohol incubated at -20°C for 1 hr and then centrifugation at 13,000 rpm for 10 min was used to sediment pellets of the genomic DNA. The resulting pellet was washed with 70% ethanol, centrifuged at 13,000 rpm for 10 min (2 times), allowed to dry at room temperature and then dissolved in 70 µL of 1X TE buffer. Agarose gel electrophoresis was used at a concentration of 1.2% to evaluate DNA quantity and quality by comparison with a 100 bp GeneRuler DNA Ladder.

PCR amplification using species-specific primers

DNA templates were amplified by PCR using specific primers *Ca*Int2 (5′GGGGAAGCCTCTCGCGG′3) (Sreenivasaprasad *et al*., 1996)/ITS4 (5′TCCTCCGCTTATTGATATGC′3) (White *et al*., 1990) and *Cg*Int (5′GG CCTCCCGCCTCCGGGCGG′3) (Mills *et al*., 1992)/ITS4 (5′TCCTCCGCTTATTGATATGC′3) (White *et al*., 1990). PCR was performed in a 25 µl total volume containing 20 ng of DNA template, 1X Dream *Taq* buffer $MgCl_2$, 2 mM dNTPs, 400 nm of each primer and 1 U of Dream *Taq* DNA polymerase (Fermentas Co., Ltd.). PCR conditions were carried out in a programmable thermal cycler as follows: pre-denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 1 min and a final extension step of 72°C for 10 min. PCR products were verified by 1.2% agarose gel electrophoresis in 1X TBE buffer with a 100-volt electronic current. The DNA bands were visualized under UV light using gel documentation (Syngene Co., Ltd.).

Inter simple sequence repeat-PCR (ISSR-PCR)

ISSR-PCR was performed in a 25 µl PCR containing 25 ng/µl DNA template, 10X *Taq* buffer, 2 mM $MgCl_2$, 1 mM dNTPs, 10 µM single primer and 5 U/µl *Taq* polymerase. PCR was carried out using TProfessional Standard Gradient (Biometra Co., Ltd.) under the following conditions: 94°C for 5 min, then 40 cycles of 94°C for 20 sec, annealing of each primer (described in Table 1) for 45 sec, 72°C for 1 min and a final extension at 72°C for 3 min. PCR products were verified by 1.4% agarose gel electrophoresis in 1X TBE buffer with a 80-volt electronic current. The polymorphic bands of the products were visualized under UV light and photographed using gel documentation. Polymorphic bands of each primer were recorded by using binary data with 1= present

Table 1: Primer used in this study, with sequences.

Primer	Sequence	Annealing temp. (°C)
AG_8C	AG AG AG AG AG AG AG AG C	53.0
CAG_5	CAG CAG CAG CAG CAG	58.0
$GACA_4$	GACA GACA GACA GACA	54.0
GTG_5	GTG GTG GTG GTG GTG	56.0
GCA_5	GCA GCA GCA GCA GCA	56.0
TCC_5	TCC TCC TCC TCC TCC	56.0

and 0= absent. The data were analyzed with NTSYSpc version 2.20e (Rohlf, 1988). Cluster analysis was performed using the Jaccard similarity coefficient and grouping by the unweighted pair-group method with averages (UPGMA). A dendrogram was generated with the cophenetic correlation (r) (Rohlf and Sokal, 1981). Bootstrap values were calculated with 1,000 replicates using Winboot (Yap and Nelson, 1996).

Pathogenicity test

Representative isolates of the *Colletotrichum* species complex were selected for pathogenicity testing on immature mango fruits (cv. Nam Dork Mai See Tong) under laboratory conditions. All isolates were cultured on PDA and incubated at 25°C under a 12 hr light/12 hr dark photoperiod for 5 days. The colony margin was then cut with a cork borer and 5-mm mycelial discs were transferred onto mango fruits that were previously washed with 1.2% sodium hypochlorite for 1 min and rinsed twice with sterile distilled water. Inoculated fruits were used with the unwounded technique, kept in a moisture box and incubated at room temperature under a 12 hr light/12 hr dark photoperiod for 5 days. Symptom development was examined. The experimental design was analyzed by R-stat X64 3.4.0 software using a completely randomized design (CRD). Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were used to detect statistical significance and differences were considered significant when $P<0.05$. Five replications were used in each isolate for this study.

RESULTS AND DISCUSSION

Fungal isolation

A total of 37 isolates were recovered from diseased samples showing typical anthracnose symptoms on the inflorescences, leaves and fruits both pre- and postharvest. All parts of the inflorescences are susceptible to *Colletotrichum* species with dark-brown spots and elongated dark lesions that enlarge and coalesce and then, the infected flower parts fall off the inflorescences. The infection of the leaves starts as small brown to black spots, often with a distinct yellow halo, that enlarge to irregular lesions with brown-gray to black necrotic spots on the leaf surfaces. While infection of mature fruits appears as small dark circular spots becoming sunken lesions, prior to ripening, the lesions are black and expand rapidly in size, producing acervuli with different colors of conidial masses under high relative humidity (Fig 1a-1c). All *Colletotrichum* isolates were isolated from mangos cv. Nam Dork Mai See Tong, which were collected from 3 provinces in Thailand: Chachoengsao, Ratchaburi and Phichit, containing 10, 15 and 12 fungal pathogens, respectively (Table 2).

