Colletotrichum Species Related To “Nam Dork Mai See Tong” Mango In Central Thailand And Risk Detection of Carbendazim-Resistant Strains

Santiti Bincader

Kasetsart University Faculty of Agriculture at Kamphaengsaen

Ratiya Pongpisutta (✉ agrryp@ku.ac.th)

Kasetsart University Kamphaeng Saen Campus <https://orcid.org/0000-0003-4161-4597>

Chainarong Rattanakreetakul

Kasetsart University Faculty of Agriculture at Kamphaengsaen

Research Article

Keywords: Colletotrichum, Fungicide resistance, Mangifera indica, PCR-RFLP

Posted Date: August 19th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-782870/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Essential title page information**Title:**

Colletotrichum species related to “Nam Dork Mai See Tong” Mango in Central Thailand and Risk Detection of Carbendazim-resistant Strains

Author information:**Affiliations**

Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Nakhon Pathom 73140, Thailand.

Santiti Bincader, Ratiya Pongpisutta & Chainarong Rattanakreetakul

Postharvest Technology Innovation Center, Ministry of High Education, Science, Research and Innovation, Bangkok 10400, Thailand.

Santiti Bincader, Ratiya Pongpisutta & Chainarong Rattanakreetakul

Santiti Bincader

E-mail: Santiti.bi@ku.th

ORCID ID: 0000-0003-2427-5394

Ratiya Pongpisutta

Email: agrryp@ku.ac.th

ORCID ID: 0000-0003-4161-4597

Chainarong Rattanakreetakul

Email: crattan99@yahoo.com

ORCID ID: 0000-0003-4616-0619

Corresponding author:

Correspondence to Ratiya Pongpisutta

Tel: +66 34 351890; Fax: +66 34 351890

***Colletotrichum* species related to “Nam Dork Mai See Tong” Mango in Central Thailand and Risk
Detection of Carbendazim-resistant Strains**

Abstract:

Nam Dork Mai See Tong mango is a deluxe commercial fruit in Thailand, however anthracnose disease becoming a major problem affecting market-driven. Fungicide treatment is the main component in the mango anthracnose management, notwithstanding the appearance of fungal-resistance has been become a factor of fungicide limitation and can affect to increasingly higher costs. Thirty-two isolates of the *Colletotrichum* species complex were obtained from anthracnose-diseased mango cv. Nam Dork Mai See Tong in 3 provinces of Thailand. Fungal identification based on morphological and molecular markers was investigated. All isolates were divided into 3 species: *C. asianum*, *C. gloeosporioides* and *C. siamense*. Pathogenicity tests revealed that all 3 species caused symptoms on artificially unwounded mango fruits and leaves. Only *C. gloeosporioides* have previously been reported on mango, while *C. asianum* and *C. siamense* were first reports associated with mango in Thailand. The responsiveness of *Colletotrichum* isolates to carbendazim was evaluated using an MIC assay. Nine isolates of *C. asianum* were highly resistant, while 5 and 8 isolates of *C. gloeosporioides* were resistant (R) and highly resistant (HR), respectively. Moreover, all isolates of *C. siamense* were HR. Mutations were detected by PCR of a partial sequence of the β -tubulin gene. The sequence of β -tubulin gene in HR strains showed a single nucleotide transversion of adenine to cytosine, resulting in a substitution at codon 198. However, R strains were found to have only an amino acid change at codon 200. Additionally, PCR-RFLP was applied as a rapid technique to detect carbendazim resistance. The results demonstrated that only the *Bsh*1236I restriction enzyme generated two bands (200 and 300 bp) in the highly resistant strain, but these bands were absent in the sensitive (S) and R strains. Hence, PCR-RFLP technique showed the potential to specifically detect benzimidazole fungicide resistance in 3 species of *Colletotrichum*. Moreover, this technique is accessible and feasible for laboratory assessment.

Keywords:

Colletotrichum; Fungicide resistance; *Mangifera indica*; PCR-RFLP

Introduction:

In 2018-2019, world mango production expanded to more than 100 countries, with more than 44,626,000 tons of fruits produced annually. Thailand is one of the top three mango-producing areas, producing almost 7.69% of global mango production, and approximately 2% of fresh mango fruit is exported (Worldatlas 2019). Anthracnose disease caused by the genus *Colletotrichum* is one of the crucial problems occurring in field, along with postharvest diseases, and affects mango quality in Thailand (Walker et al. 1991). The mango cv. 'Nam Dork Mai See Tong' is highly susceptible to disease and can be affected by a latent infection during transportation (Sangchote 1987). The infection occurs via an appressorium that develops from germinating spores on the plant surface followed by turgor pressure penetration in the cuticle and, in some cases, infection of the epidermal cells by infective hyphae.

The studies of genus *Colletotrichum* on mango have been reported and classified into many different species such as *C. acutatum* species complex, *C. boninense* species complex and *C. gloeosporioides* species complex (Damm et al. 2012a, b; Weir et al. 2012). Previously, *Colletotrichum* species complex has been identified using morphological markers and ITS sequences but sometimes the ITS-region information alone is insufficient to distinguish closely related taxa. Hence, ACT gene, CHS-1 gene including TUB gene were used to clarify for species identification in the genus *Colletotrichum*, accurately. Moreover, species identification of fungal plant pathogen will lead to decide chemical fungicides for protecting and controlling of anthracnose disease, appropriately.

The first report of anthracnose disease management in mango was by Jeffries et al. (1990), in which fungicide application was suggested for the management of this disease due to its ease of use and success in disease control (Cools et al. 2005). Carbendazim, a member of the benzimidazole class, is a fungicide that is widely used for anthracnose disease control and can be managed both in the field and postharvest. The mode of action of carbendazim is the inhibition of mitosis and cell division (β -tubulin assembly in mitosis), but recently, the appearance of fungal resistance has become important and has affected the ability to use the fungicide for anthracnose management in the mango industry (Brent 1995). The report of the Fungicide Resistance Action Committee (FRAC) (2020) explained that fungicides have become an integral part of efficient food production and that the loss of a fungicide through resistance is a problem that affects everyone.

The methods for fungicide resistance detection require isolation of pathogens treated with each fungicide (Anon 1991), but these processes are time-consuming. Currently, molecular biology is a new method for fungicide detection once the other mechanisms have been exhausted. For carbendazim, Ma and Michailides (2005) reviewed the molecular mechanisms of fungicide resistance in four fungicide classes, including the benzimidazole class. The results indicated that resistance was correlated with point mutations in the β -tubulin gene, resulting in altered amino acid sequences at the benzimidazole site, and most of the results from numerous studies have shown that changes at codons 198, 200 and 240 in the β -tubulin gene could cause benzimidazole resistance (Ma et al. 2003; Nalumpang et al. 2010; Peres et al. 2004).