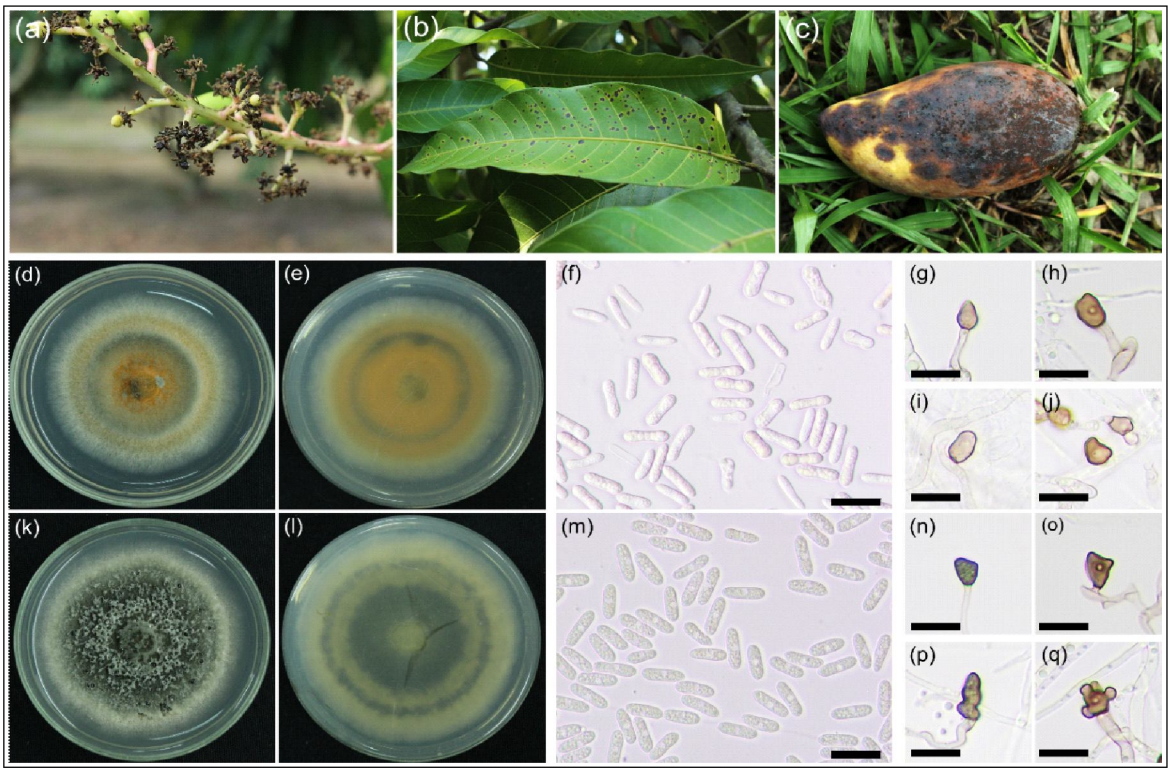

Fig 1: Anthracnose symptoms from mango plantations in 3 provinces of Thailand (a-c) and the morphological characteristics of *the* colonies, conidia and appressoria of the mango. Upper (d) and reverse (e) sides of *Colletotrichum acutatum* species complex incubated on PDA at 25°C for 5 days, conidia of *C. acutatum* species complex (f) and appressoria of *C. acutatum* species complex (g-j). Upper (k) and reverse (l) sides of the *C. gloeosporioides* species complex incubated on PDA at 25°C for 5 days, conidia of the *C. gloeosporioides* species complex (m) and appressoria of the *C. gloeosporioides* species complex (n-q). Scale bar = 20 µm.

Generally, anthracnose disease can invade preharvest in leaves, shoots, inflorescences and fruits, including postharvest (latent infection) fruits. The postharvest phase is the most damaging and economically significant phase of the disease worldwide. This disease stage directly affects the marketability of the fruit, rendering it worthless. Akem (2006) found that anthracnose is recognized as the most important disease in mangos, especially where high humidity prevails during the cropping season and directly affects marketability of the fruit. Apart from this, the study by Ploetz *et al*. (1994) explained that mango anthracnose disease is found in all mango product areas of the world and is most important where high rainfall and humidity occur. Although some mango cultivars possess moderate resistance to anthracnose, but cv. Nam Dork Mai See Tong is different according to the report by Sangchote (1987) and the Department of Agriculture and Fisheries, Queensland (2014), which described the mango cv. Nam Dork Mai group to be more susceptible to fungal diseases, such as *Botryosphaeria* spp., *Lasiodiplodia theobromae* and

Table 2: Details of isolates, including geographical information, from anthracnose legions on mango cv. Nam Dork Mai See Thong from 3 provinces in central Thailand.

Location of origin	Geographic coordinates[1]	Isolate	Disease infected level		Specific primers		ISSR
			% Disease	Level[2]	*Ca*Int	*Cg*Int	subgroup
Plaeng Yao 1, Chachoengsao	13°36′28.6″N 101°17′53.5″E	CS001	55.85	+++	-	✓	1
		CS002	41.72	++	-	✓	2
		CS003	46.06	++	-	✓	3
Plaeng Yao 2, Chachoengsao	13°36′42.2″N 101°17′41.6″E	CS004	42.05	++	-	✓	3
		CS005	80.90	+++	-	✓	4
		CS006	62.71	+++	-	✓	3
		CS007	53.00	+++	-	✓	3
Plaeng Yao 3, Chachoengsao	13°36′50.2″N 101°17′24.8″E	CS008	45.05	++	-	✓	1
		CS009	8.84	+	-	✓	1
		CS010	58.20	+++	-	✓	4
Mueang Ratchaburi, Ratchaburi	13°35′50.0″N 99°49′57.1″E	RB001	57.82	+++	-	✓	4
		RB002	73.56	+++	-	✓	5
Paktho, Ratchaburi	13°24′47.5″N 99°45′32.9″E	RB003	8.72	+	-	✓	7
		RB004	6.13	+	-	✓	1
		RB005	48.98	++	-	✓	4
		RB006	8.54	+	-	✓	1
		RB007	9.34	+	-	✓	4
		RB008	8.13	+	-	✓	8
		RB009	66.83	+++	-	✓	8
		RB010	68.15	+++	-	✓	4
		RB011	47.92	++	-	✓	4
		RB012	9.62	+	-	✓	4
Bang Phae, Ratchaburi	13°39′50.5″N 99°57′54.4″E	RB013	6.58	+	-	✓	4
		RB014	8.26	+	✓	-	6
		RB015	44.24	++	-	✓	4
Sak Lek 1, Phichit	16°28′20.9″N 100°33′42.4″E	PC001	56.44	+++	-	✓	4
		PC002	7.95	+	-	✓	4
		PC003	41.53	++	-	✓	4
		PC004	9.64	+	-	✓	4
		PC005	6.54	+	-	✓	4
		PC006	84.74	+++	✓	-	6
		PC007	56.80	+++	✓	-	6
Sak Lek 2, Phichit	16°28′41.7″N 100°33′52.4″E	PC008	34.82	++	-	✓	3
		PC009	9.86	+	-	✓	3
		PC010	5.53	+	-	✓	3
		PC011	8.42	+	✓	-	6
		PC012	8.82	+	✓	-	6

[1]Geographic location was detected by GPS status version 8.0.170.

[2]Diseased levels were assessed following Corkidi *et al*. (2006).

Colletotrichum. The major mango cultivars in Thailand are Nam Dork Mai No.4 and Nam Dork Mai See Tong, which is spread throughout the country. Hence, anthracnose disease is a very common disease in mango production. The distribution of mango anthracnose in Thailand is in several important mango product areas, such as Phitsanulok, Chiang Mai, Prachuap Khiri Khan, Suphan Buri, Phetchabun, Chachoengsao, Phichit and Ratchaburi (Thailand's Department of Agricultural Extension, 2018). In the field, anthracnose can cause a direct loss of fruit quality, leading to a loss of the mango fruits. Therefore, field management is crucial to control mango anthracnose to reduce severe decline in fruit quality and to assure customer satisfaction.

Fungal identification

Based on morphological and colony characteristics, a total of 37 isolates were divided into 2 groups, of which 5 isolates were identified as *C. acutatum* with white to gray mycelia covered with orange to bright orange conidial masses (Fig 1d-1e); the conidia were hyaline, single celled and cylindrical to clavate constricted at the middle being rounded at one end and tapered at the other (3.29-6.57×9.91-22.79 µm) (Fig 1f), setae absent; the appressoria were mostly single, medium brown, smoot-walled, ellipsoidal to obovate sometimes undulate (2.30-7.92×4.42-12.69 µm) (Fig 1g-1j). Thirty-two isolates were identified as *C. gloeosporioides* with white, gray to green olive cottony aerial mycelia and pale to orange salmon conidial masses (Fig 1k-1l); conidia hyaline, 1 cell, straight to cylindrical with round shapes at both ends (3.59-6.91×11.02-19.52 µm) (Fig 1m) and the appressoria varied in shape, deeply lobed to irregular shapes (4.37-7.31×6.49-11.59 µm) (Fig 1n-1q).

Additionally, PCR assays confirmed the morphological identification of *Colletotrichum* in both species. Species-specific primer sets using CaInt2/ITS4 and CgInt/ITS4 were designed to prosperously distinguish between *C. acutatum* and *C. gloeosporioides*. Of the 37 isolates evaluated, 32 were positive for *C. gloeosporioides* when the CgInt/ITS4 primers were applied, while only 5 (isolates RB014 from Ratchaburi and PC006, PC007, PC011 and PC012 from Phichit) were positive for *C. acutatum* when CaInt2/ITS4 was used. PCR products of approximately 500 bp in both species were obtained and visualized in an agarose electrophoresis gel (Table 2). Furthermore, molecular identification was similar to that based on morphological characteristics.

Many researchers have reported that anthracnose caused by *Colletotrichum* is one of the most severe diseases in mangos. In particular, *C. gloeosporioides* has been reported to be one of the dominant pathogens infecting mangos in Thailand. Later, it was revealed that other species of *Colletotrichum,* such as *C. acutatum*, *C. asianum* and *C. siamense,* also attacked mangos (Đinh *et al*., 2003; Than *et al*., 2008; Prihastuti *et al*., 2009; Rungjindamai, 2016; Pongpisutta *et al*. 2019).

Inter simple sequence repeat-PCR (ISSR-PCR)

Six ISSR primers were used to reveal the genetic diversity of *Colletotrichum* species causing mango anthracnose and all primers produced interpretable and variable banding patterns (Table 3). These primers amplified 46 total DNA bands and 35 polymorphic bands from 37 isolates with sizes ranging from 200 to 2,500 bp (Fig 2). The number of total bands scored per primer ranged from 5 ($GACA_4$) to 11 (GTG_5), with an average of 8 bands (Table 3). The most informative primers were CAG_5, GCA_5 and GTG_5 generating 100% polymorphic loci (PL), whereas the least informative primer was $GACA_4$ producing 72.97% polymorphic loci (Table 3).

Certainly, the number of polymorphic loci and percentage of polymorphic loci (PPL) are important factors that affect the level of genetic variation in a population. When using ISSR markers, values for the observed number of alleles (Na), effective number of alleles (Ne) and genetic diversity (h) within the 37 isolates are important indicators that influence the level of genetic variation in the population found. Dominant primers were able to detect the highest level of variability, which was demonstrated by the highest values, such as CAG_5 (Na=2.0000, Ne=1.8418, h=0.4530) and GTG_5 (Na=2.0000, Ne=1.7391, h=0.4137), compared to other markers. The values were calculated by using GenAlEx software version 6.5 (Table 3).