Apart from this, RFLP markers are codominant and highly locus-specific because of the restriction site and cutting site of each restriction enzyme (National Center for Biotechnology Information 2017). For example, N Ziogas et al. (2009) studied gray mold disease of strawberry in Greece and examined the sensitivity to benzimidazoles (carbendazim and diethofencarb) by using a set of PCR-specific primers designed to amplify a portion of β -tubulin and digestion with *Bsa*I. The results of these studies showed that the differentiation between sensitive and resistant

strains based on digested products could be detected via; PCR-RFLP of benomyl resistant strains of *Mycosphaerella fijiensis* in Colombia (Cañas-Gutiérrez et al. 2006), and many reports applied this method to detect other fungicide resistance of plant pathogenic fungi within a short time (Cunha and Rizzo 2003; Ishii et al. 2002; Ma et al. 2003)

Although the RFLP technique is a simple method and the markers are codominant for the detection of point mutations, some target fragments may not contain a restriction endonuclease recognition sequence at the site of point mutation. However, preliminary restriction enzyme cutting analysis has become a useful method to create and simulate RFLP patterns. A virtual RFLP assay was developed for classifying over 800 publicly available Phytoplasma 16S rRNA gene sequences, and the results showed that computer-simulated RFLP was highly effective in classifying and separating the 28 groups with the highest similarity coefficients (Wei et al. 2007). Hence, the rapid detection method was applied as an essential tool for fungicide resistance inspection to improve fungicide utilization for anthracnose control. The aim of this research was to detect carbendazim resistance in anthracnose disease in the mango cv. Nam Dork Mai See Tong using molecular characterization and a modified PCR-RFLP as a rapid technique.

Materials and Methods

Fungal isolation

Thirty-two isolates of *Colletotrichum* species were obtained from mango fruits with typical anthracnose symptoms in 3 mango plantation provinces of Thailand, Chachoengsao, Phichit and Ratchaburi, in 2016 – 2017 and cultured using a tissue transplanting technique on potato dextrose agar (PDA). Later, mycelial cutting was performed by placement on fresh PDA, and samples were incubated at 25°C under a photoperiod of 12 hr light / 12 hr dark for 5 days. Then, mycelia were transferred onto potato carrot agar (PCA), and finally, the cultures were kept at 14°C for further study.

Morphological identification

Petri dished containing 15 mL of PDA were inoculated with a 5 mm in diameter core taken from the edge of an activity growing 5-day old culture. The culture was incubated at 25°C under a photoperiod of 12 hr light/12 hr dark. Colony type and spore mass color were recorded at day 5. Fifty conidia were randomly selected from each replicate to measure their length and width under compound microscope with Olympus CellSens Standard software version 1.6. Appressoria were induced with modified slide culture technique (Johnston and Jones, 1997). The isolates 5-day old cultures on PDA were transferred onto 25.4×76.2 mm sterile microscope slides and covered with 22×22 mm cover slips incubated in petri dish to serve as a moisture chamber under 25°C for 4 days. Length and width of 30 appressoria per isolate were measured under compound microscope with Olympus CellSens Standard software version 1.6.

DNA extraction and fungal confirmation

Genomic DNA of each isolate was extracted using the method of Pongpisutta et al. (2013) and the modified protocol of Zimand et al. (1994). PCR amplification was performed for the partial internal transcribed spacer (ITS region), actin gene (ACT), chitin synthase 1 gene (CHS-1) and β -tubulin (TUB) regions, as well as with specific primers for *C. acutatum* and *C. gloeosporioides* (Table 1). The PCR master mix had a total volume of 25 μ L. The PCR mixture contained 2 μ L of 20 ng genomic DNA, 0.48 μ M of each primer, 0.69X *Taq* buffer (Thermo Fisher

Scientific, Massachusetts, USA), 2.4 mM MgCl₂, 10 µM of each dNTP, 1 U *Taq* polymerase (Thermo Fisher Scientific, Massachusetts, USA) and 14.4 µL of sterile distilled water.

PCR thermal cycling (Sensoquest GmbH, Göttingen, Germany) was carried out using the following program: predenaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30s, annealing for 30s (the temperature each of primer is shown in Table 1) and extension at 72°C for 1 min, with a final extension step of 72°C for 10 min. PCR products were stained with GelStar® and verified on 1.2% agarose gels via electrophoresis in 1X TBE buffer at a constant power of 80 volts with the GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Massachusetts, USA) used for comparison. The gels were photographed under UV radiation with a 365 nm wavelength using a GeneFlash Gel Documentation System (Syngene, Cambridge, UK). Sequencing of the PCR products was performed by 1st Base Laboratory Co., Ltd. in Malaysia. Consensus sequences were produced using the EditSeq program of the sequence analysis software (DNASTar Inc., WI, USA) by careful trimming and editing and then compared by Basic BLASTn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to the GenBank National Center for Biotechnology Information (NCBI) database.

Phylogenetic analyses

Analysis of the sequence data was performed by the MEGA version X software program (Kumar et al. 2018) for phylogenetic tree confirmation. A comparison of the sequences of 4 loci (ACT, CHS-1, ITS, TUB) was carried out by choosing *Colletotrichum* species from the Genbank database. Data alignments were achieved by using the clustalW alignment routine (Thompson et al. 1994). The phylogenetic tree was generated using the maximum likelihood (Tamura et al. 2011), and the confidence of the groupings was determined by bootstrap analysis (Felsenstein 1985), which was performed using 1,000 replications to test the significance the trees.

Pathogenicity test

Representative isolate of *Colletotrichum* species complex were used for pathogenicity test on immature mango fruits and third branch leaves from 6-month seedling (cv. Nam Dork Mai See Tong) under laboratory conditions. All isolates were cultured on PDA and incubated at 25°C under a photoperiod of 12 hr light / 12 hr dark for 5 days, then cut the colony margin with cork borer and transferred 5 mm mycelial plug onto mango fruits and leaves which washed with 1.2% sodium hypochlorite for 1 min and sterile distilled water. Inoculated fruits and leaves were used unwounded technique, then kept in moisture box and incubated at room temperature in the photoperiod of 12 hr light / 12 hr dark for 5 days symptom developing.

Carbendazim resistibility assays

The minimum inhibitory concentration (MIC) was used to determine fungal resistibility. *Colletotrichum* isolates were grown on PDA incubated at 25°C under a photoperiod of 12 hr light / 12 hr dark for 5 days. Then, 5 mm mycelial plugs were cut from the colony margin with cork borer and transferred to a 24-well cell culture cluster of PDA augmented with carbendazim at concentrations of 0.1, 1, 10, 100, and 1,000 µg/mL and the recommended rate in the field (500 µg/mL); all samples were incubated under the same conditions. Four levels of evaluation were examined as described in Table 2.

Partial sequencing of the β-tubulin gene and sequence alignment

Primers TB2L (5' GYTTCCAGATYACCCACTCC '3) and TB2R (5' -TGAGCTCAGGAACRCTGA CG '3) (Peres et al. 2004) were used to amplify a portion of the β-tubulin gene for the detection of point mutations associated with resistance. The PCR master mix had a total volume of 25 µL. The PCR mixture contained 2 µL of 20 ng genomic DNA, 0.48 µM of each primer, 0.69X *Taq* buffer (Thermo Fisher Scientific, Massachusetts,

USA), 2.4 mM MgCl₂, 10 µM of each dNTP, 1 U *Taq* polymerase (Thermo Fisher Scientific, Massachusetts, USA) and 14.4 µL of sterile distilled water. PCR was carried out in a programmable thermal cycle as follows: pre-denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min and extension at 72°C for 1 min; and a final extension step of 72°C for 5 min. PCR products were stained with GelStar and separated by electrophoresis on a 1.2% agarose gel in 1X TBE buffer at 80 volts. The gels were recorded under UV radiation with a 365 nm wavelength using the GeneFlash Gel Documentation System. PCR product sequencing was performed at 1st Base Laboratory Co., Ltd. in Malaysia.

Nucleotide sequences were translated into amino acid sequences using Expasy by the SIB Swiss Institute of Bioinformatics (<https://web.expasy.org/translate/>) and The Sequence Manipulation Suite (https://www.bioinformatics.org/sms/show_trans.html). The data were aligned using EMBL-EBI clustal omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) and edited by MEGA version X software. Protein homology modeling was applied using CPH model version 3.2 (<http://www.cbs.dtu.dk/services/CPHmodels/>) in PDB mode, and molecular graphics visualization was performed using RasMol Molecular Graphics version 2.7.5.2 for Windows to study protein structure differentiation between sensitive and resistant strains to carbendazim fungicides.

RFLP analysis

Computer-simulated RFLP analysis of the sequences was conducted. Before the experiment, all nucleotide sequences were edited using MEGA version X software (Kumar et al. 2018) to ensure that the start and end points of all samples were the same. Over 86 restriction enzymes were screened for *in silico* restriction digestion using pDRAW32 DNA analysis software (AcaClone Software, <https://www.acaclone.com/>). After digestion, 2.0% agarose gel electrophoresis was performed by comparison with the Fermentas 100 bp Plus DNA Ladder. Digested products were generated by adding the restriction enzymes according to the manufacturer's instructions, and the banding patterns of the products were determined. Each digestion reaction had a final volume of 30 µl and contained 17 µl of nuclease-free water, 2 µl of 10X FastDigest Green Buffer, 10 µl (~0.2 µg) of PCR product and 1 µl of FastDigest enzyme. Samples were mixed gently and incubated at 37°C in a heat block for 5 min. The digested products were stained with GelStar® and separated by 2.0% agarose gel electrophoresis in 1X TBE buffer at 80 volts; gels were observed under 365 nm wavelength UV radiation via the GeneFlash Gel Documentation System.

Results

Fungal isolation and identification

During disease survey, anthracnose symptoms in the mango orchards were observed (Table S1). On mango, disease symptoms of anthracnose were separated into 3 types: 1) 6.25% of the symptom on inflorescences are dark-brown spots to elongated dark lesions which can enlarge, coalesce, and blighted flowers affecting small emerging fruits that could fall out, then (Fig. 1a-1b), 2) 21.87% of lesions on leaves start as brown to black small spot, then developing to irregularly shaped lesions with brown-gray to black necrotic spots on leaf surfaces (Fig. 1c-1d), and 3) 71.88% small black circular spots becoming sunken, rounded brown to black lesions on ripe fruits and producing acervuli on the lesions with bright salmon, salmon-orange to pale colored spore masses at high relative humidity. Premature fruits can be dropped from the trees. Furthermore, the spot decay on mature-fruit during harvesting or transportation can penetrate deep into the fruit, resulting in extensive fruit rotting (Fig. 1e-1f). Morphological markers of 32 isolates from Sutton (1980) were identified as only belonging to the *C.*

gloeosporioides species complex due to their cottony aerial mycelia and white to gray mycelia covered with pale to orange spore masses (Fig. 2a-2j). Conidia were straight to cylindrical with round ends ((3.59)6.91 × 11.02(19.52) μm) (Fig. 2k-2t), no setae, and appressoria pale to dark brown with various shapes such as clavate, long clavate to irregular ((4.37)7.31 × 6.49(11.59) μm) (Table S2; Fig. 2u-2ad; Fig. S1). Then, identification of species complexes using PCR techniques based on multilocus phylogenetic analyses was performed.

Phylogenetic analyses

Total genomic DNA was used as the template for identification using PCR. Molecular detection with the species-specific primers *CaInt*/ITS4 and *CgInt*/ITS4 was utilized to detect the *Colletotrichum* samples in this study. The species-specific primers *CgInt*/ITS4 amplified an approximately 500 bp fragment from all isolates, while *CaInt*/ITS4 did not amplify this fragment. Blast comparison showed that ten isolates were identified as *C. asianum* with 96.62 – 100.00%, three isolates were determined to be *C. gloeosporioides* with 86.00- 100.00%, while three isolates (RB003, RB006 and PC002) were accurately classified as *C. siamense* with 96.00- 100.00%.

Phylogenetic analysis of multiple loci (ACT gene, CHS-1 gene, ITS region and TUB gene) used ClustalW as implemented in MEGA version X (Kumar et al. 2018), and manually adjusted to allow high sequence similarity. Additionally, the same software was used to carry out the statistical selection of best-fit models of nucleotide substitution, the result showed that all nucleotide sequence alignments of genes were conducted using a maximum likelihood (ML) method supported by the Kimura 2-parameter model combined with gamma distribution (G) (Archibald et al. 2003) and compared with the ex-holotype/epi-type in Table 3. Ten isolates were absolutely identified as *C. asianum* with 95% bootstrap values. Furthermore, three representative isolates were determined to be *C. gloeosporioides* with approximately bootstrap value 86%, while two representative isolates (RB003, RB006 and PC002) were accurately classified as *C. siamense* with high bootstrap values 96% shown in the branch of phylogenetic tree (Fig. 3). All the sequences (excepted results of specific-primer) generated in this study were deposited in GenBank and DDBJ for assigning accession numbers (Table 3).