Based on the ISSR-PCR products, the dendrogram clustered all the isolates (from 3 geographic populations)

Table 3: Frequency of bands and alleles and estimated population diversity for binary data using GenAlEx software version 6.5.

Pop	n	Na[1]	Ne[2]	I[3]	h[4]	uh[5]	Polymorphic loci
AG_8C	6	1.8649	1.5846	0.4998	0.3393	0.4072	86.4900%
CAG_5	10	2.0000	1.8418	0.6443	0.4530	0.5033	100.0000%
$GACA_4$	5	1.4865	1.4657	0.4118	0.2768	0.3459	72.9700%
GCA_5	8	2.0000	1.3915	0.4335	0.2677	0.3060	100.0000%
GTG_5	11	2.0000	1.7391	0.6003	0.4137	0.4550	100.0000%
TCC_5	6	1.9459	1.6757	0.5662	0.3859	0.4631	97.3000%
Mean	8	1.8829	1.6164	0.5260	0.3561	0.4134	92.7933%

[1]Na = No. of different alleles.

[2]Ne = No. of effective alleles = 1/(p^2 + q^2).

[3]I = Shannon's information index = -1* [p * Ln (p) + q * Ln (q)].

[4]h = Diversity = 1 - (p^2 + q^2).

[5]uh = Unbiased diversity = [N/(N-1)] * h.

into 3 main groups and 8 subgroups at similarity coefficients ranging from 61 to 73% with a 100% bootstrap value and a cophenetic value of (*r*) = 0.6781. The *C. gloeosporioides* species complex represented 7 subgroups showing higher variation than the *C. acutatum* species complex, which found only 1 subgroup (Fig 3). Analysis of diversity is an important platform to understand the development and evolution of pathogen populations, in particular the genus *Colletotrichum*, which can invade various plants. ISSR-PCR is one of the techniques for detection (McDonald *et al*., 1997) because of the multilocus fingerprinting profiles obtained. ISSR analysis can be applied in several investigations concerning genetic identity, parentage, clone and strain identification and pathogen taxonomy of closely related species. In addition, ISSRs are considered useful in gene mapping studies (Zietkiewicz *et al*., 1994; Godwin *et al*., 1997; Katkar *et al*., 2015; Abirami *et al*., 2018; Prasannan and Jose, 2021) including application markers for selection of plant disease resistance variety (Babayeva *et al*. 2018). However, the isolates of each subgroup blended from different geographic