Pathogenicity test

Colletotrichum isolates RB005, CS008 and RB003 were selected from *C. asianum*, *C. gloeosporioides* and *C. siamense*, respectively, in order to test their pathogenicity. Symptom on unwounded fruits and leaves developed sunken spot, dark brown to brown lesion at day 5 after inoculation (Fig. 4). The damage of *Colletotrichum gloeosporioides* had the highest infection incidence reached to 100% in both inoculated fruits and leaves, while the *C. asianum* and *C. siamense* were about 75 and 60% on fruits and each absolutely counting 100% on leaves (Table 4).

Carbendazim resistibility assays

Thirty-two isolates of *Colletotrichum* separated into 3 species were tested for carbendazim responsiveness by MIC assay on PDA amended with carbendazim at concentrations of 0.1 - 1,000 mg/L, including the recommended rate. The results indicated that 9 isolates (90.00%) of *C. asianum* were found to be HR strains, while one isolate (10.00%) was found to be an R strain. Six isolates (31.58%) of *C. gloeosporioides* were clustered as sensitive strains, and 5 isolates (26.32%) were proven to be R strains; moreover, 8 isolates (42.11%) were revealed to be HR strains. Ultimately, all 3 isolates (100.00%) of *C. siamense* were evaluated to be HR strains (Table 5).

Partial sequencing of β -tubulin genes and sequence alignment

PCR products (approximately 450 bp) of the partial β -tubulin sequence from all 32 isolates were generated with TB2L/TB2R primers and electrophoresed on a 1.2% agarose gel (Fig. 5c). The S strains showed the nucleotides GAG (glutamic acid; Glu (E)) at codon 198 and TTC (phenylalanine; Phe (F)) at codon 200, while the HR strains showed the nucleotides GCG (alanine; Ala (A)) at codon 198. The R strains had a point mutation at codon 200, resulting in the nucleotides TAC (tyrosine; Tyr (Y)) (Fig. 5a 5b, 5f). Other point mutation within the remaining codons, such as codons 167 and 240, were studied with the same pair of primers, and no differentiation was detected. The results indicated that the mutated codons E198A and F200Y of the β -tubulin gene were related to the responsiveness phenotype of the 3 *Colletotrichum* species causing mango anthracnose and were associated with resistance to MBC fungicides, such as carbendazim, in this study.

RFLP analysis

The partial β -tubulin gene sequence amplified with the TB2L/TB2R primers was subjected to *in silico* RFLP using pDRAW32 software to assess HR, R and S strains. The results showed that 37 of 86 restriction enzymes in the program did not cut the PCR fragment. However, the *Bst*UI restriction enzyme revealed distinctly different fragments among the 3 strains (Fig. 6c, 6d). Digestion of the HR strain with *Bsh*1236I (isoschizomers: *Acc*II, *Bsp*FNI, *Bst*FNI, *Bst*UI, *Mvn*I) generated bands of approximately 200 and 300 bp, while the S strain and R strain products were not cut (Fig. 6b, 6e). The *Bsh*1236I enzyme recognizes the CG[^]CG site and cuts at the position of codon 198, which was useful for distinguishing between HR and S strains. For the R strain, the *Hpy*AV restriction enzyme (isoschizomers: *Hin*4II) cut and showed differentiation between R and HR or S strains, but the cut and recognition sites were not positioned at codon 200 (Fig. 6). Hence, RFLP using the *Bsh*1236I restriction enzyme was a useful and rapid technique for preliminary detection of strains highly resistant to carbendazim. Thus, the PCR-RFLP technique can be used to assess the fungicide resistance of *Colletotrichum* causing mango anthracnose disease.

Discussion

In Thailand, most reports in the past have described and identified fungi causing anthracnose disease of mango in plantation areas as *Colletotrichum gloeosporioides*. Nonetheless, there is a report in the years 2010 showed that *C. gloeosporioides* was actually not a common pathogen in the tropics and it was not the main causal pathogen of mango anthracnose in Laos and Thailand, in spite of first understanding that anthracnose of most tropical fruits is caused by *C. gloeosporioides* but molecular analysis, showed that most of the fungal samples still belong into the “*C. gloeosporioides* species complex” (Phoulivong et al. 2010). So far as anyone know, molecular classification based on ITS sequences was typically used for identified anthracnose-pathogens including other fungi. Recently; it was found that ITS-region were unable to distinctly resolve the species identification of individual *Colletotrichum*. The information of Wei et al. (2012) reported that ITS sequences are insufficient to separate *C. aenigma*, *C. aeshynomenes*, *C. alienum* and some *C. siamense* isolates. These taxa are best differentiated by using TUB, gluta-mine synthetase (GS) or manganese-superoxide dismutase (SOD2). For *C. asianum* or *C. gloeosporioides* are distinguished from all other taxa using any of the genes tested, excluding ITS.

However, this study investigated *Colletotrichum* 32 isolates obtained from 3 important mango plantation areas based on morphological characteristics and pathogenicity tests performance combined with multiloci molecular phylogenetic analysis, the fungal pathogens were clearly divided into 3 species: *C. asianum*, *C. gloeosporioides* and *C. siamense*. Pathogenicity tests revealed that all 3 species caused symptoms on artificially unwounded mango

fruits and leaves. The typically symptom of each species infected mango shown similar, there are depressed black lesion which are sub circular and in which spore mass develop with high humidity (Bailey and Jeger. 1992). The pathogenicity assay indicated that 3 species were causal agents on mango anthracnose disease, Some Thai researchers have previously reported that *C. gloeosporioides* is mainly causal agent of mango and chilli anthracnose while *C. asianum* and *C. siamense* are also cause coffee berry disease (Đinh et al. 2003; Dinh and Sangchote 2002; Prihastuti et al. 2009; Rungjindamai. 2016; Sangchote. 2013; Than et al. 2008). Other from this, in Brazil, China or India, important mango producer, have reported *Colletotrichum* species more than 5 species; *C. cliviicola*, *C. cordylinicola*, *C. endophytica*, *C. fructicola*, *C. karstii*, *C. tropicale* etc. can be pathogenic to mango (Li et al., 2019; Lima et al. 2013; Mo et al. 2018; Zakaria. 2021). Our results showed that the most common fungal isolates related to mango involved 3 species: *C. asianum*, *C. gloeosporioides* and *C. siamense*. Only *C. gloeosporioides* have previously been reported on mango, while *C. asianum* and *C. siamense* were first reports associated with mango in Thailand.

Anthracnose control and fungicide application are crucial for treating plants and producing high-quality agricultural products. Most report addressing several new products have been introduced, including single-site and combination fungicides classes for profitable against anthracnose fungal pathogens and also effective disease control (Tredway and Wong. 2012). Carbendazim, a member of the benzimidazole group of fungicides, is a major fungicide used to control mango anthracnose and has been reported by many researchers (Kim et al. 2007, 2020; Nagaraju et al. 2019). Although carbendazim is a fungicide affecting mango anthracnose control, the appearance of fungicide resistance is currently becoming a factor limiting the potential of this drug (Ma and Michailides. 2005).

The mode of action (MOA) of carbendazim, a member of the benzimidazole group of fungicides, is associated with interference in the biosynthesis of DNA during fungal cell division, biosynthesis of the β -tubulin gene and the polymerization of β 2-tubulin, which is the main mechanism of action of benzimidazole compounds, such as benomyl and thiophanate-methyl fungicides (Qiu et al. 2011; Zhou et al. 2016). Several mutations in the β -tubulin gene can lead to benzimidazole fungicide resistance.

The codon changes in most field-resistant isolates that have arisen for several fungal plant pathogens seem to be restricted to positions 6, 50, 167, 198, 200 and 240 (Zhan et al. 2014). For mutation or resistance detection, several studies, including this research, have detected mutations in the β -tubulin gene at codon 198, which resulted in a substitution of glutamic acid (GAG) in the S strain to alanine (GCG) in the R strain (Nalumpang et al. 2010; Peres et al. 2004). This mutation is present not only in the genus *Colletotrichum* but also in other plant pathogens, such as *Botrytis cinerea*, *Penicillium aurantiogriseum* and *Helminthosporium solani* (Cunha and Rizzo 2003; Koenraad et al. 1992; Luck and Gillings. 1995). Some reports have also found different affected codons in some plant pathogenic fungi, such as codon 6 in *Monilinia fructicola*, a substitution resulting in a change of histidine to tyrosine at codon 200 in *C. gloeosporioides* isolated from tropical fruit in Taiwan, and a nucleotide substitution resulting in a change of phenylalanine to tyrosine (Chung et al. 2010).

In this study, we found *C. asianum* isolates (10 isolates) showing HR (9 isolates) and R (1 isolate) to carbendazim, and all the isolates (3 isolates) of *C. siamense* showed HR. However, only *C. gloeosporioides* isolates (6 isolates) were sensitive to carbendazim. The results as mentioned in Table 5, most of *Colletotrichum*, isolated from mango cv. Nam Dork Mai See Tong showed highly resistance to carbendazim fungicide to 62.5% (20 isolates) of the fungal samples. The MIC values showed more than 1,000 ppm for highly resistance strains of three *Colletotrichum*

species which was exceeding the recommended rate of carbendazim (approximately 500 ppm; 20 cc/20L with active ingredient 50 w/v in suspension concentrate type). Although, other researchers have informed development of carbendazim or fungicide in class benzimidazole from several areas or host plants including various mango cultivars in Thailand (Nalumpang et al. 2010; Poti et al. 2020); but this is the first report of technique for carbendazim-resistant detection in mango cv. Nam Dork Mai See Tong that caused by *Colletotrichum* 3 species. Usually, fungicide resistance studies require isolation of the pathogen in a pure culture and subsequent plating on a medium containing fungicide or a "poisoned food" (Anon 1982, 1991). The conventional method is time-consuming, which is why molecular techniques provide a new opportunity for rapid detection. Although molecular tools are rapid techniques, most studies differentiate between S and R strains by using nucleotide sequencing analysis as showed in Fig. 5a and 5b.

However, the fungicide detection method is expensive. Hence, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) has been used to detect fungicide resistance. In this research, we indicated that these techniques are highly specific and prompt for detecting fungicide responsiveness. Rosenzweig et al. (2015) developed PCR-RFLP to detect the G143A and E198A point mutations of the cytochrome b and β -tubulin genes in *Cercospora* leaf spot (CLS) caused by *Cercospora beticola* in Michigan sugar beet (*Beta vulgaris*) fields. The results showed that the PCR-RFLP assay was effective in detecting the presence of the G143A and E198A mutations in *C. beticola* from field populations in Michigan. For this research, restriction enzyme *Bsh1236I* can use to digest PCR products of the *Colletotrichum* HR strains, excellently. Whilst in R strain had a point mutation at codon 200, the result revealed that some restriction enzymes appeared *in silico* can cut product but the cut and recognition sites were not positioned at target codon. So, a modification of PCR-RFLP assay must be developed as the report of Chung et al. (2010), which study benzimidazole-resistant with designed specific primers for scope the codon or utilized wild-type strain (N Ziogas et al. 2009). Moreover, PCR-RFLP is a rapid technique for fungicide resistance detection in the case of carbendazim and azoxystrobin (Cañas-Gutiérrez et al. 2006; Chung et al. 2010; Inada et al. 2008), but some microorganisms, especially the genus *Colletotrichum*, are highly variable phenotypic diversity within taxa/species complex and may not contain a recognition site sequence at the site of point mutation. Therefore, virtual RFLP analysis (*in silico*) is a convenient method for preliminary potential digestion of each restriction enzyme as a reliable alternative to conventional PCR-RFLP, as shown in Fig. 5d and Fig. 6.

Furthermore, RFLP or virtual RFLP techniques are considered to be essential tools to solve the problems of species differentiation in microorganisms, such as the identification of 3 species of *Colletotrichum* causing chili anthracnose in Thailand, pineapple spoilage fungi, and three major *Meloidogyne* species in Korea, as well as the classification of phytoplasma using *cpn60* universal target sequences (Han et al. 2004; Koffi et al. 2019; Pérez-López et al. 2016; Pongpisutta et al. 2013) Apart from this, *In silico* testing has reported that this method can be solve the error of PCR-RFLP method, caused by primers that may not amplify PCR products and low PCR product concentration that affect availability of template for cleavage by restriction enzymes and affect visibility of digested product on agarose gels, including PCR master mixed such as $MgCl_2$, *Taq* buffer contained with KCl or $(NH_4)_2SO_4$ and primer dimers that can inhibit activity of restriction enzyme (Ramdeen and Rampersad 2014). This research is not only identification but also differentiation among sensitive, resistant and highly resistant strains in population of *Colletotrichum* species obtained from mango anthracnose disease by using virtual PCR-RFLP and confirm with PCR-RFLP in gel electrophoresis. The method can be performed in a very short time and

achieved reliable data comparing to the old assay such as poisoned food or ED₅₀ calculation, then PCR-RFLP technique illustrated the potential to specifically detect benzimidazole fungicide resistance in 3 species of *Colletotrichum* like *C. asianum*, *C. gloeosporioides* and *C. siamense*. Furthermore, this technique is accessible and feasible for laboratory assessment. It is a crucial caution and signal for precise management using fungicide utilization with the target to achieve effective control. Once, the mutation fungal strains invade in a field, it is quite tough to reduce the population of these strains. Hence, a rotation of different fungicide groups is needed. Moreover, this is the first report of carbendazim resistance of three species of *Colletotrichum*, causal agents of mango anthracnose in Thailand using PCR-RFLP for benzimidazole fungicide resistant detection.