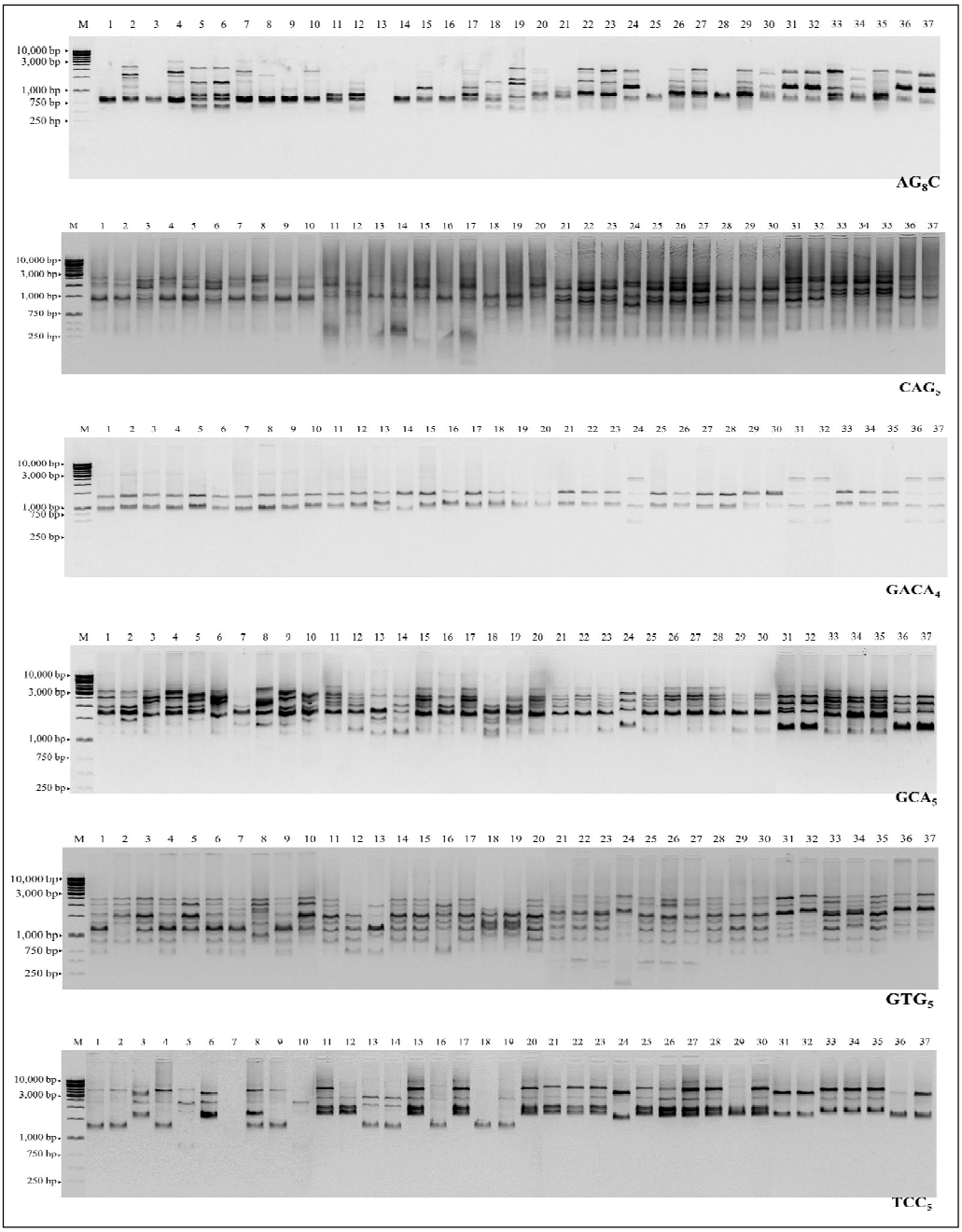

Fig 2: DNA fingerprinting on 1.4% agarose gel electrophoresis obtained by ISSR primers AG_8C, CAG_5, $GACA_4$, GCA_5, GTG_5 and TCC_5. Lane M: 1 kb DNA ladder, Lanes 1-10: Isolates from Chachoengsao Province, Lanes 11-25: Isolates from Ratchaburi Province and Lanes 26-37: Isolates from Phichit Province.

origins were observed. There was no correlation between genetic diversity and geographical origins, in contrast to the report by Palacıoğlu *et al*. (2020), which found that the isolates of *C. lindemuthianum* causing Turkish bean anthracnose belonging to the same geographic origin were observed to be more closely related to one another.

The statistical results showed the percentage of variation within the population of these genera with a high level of genetic differentiation. Additionally, in Thailand, the genus *Colletotrichum* has been reported to have a high level of population differentiation and genetic diversity, such as *C. capsici* or *C. gloeosporioides* causing chilli anthracnose (Ratanacherdchai *et al*., 2010; Rodkate, 2012). There may be multiple clarifications for this event; for example, dispersal, wind currents and other abiotic factors (*e.g*., geographical isolation) play roles in the dissemination of spores and infected fruits/plant parts and agricultural systems play an important role, there by resulting in gene exchange within populations (Freeman *et al*., 2013). The difference within the populations indicated that genetic recombination in pathogen reproduction may play an important role in the population evolution of *C. gloeosporioides* in Thailand. In particular, conidial anastomosis between different strains may contribute to a high level of genetic diversity within the population. Additionally, the levels of genetic diversity observed between the isolates within the same population may be attributed to sexual recombination and the parasexual cycle, which play a role in the life cycle of the pathogen (Padder *et al*., 2017).

Therefore, genetic diversity studies using ISSR markers are reliable and effective tools. The results showed that the *C. gloeosporioides* species complex occupied a high level of genetic polymorphism and clustered into 2 main groups and 7 subgroups.

Pathogenicity test

Pathogenicity was tested using Koch's postulates for all *C. gloeosporioides* and *C. acutatum* isolates. These isolates all caused sunken spots and dark brown to brown lesions at Day 5 after the inoculation period. Ten *C. gloeosporioides* isolates (CS001, CS005, CS006, CS007, CS010, RB001, RB002, RB009, RB010 and PC001) and 2 *C. acutatum* isolates (PC006 and PC007) caused the most severe symptoms, with level 3 disease incidence in particular. PC006 (*Ca*) and CS005 (*Cg*) showed the highest aggressiveness with disease incidences of 84.74 and 80.90%, respectively. However, there were few visible symptoms, with level 1 disease incidence from 13 *C. gloeosporioides* isolates (CS009, RB003, RB004, RB006, RB007, RB008, RB012, RB013, PC002, PC004, PC005, PC009 and PC010) and 3 *C. acutatum* isolates (RB014, PC011 and PC012). The isolates representing the lowest disease incidence, PC011 (*Ca*) and RB004 (*Cg*), had disease incidences of 8.42 and 6.13%, respectively (Table 2; Fig 4).

The predominance of *C. gloeosporioides* (31.25%) and *C. acutatum* (40%) in the mango anthracnose pathogen population from 3 provinces in Thailand can be attributed to their high levels of pathogenicity and aggressiveness.