Acknowledgments:

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.

Declarations:

Funding: 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].

Conflicts of interest/Competing interests: The authors declare no conflict of interest.

Code availability: Not applicable.

Authors' contributions: R.P. and C.R. Conceptualization; S.B., R.P and C.R. methodology, software, formal analysis, writing—original draft preparation; R.P. and C.R. supervision project administration, funding acquisition; S.B., R.P. and C.R. investigation, data curation; R.P. and C.R. validation, resource, writing-review and editing. All authors have read and agreed to the published version of the manuscript.

Ethics approval: Not applicable.

References:

- Anon (1982) Recommended methods for the detection and measurement of resistance of agricultural pests to pesticides. FAO Plant Prot Bull 30: 36–143.
- Anon (1991) FRAC methods for monitoring fungicide resistance. Bull OEPP 21: 291–354.
- Archibald JK, Mort ME, Crawford DJ (2003) Bayesian inference of phylogeny: a non-technical primer. Taxon 52: 187–191. <https://doi.org/10.2307/3647388>
- Bailey JA, Jeger MJ (1992) *Colletotrichum*: biology, pathology and control. Wallingford, UK
- Brent KJ (1995) Fungicide resistance in crop pathogens: How can it be managed?. GIFAP, Brussels: FRAC Monograph No 1.
- Buhr TL, Dickman MB (1993) Isolation and characterization of a β -tubulin-encoding gene from *Colletotrichum gloeosporioides* f. sp. *aeschynomene*. Gene 124: 121–125. [https://doi.org/10.1016/0378-1119\(93\)90771-T](https://doi.org/10.1016/0378-1119(93)90771-T)
- Cañas-Gutiérrez GP, Patiño LF, Rodríguez–Arango E, Arango R (2006) Molecular characterization of benomyl-resistant isolates of *Mycosphaerella fijiensis*, collected in Colombia. J Phytopathol 154: 403–409. <https://doi.org/10.1111/j.1439-0434.2006.01113.x>

- Carbone I, Kohn LM (1999) A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91:553-556. <https://doi.org/10.2307/3761358>
- Chung W-H, Chung W-C, Peng M-T, Yang H-R., Huang J-W (2010) Specific detection of benzimidazole resistance in *Colletotrichum gloeosporioides* from fruit crops by PCR-RFLP. *New Biotechnol* 27: 17-24. <https://doi.org/10.1016/j.nbt.2009.10.004>
- Cools HJ, Fraaije B, Lucas JA (2005) Molecular examination of *Septoria tritici* isolates with reduced sensitivity to triazoles. Paper presented at the 14th International Reinhardtsbrunn Symposium; Modern Fungicides and Antifungal Compounds, Friedrichroda, Germany.
- Cunha MG, Rizzo DM (2003) Development of fungicide cross resistance in *Helminthosporium solani* populations from California. *Plant Dis* 87: 798-803. <https://doi.org/10.1094/PDIS.2003.87.7.798>
- Damm U, Cannon PF, Woudenberg JH, Crous PW (2012a) The *Colletotrichum acutatum* species complex. *Stud Mycol* 73: 37-113. <https://doi.org/10.3114/sim0010>
- Damm U, Cannon PF, Woudenberg JH, et al. (2012b) The *Colletotrichum boninense* species complex. *Stud Mycol* 73: 1-36. <https://doi.org/10.3114/sim0002>
- Đinh Q, Chongwungse J, Pongam P, Sangchote S (2003) Fruit infection by *Colletotrichum gloeosporioides* and anthracnose resistance of some mango cultivars in Thailand. *Australas Plant Pathol* 32: 533-538. <https://doi.org/10.1071/AP03053>
- Đinh Q, Sangchote S (2002) Infection of *Colletotrichum gloeosporioides* on mango fruit and its resistance. *J Agric Sci* 33(Suppl.): 68-70.
- Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evol* 39: 783-791. <https://doi.org/10.2307/2408678>
- Fungicide resistance action committee (FRAC) (2020) FRAC Code List©*2020: Fungal control agents sorted by cross resistance pattern and mode of action (including FRAC Code numbering). <https://cpb-us-w2.wpmucdn.com/u.osu.edu/dist/b/28945/files/2020/02/frac-code-list-2020-final.pdf>. Accessed 3 January 2020
- Han H, Cho M, Jeon H, Lim C, Jang H (2004) PCR-RFLP identification of three major *Meloidogyne* species in Korea. *J Asia Pac Entomol* 7: 171-175. [https://doi.org/10.1016/S1226-8615\(08\)60212-5](https://doi.org/10.1016/S1226-8615(08)60212-5)
- Inada M, Ishii H, Chung W, Yamada T, Yamaguchi J, Furuta A (2008) Occurrence of strobilurin-resistant strains of *Colletotrichum gloeosporioides* (*Glomerella cingulata*), the causal fungus of strawberry anthracnose. *Japanese Journal of Phytopathology* 74: 114-117. <https://doi.org/10.3186/jjphytopath.74.114>
- Ishii H, Fraaije B, Sugiyama T, Noguchi K, Nishimura K, et al. (2002) Occurrence and molecular characterization of strobilurin resistance in cucumber powdery mildew and downy mildew. *Phytopathology* 91: 1166-1171. <https://doi.org/10.1094/PHYTO.2001.91.12.1166>
- Jeffries P, Dodd JC, Jeger MJ, Plumbley RA (1990) The biology and control of *Colletotrichum* species on tropical fruit crops. *Plant Pathol* 39: 343-366. <https://doi.org/10.1111/j.1365-3059.1990.tb02512.x>
- Johnston P, 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>
- Kim CH, Hassan O, Chang T (2020) Diversity, pathogenicity, and fungicide sensitivity of *Colletotrichum* species associated with apple anthracnose in South Korea. *Plant Dis* 104: 2866-2874. <https://doi.org/10.1094/PDIS-01-20-0050-RE>