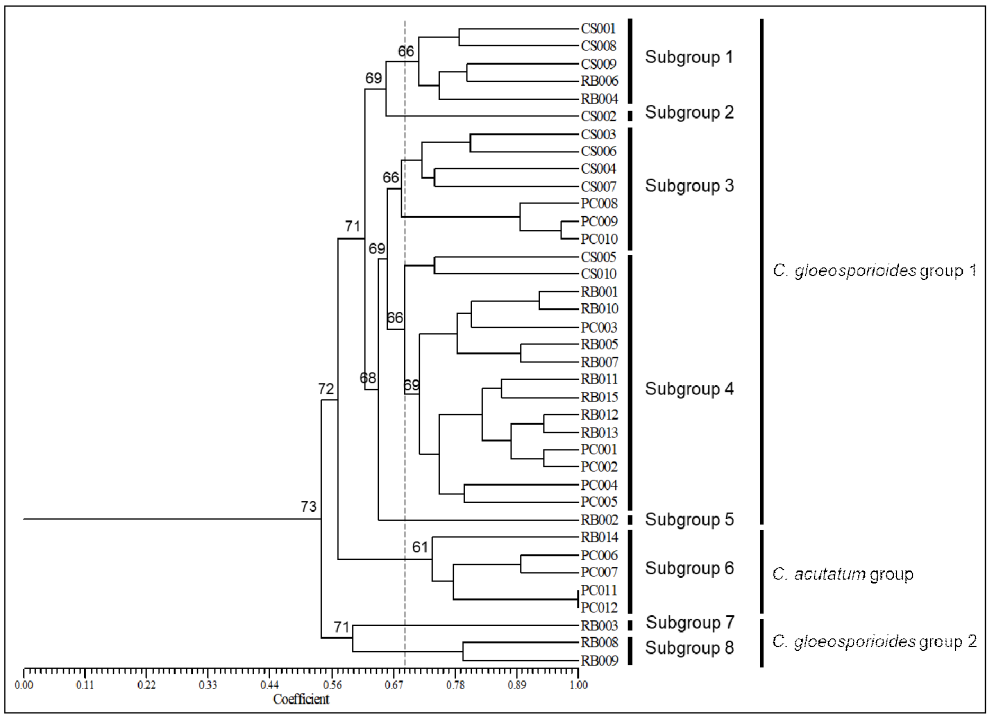

Fig 3: UPGMA dendrogram constructed using NTSYS pc (version 2.02) for 37 isolates from *Colletotrichum* species (3 provinces) using ISSR data and Jaccard's coefficient (r = 0.6781).

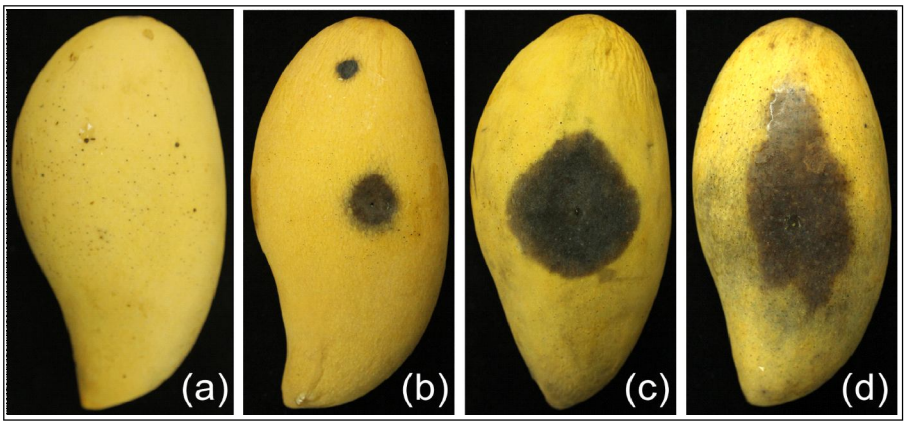

Fig 4: Disease severity levels on mango fruits cv. Nam Dork Mai See Tong after 5 days of inoculation with mycelium plugs of *Colletotrichum* species using the unwounded technique: (a) 0-1% disease incidence (level = non diseased), (b) 1-10% disease incidence (level + = slightly diseased), (c) 11-49% disease incidence (level ++ = moderately diseased) and (d) 50-100% disease incidence (level +++ = very severely diseased). Disease levels were assessed following Corkidi *et al*. (2006).

Although, *C. acutatum* was found in only 5 isolates in mango plantations in Ratchaburi and Phichit provinces, 40% of the population exhibited aggressiveness higher than the population of *C. gloeosporioides*. Temperature is among the key environmental factors affecting pathogen survival. Culture of *Colletotrichum* on PDA in both species was performed at 25-30°C under a 12 hr light/12 photoperiod hr dark for 5 days. Mycelial growth was measured and the growth diameter of *C. gloeosporioides* was found to be greater than that of *C. acutatum*. However, there was no correlation between the diversity and geographical origin or aggressiveness and this research found that PC006 (*Ca*) showed more aggressiveness than CS005 (*Cg*). Similar to some reports, several *Colletotrichum* spp. were found to have the capacity to cause strawberry anthracnose in temperate regions (Damm *et al*., 2012; Weir *et al*., 2012). Chung *et al*. (2020) found that the predominance of *C. siamense* (75%) and *C. fructicola* (11%) in the strawberry anthracnose pathogen population in Taiwan can be attributed to their higher levels of pathogenicity and aggressiveness. Due to rising global temperatures, it will be particularly imperative to monitor the dissemination of *C. acutatum* toward mango plantations in Thailand. The results provide precise information about pathogen identification, which is valuable for the development of effective disease management strategies.

CONCLUSION

Anthracnose is a crucial limiting factor for mango production in Thailand and many other areas in the world. This study demonstrated the diversity of pathogenic *Colletotrichum* species associated with mango cv. Nam Dork Mai See Tong obtained from 3 provinces in Thailand. Regarding nonwounded fruit inoculation, the predominant pathogens were found in both species; *C. gloeosporioides* and *C. acutatum* caused larger lesions with 50-100% disease incidence with 31.25 and 40% of each species population, respectively. ISSR markers were reliable and effective tools for determining the diversity of *Colletotrichum* species and higher diversity was found in *C. gloeosporioides* than in *C. acutatum*. Moreover, there was no correlation between diversity and geographical origin or aggressiveness. This research will lead to the development of effective disease management strategies. Future work will focus on investigating the sensitivity of different *Colletotrichum* species to various levels of fungicide.

ACKNOWLEDGEMENT

This research was funded by the Postharvest Technology Innovation Center, Ministry of Higher Education, Science, Research and Innovation, Bangkok, Thailand. [Grant No. CRP.3. NOV11.8]. We would also like to thank the Mycology Laboratory and Physiology of Plant Disease Laboratory, Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand for their laboratory equipment in plant pathology support.

REFERENCES

Abirami, K., Baskaran, V. and Simhachalam, P. (2018). ISSR marker-based diversity assessment of *Piper* spp. in Bay Islands, India. Indian Journal of Agricultural Research. 52: 434-438. doi: htttp:// 10.18805/IJARe-4941.

Akem, C.N. (2006). Mango anthracnose disease: Present status and future research priorities. Plant Pathology Journal. 5: 266-273. doi: http://10.3923/ppj.2006.266.273.

Babayeva, S.M., Nasibova, J.A., Akparov, Z.I. Shikhaliyeva, K.B., Mammadova, A.D., Izzatullayeva, V.I. and Abbasov, M.A. (2018). Application of DNA markers in determination of *fusarium* resistance and genetic diversity in chickpea. Legume Research. 41(4): 537-542. doi: http://10.18805/LR-407.

Bailey, J.A. and Jeger, M.J. (1992). *Colletotrichum*: Biology, Pathology and Control. CAB International: Wallingford, UK.

Cannon, P.F., Damm, U., Johnston, P.R. and Weir, B.S. (2012). *Colletotrichum*- Current status and future directions. Studies in Mycology. 73: 181-213. doi: https://doi.org/10.3114/sim0014.

Chung, P.C., Wu, H.Y., Wang, Y.W., Ariyawansa, H.A., Hu, H.P., Hung, T.H., Tzean, S.S. and Chung, C.L. (2020). Diversity and pathogenicity of *Colletotrichum* species causing strawberry anthracnose in Taiwan and description of a new species, *Colletotrichum miaoliense* sp. nov. Scientific Reports. 10: 14664. doi: https://doi. org/10.1038/s41598-020-70878-2.

Corkidi, G., Balderas-Ruiz, K.A., Taboada, B., Serrano-Carreon, L. and Galindo, E. (2006). Assessing mango anthracnose using a new three-dimensional image-analysis technique to quantify lesions on fruit. Plant Pathology. 55: 250-257. doi: https://doi.org/10.1111/j.1365-3059.2005.01321.x.

Damm, U., Cannon, P.F., Woudenberg, J.H.C. and Crous, P.W. (2012). The *Colletotrichum acutatum* species complex. Studies in Mycology. 73: 37-113. doi: http://10.3114/sim00 10.

Deising, H.B., Reimann, S. and Pascholati, S.F. (2008). Mechanisms and significance of fungicide resistance. Brazilian Journal of Microbiology. 39(2): 286-295. doi: http://10.1590/S1517-838220080002000017.

Department of Agriculture and Fisheries, Queensland. (2014). Nam Doc Mai. https://www.daf.qld.gov.au/business-priorities/agriculture/plants/fruit-vegetable/fruit-vegetable-crops/mangoes/mango-varieties/nam-doc-mai. (Accessed on 01 August 2021).

Đinh, Q., Chongwungse, J., Pongam, P. and Sangchote, S. (2003). Fruit infection by *Colletotrichum gloeosporioides* and anthracnose resistance of some mango cultivars in Thailand. Australasian Plant Pathology. 32: 533-538. doi: https://doi.org/10.1071/AP03 053.

Freeman, S., Horowitz-Brown, S., Afanador-Kafuri, L., Maymon, M. and Minz, D. (2013). *Colletotrichum*: host specificity and pathogenicity on selected tropical and subtropical crops. Acta Horticulturae. 975: 209-216. doi: http://10.17660/ActaHortic.2013.975.22.

Godwin, I.D., Aitken, E.A.B. and Smith, L.W. (1997). Application of inter simple sequence repeat (ISSR) markers to plant genetics. Electrophoresis. 18: 1524-1528. doi: http://10.1002/elps.1150180906.

Hettwer, U. and Gerowitt, B. (2004). An investigation of genetic variation in *Cirsium arvense* field patches. Weed Research. 44: 289-297.doi: http://10.1111/J.1365-3180.2004.004 02.X.

Johnston, P. and Jones, D. (1997). Relationships among *Colletotrichum* isolates from fruit-rots assessed using rDNA sequences. Mycologia. 89: 420-430. https://doi.org/10.1080/00275514.1997.12026801.

Katkar, M., Mane, S.S. and Kadam, N. (2015). Molecular characterization races of *Fusarium oxysporum* f.sp. *ciceri* using RAPD and ISSR markers. Legume Research. 38(2): 246-252. doi: http://10.5958/0976-0571.2015.00083.1.

Lima, N.B., Lima, W.G., Tovar-Pedraza, J.M., Michereff, S.J. and Câmara, M.P.S. (2015). Comparative epidemiology of *Colletotrichum* species from mango in northeastern Brazil. European Journal of Plant Pathology. 141: 679-688. doi: http://10.1007/s10658-014-0570.

Masel, A.M., He, C., Poplawski, A.M., Irwin, J.A.G. and Manners, J.M. (1996). Molecular evidence for chromosome transfer between biotypes of *Colletotrichum gloeosporioides*. Molecular Plant-Microbe Interactions. 5: 339-348. doi: http://10.1094/MPMI-9-0339.

McDonald, B.A. (1997). The population genetics of fungi: Tools and techniques. Physiopathology. 87(4): 448-453. doi: http://10.1094/PHYTO.1997.87.4.448.

Mills, P.R., Sreenivasaprasad, S. and Brown, A.E. (1992). Detection and differentiation of *Colletotrichum gloeosporioides* isolates using PCR. FEMS Microbiology Letters. 98: 137-143. https://doi.org/10.1111/j.1574-6968.1992.tb05503.x.

Mort, M.E., Crawford, D.J., Santos-Guerra, A., Francisco-Ortega, J., Esselman, E.J. and Wolfe, A.D. (2003). Relationships among the macaronesian members of *Tolpis* (Asteraceae: Lactuceae) based upon analyses of inter-simple sequence repeat (ISSR) markers. Taxon. 52: 511-518. doi: https://doi.org/10.2307/3647449.

Padder, B.A., Sharma, P.N., Awale, H.E. and Kelly, J.D. (2017). *Colletotrichum lindemuthianum*, the causal agent of bean anthracnose. Journal of Plant Pathology. 99(2): 317-330. doi: http://dx.doi.org/10.4454/jpp.v99i2.3867.