- 477 Kim Y-S, Min JY, Kang BK, Bach NV, Choi WB, Park EW, Kim HT (2007) Analyses of the less
478 benzimidazole-sensitivity of the isolates of *Colletotrichum* spp. causing the anthracnose in pepper and
479 strawberry. Plant Pathol J 23: 187-192. <https://doi.org/10.5423/PPJ.2007.23.3.187>
- 480 Koenraadt H, Somerville SC, Jones AL (1992) Characterization of mutations in the β -tubulin gene of benomyl-
481 resistant field strains of *Venturia inaequalis* and other plant pathogenic fungi. Phytopathology 82: 1348-
482 1354.
- 483 Koffi Y, Diguta C, Alloue-Boraud M, Koffi L, Dje M, Gherghina E, (Radoi) Matei F (2019) PCR-ITS-RFLP
484 identification of pineapple spoilage fungi. Rom Biotechnol Lett 24: 418-424. <https://doi.org/10.25083/rbl/24.3/418.424>
- 485
- 486 Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular evolutionary genetics analysis
487 across computing platforms. Mol Biol Evol 35: 1547-1549. <https://doi.org/10.1093/molbev/msy096>
- 488 Li Q, Bu J, Shu J, Yu Z, Tang L, Huang S et al. (2019) *Colletotrichum* species associated with mango in
489 southern China. Sci Rep 9: 18891. <https://doi.org/10.1038/s41598-019-54809-4>
- 490 Lima NB, de A Batista MV, de Moraes MA, Barbosa MAG, Michereff SJ, Hyde KD, Câmara MPS (2013) Five
491 *Colletotrichum* species are responsible for mango anthracnose in northeastern Brazil. Fungal Divers 61: 75-
492 88. <https://doi.org/10.1007/s13225-013-0237-6>
- 493 Luck JE, Gillings MR (1995) Rapid identification of benomyl resistant strains of *Botrytis cinerea* using the
494 polymerase chain reaction. Mycol Res 99: 1483-1488. [https://doi.org/10.1016/S0953-7562\(09\)80797-1](https://doi.org/10.1016/S0953-7562(09)80797-1)
- 495 Ma Z, Michailides T (2005) Advances in understanding molecular mechanisms of fungicide resistance and
496 molecular detection of resistant genotypes in phytopathogenic fungi. Crop Prot 24: 853-863.
497 <https://doi.org/10.1016/j.cropro.2005.01.011>
- 498 Ma Z, Yoshimura M, Michailides TJ (2003) Identification and characterization of benzimidazole resistance in
499 *Monilinia fructicola* from stone fruit orchards in California. Appl Environ Microbiol 69: 7145–7152.
500 <https://doi.org/10.1128/AEM.69.12.7145-7152.2003>
- 501 Mills PR, Sreenivasaprasad S, Brown AE (1992) Detection and differentiation of *Colletotrichum*
502 *gloeosporioides* isolates using PCR. FEMS Microbiol Lett 98: 137-143. <https://doi.org/10.1111/j.1574-6968.1992.tb05503.x>
- 503
- 504 Mo J, Zhao G, Li Q, Solangi G, Tang L, Huang S et al. (2018). Identification and characterization of
505 *Colletotrichum* species associated with mango anthracnose in Guangxi, China. Plant Dis 102.
506 <https://doi.org/10.1094/PDIS-09-17-1516-RE>
- 507 N Ziogas B, Nikou D, N Markoglou A, A Malandrakis A, Vontas J (2009) Identification of a novel point
508 mutation in the β -tubulin gene of *Botrytis cinerea* and detection of benzimidazole resistance by a diagnostic
509 PCR-RFLP assay. Eur J Plant Pathol 125: 97-107. <https://doi.org/10.1007/s10658-009-9462-y>
- 510 Nagaraju R, Sriram R, Achur R (2019) Antifungal activity of carbendazim-conjugated silver nanoparticles
511 against anthracnose disease caused by *Colletotrichum gloeosporioides* in mango. Plant Pathol J 102: 39-46.
512 <https://doi.org/10.1007/s42161-019-00370-y>
- 513 Nalumpang S, Miyamoto Y, Miyake C, Izumi Y, Akitmitsu K, Kongtragoul P (2010) Point mutations in the
514 beta-tubulin gene conferred carbendazim-resistant phenotypes of *Colletotrichum gloeosporioides* causing
515 ‘Nam Dok Mai’ mango anthracnose. J Agric Sci Technol 6: 365-378.