Palacıoğlu, G., Bayraktar, H. and Özer, G. (2020). Genetic variability of *Colletotrichum lindemuthianum* isolates from Turkey and resistance of Turkish bean cultivars. Spanish Journal of Agricultural Research. 18(3): 1-12. http://10.5424/sjar/2020183-16398.

Ploetz, R.C., Zentmeyer, G.A., Nishijima W.T., Rohrbach, K.G. and Ohr, H.D. (1994). Compendium of Tropical Fruit Diseases. APS Press. St. Paul, Minnesota, US.

Pongpisutta, R., Rattanakreetakul, C. and Bincader, S. (2019). Responsiveness to fungicide chemicals of *Colletotrichum gloeosporioides* and *Colletotrichum siamense* associated with postharvest disease of mango. Agricultural Science Journal. 50: 3(Suppl.): 175-178. https://doi.org/10.13140/RG.2.2.27854.82244.

Pongpisutta, R., Winyarat, W. and Rattanakreetakul, C. (2013). RFLP identification of *Colletotrichum* species isolated from chilli in Thailand. Acta Horticulturae. 973: 181-186. https://doi.org/10.17660/ActaHortic.2013.973.24.

Prasannan, A. and Jose, S. (2021). Assessment of ISSR markers for tagging genetic variability for yield components in small cardamom (*Elettaria cardamomum* Maton). Indian Journal of Agricultural Research. 55(2): 212-216. doi: http://10.18805/IJARe.A-5414.

Prihastuti, H., McKenzie, E., Hyde, K., Cai, L., Chen, H., McKenzie, E.H.C. and Hyde, K.D. (2009). Characterization of *Colletotrichum* species associated with coffee berries in northern Thailand. Fungal Diversity. 39: 89-109.

Ratanacherdchai, K., Wang, H.K., Lin, F.C. and Soytong, K. (2010). ISSR for comparison of cross-inoculation potential of *Colletotrichum capsici* causing chilli anthracnose. African Journal of Microbiology Research. 4: 76-83.

Rodkate, C. (2012). Variation of *Colletotrichum gloeosporioides* causing chilli anthracnose. Master Degree Thesis (Plant Pathology), Kasetsart University. Bangkok, Thailand.

Rohlf, F. (1988). NTSYS-pc - Numerical Taxonomy and Multivariate Analysis System. Applied Biostatistics Inc. New York, US.

Rohlf, F.J. and Sokal, R.R. (1981). Comparing numerical taxonomic studies. Systematic Biology. 30(4): 459-490. doi: http://10.1093/sysbio/30.4.459.

Rungjindamai, N. (2016). Isolation and evaluation of biocontrol agents in controlling anthracnose disease of mango in Thailand. Journal of Plant Protection Research. 56: 306-311. doi: https://doi.org/10.1515/jppr-2016-0034.

Sangchote, S. (1987). Postharvest diseases of mango fruits and their losses. Kasetsart Journal, Natural Sciences. 21(1): 81-85.

Schena, L., Mosca, S., Cacciola, S.O., Faedda, R., Sanzani, S., Agosteo, G., *et al*. (2013). Species of the *Colletotrichum gloeosporioides* and *C. boninense* complexes associated to olive anthracnose. Plant Pathology. 63: 437-446. https://doi.org/10.1111/ppa.12110.

Sreenivasaprasad, S., Sharada, K., Brown, A.E. and Mills, P.R. (1996). PCR-based detection of *Colletotrichum acutatum* on strawberry. Plant Pathology. 45: 650-655. https://doi.org/10.1046/j.1365-3059.1996.d01-3.x.

Sutton, B.C. (1980). The Coelomycetes: Fungi Imperfecti with Pycnidia, Acervuli and Stromata. Commonwealth Mycological Institute: Kew, UK.

Thailand's Department of Agricultural Extension. (2018). https://www.oae.go.th/assets/portals/1/files/ebook/2563/commodity2562.pdf. (Accessed on 27 July 2021).

Than, P.P., Jeewon, R., Hyde, K.D., Pongsupasamit, S., Mongkolporn, O. and Taylor, P.W.J. (2008). Characterization and pathogenicity of *Colletotrichum* species associated with anthracnose on chilli (*Capsicum* spp.) in Thailand. Plant Pathology. 57: 562-572. doi: https://doi.org/10.1111/j.1365-3059.2007.01782.x.

Walker, J., Nikandrow, A. and Millar, G.D. (1991). Species of *Colletotrichum* on *Xanthium* (asteraceae) with comments on some taxonomic and nomenclatural problems in *Colletotrichum*. Mycological Research. 95(10): 1175-1193. doi: https://doi.org/10.1016 /S0953-7562(09)80008-7.

Weir, B. S., Johnston, P. R. and Damm, U. (2012). The *Colletotrichum gloeosporioides* species complex. Studies in Mycology. 73: 115-180. doi: https://doi.org/10.3114/sim 0011.

White, T.J, Bruns, T., Lee, S. and Taylor, J. (1990). Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: PCR pProtocols: A Guide to Method and Application [M.A. Innis, D.H. Gelfand, J. Sninsky, T.J. White (Eds.)], Academic Press: New York, US.

Yap, I. and Nelson, R.J. (1996). Winboot: A Program for Performing Bootstrap: Analysis of Binary Data to Determine the Confidence Limits of UPGMA based Dendrograms. International Rice Research Institute. Manila, Philippines.

Zietkiewicz, E., Rafalski, A. and Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics. 20: 176-183. doi: http://10.1006/geno.1994.1151.