- 516 National Center for Biotechnology Information (2017) Restriction Fragment Length Polymorphism (RFLP).
 517 <https://www.ncbi.nlm.nih.gov/probe/docs/techrflp/>. Accessed 3 January 2020
- 518 O'Donnell K, Cigelnik E (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage
 519 of the fungus *Fusarium* are nonorthologous. *Mol Phylogenet* 7: 103-116. [https://doi.org/10.1006/mpev.](https://doi.org/10.1006/mpev.1996.0376)
 520 [1996.0376](https://doi.org/10.1006/mpev.1996.0376)
- 521 Peres NAR, Souza NL, Peever TL, Timmer LW (2004) Benomyl sensitivity of isolates of *Colletotrichum*
 522 *acutatum* and *C. gloeosporioides* from citrus. *Plant Dis* 88: 125-130. [https://doi.org/10.1094/PDIS](https://doi.org/10.1094/PDIS.2004.88.2.125)
 523 [.2004.88.2.125](https://doi.org/10.1094/PDIS.2004.88.2.125)
- 524 Pérez-López E, Olivier CY, Luna-Rodríguez M, Dumonceaux TJ (2016) Phytoplasma classification and
 525 phylogeny based on *in silico* and *in vitro* RFLP analysis of *cpn60* universal target sequences. *Int. J. Syst.*
 526 *Evol* 66: 5600-5613. <https://doi.org/10.1099/ijsem.0.001501>
- 527 Phoulivong S, Cai L, Chen H, McKenzie EHC, Abdelsalam K, Chukeatirote E, Hyde KD (2010) *Colletotrichum*
 528 *gloeosporioides* is not a common pathogen on tropical fruits. *Fungal Divers* 44: 33-43. [https://doi.](https://doi.org/10.1007/s13225-010-0046-0)
 529 [org/10.1007/s13225-010-0046-0](https://doi.org/10.1007/s13225-010-0046-0)
- 530 Pongpisutta R, Winyarat W, Rattanakreetakul C (2013) RFLP identification of *Colletotrichum* species isolated
 531 from chilli in Thailand. *Acta Hort* 973: 181-186. <https://doi.org/10.17660/ActaHortic.2013.973.24>
- 532 Poti T, Mahawan K, Cheewangkoon R, Arunothayanan H, Akimitsu K, Nalumpang S (2020) Detection and
 533 molecular characterization of carbendazim-resistant *Colletotrichum truncatum* Isolates causing anthracnose
 534 of soybean in Thailand. *J Phytopathol* 168: 267-278. <https://doi.org/10.1111/jph.12888>
- 535 Prihastuti H, McKenzie E, Hyde K, Cai L, Chen H, McKenzie EHC, Hyde KD (2009) Characterization of
 536 *Colletotrichum* species associated with coffee berries in northern Thailand. *Fungal Divers* 39: 89-109.
- 537 Qiu J, Xu J, Yu J, Bi C, Chen C, Zhou M (2011) Localization of the benzimidazole fungicide binding site of
 538 *Gibberella zeae* β 2-tubulin studied by site-directed mutagenesis. *Pest Manag Sci* 67: 191-198.
 539 <https://doi.org/10.1002/ps.2050>
- 540 Ramdeen S, Rampersad SN (2013) Intraspecific differentiation of *Colletotrichum gloeosporioides* sensu lato
 541 based on *in silico* multilocus PCR-RFLP fingerprinting. *Mol Biotechnol* 53: 170-181.
 542 <https://doi.org/10.1007/s12033-012-9509-8>
- 543 Rosenzweig N, Hanson LE, Clark G, Franc GD, Stump WL, Jiang Q, et al. (2015) Use of PCR-RFLP analysis
 544 to monitor fungicide resistance in *Cercospora beticola* populations from Sugarbeet (*Beta vulgaris*) in
 545 Michigan, United States. *Plant Dis* 99: 355-362. <https://doi.org/10.1094/PDIS-03-14-0241-RE>
- 546 Rungjindamai N (2016) Isolation and evaluation of biocontrol agents in controlling anthracnose disease of
 547 mango in Thailand. *J Plant Prot Res* 56: 306-311. <https://doi.org/10.1515/jppr-2016-0034>
- 548 Sangchote S (2013) Integrated control of anthracnose (*Colletotrichum gloeosporioides*) of mango for export.
 549 *Acta Hort* 973: 55-58. <https://doi.org/10.17660/ActaHortic.2013.973.5>
- 550 Sangchote S (1987) Postharvest diseases of mango fruits and their losses. *Kasetsart Journal-Natural Sciences* 21:
 551 81-85.
- 552 Sreenivasaprasad S, Sharada K, Brown AE, Mills PR (1996) PCR-based detection of *Colletotrichum acutatum*
 553 on strawberry. *Plant Pathol* 45: 650-655. <https://doi.org/10.1046/j.1365-3059.1996.d01-3.x>
- 554 Sutton BC (1980) The Coelomycetes: fungi imperfecti with pycnidia, acervuli and stromata. Kew, UK

- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28: 2731–2739. <https://doi.org/10.1093/molbev/msr121>
- Than PP, Jeewon R, Hyde KD, Pongsupasamit S, Mongkolporn O, Taylor PWJ (2008) Characterization and pathogenicity of *Colletotrichum* species associated with anthracnose on chilli (*Capsicum* spp.) in Thailand. *Plant Pathol* 57: 562-572. <https://doi.org/10.1111/j.1365-3059.2007.01782.x>
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673-4680. <https://doi.org/10.1093/nar/22.22.4673>
- Tredway L, Wong F (2012) Managing anthracnose with fungicides. <https://www.gcsaa.org/uploadedfiles/course/pests-and-diseases/diseases/anthracnose/managing-anthracnose-with-fungicides.pdf>. Accessed 1 March 2020
- Walker J, Nikandrow A, Millar GD (1991) Species of *Colletotrichum* on Xanthium (*Asteraceae*) with comments on some taxonomic and nomenclatural problems in *Colletotrichum*. *Mycol Res* 95:1175-1193. [https://doi.org/10.1016/S0953-7562\(09\)80008-7](https://doi.org/10.1016/S0953-7562(09)80008-7)
- Wei W, Davis RE, Lee I-M, Zhao Y (2007) Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. *Int J Syst Evol* 57: 1855-1867. <https://doi.org/10.1099/ijs.0.65000-0>
- Weir BS, Johnston PR, Damm U (2012) The *Colletotrichum gloeosporioides* species complex. *Stud Mycol* 73: 115-180. <https://doi.org/10.3114/sim0011>
- White T.J, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In MA Innis, DH Gelfand, J Sninsky, TJ White (Eds.), *PCR protocols: A guide to method and application* (pp. 315-322). New York, US
- Worldatlas (2019) The top mango producing countries in the world. <https://www.worldatlas.com/articles/the-top-mango-producing-countries-in-the-world.html>. Accessed 3 January 2020
- Zakaria L (2021) Diversity of *Colletotrichum* species associated with anthracnose disease in tropical fruit Crops-A review. *Agriculture* 11: 297. <https://doi.org/10.3390/agriculture11040297>
- Zhan JS, Wu EJ, Liuand XL, Chen FP (2014) Molecular basis of resistance of phytopathogenic fungi to several site-specific fungicides. *Sci Agric Sin*, 47: 3392-3404. <https://doi.org/10.3864/j.issn.0578-1752.2014.17.007>
- Zhou Y, Xu J, Zhu Y, Duan Y, Mingguo Z (2016) Mechanism of action of the benzimidazole fungicide on *Fusarium graminearum*: interfering with polymerization of monomeric tubulin but not polymerized microtubule. *Phytopathology* 106. <https://doi.org/10.1094/PHYTO-08-15-0186-R>
- Zimand G, Valinsky L, Elad Y, Chet I, Manulis S (1994) Use of the RAPD procedure for the identification of *Trichoderma* strains. *Mycol Res* 98: 531-534. [https://doi.org/10.1016/S0953-7562\(09\)80474-7](https://doi.org/10.1016/S0953-7562(09)80474-7)

Table 1. Primers used in this study with sequences and sources.

Gene/Region	Primer	Sequence (5'-3')	Ta (°C)	References
Actin	ACT-512F	ATGTGCAAGGCCGGTTTCGC	58	Carbone and Kohn (1999)
	ACT-783R	TACGAGTCCTTCTGGCCCAT		
Chitin Synthase 1	CHS-79F	TGGGGCAAGGATGCTTGGGAAGAAG	58	Carbone and Kohn (1999)
	CHS-345R	TGGAAGAACCATCTGTGAGAGTTG		
ITS region	ITS5	GGAAGTAAAAGTCGTAACAAGG	56	White et al. (1990)
	ITS4	TCCTCCGCTTATTGATATGC		
β -tubulin	T1	AACATGCGTGAGATTGTAAGT	55	O'Donnell and Cigelnik (1997)
	T2	TAGTGACCCCTTGGCCCAGTTG		
<i>Ca</i> -specific primer	CaInt	GGGGAAGCCTCTCGCGG	58	Sreenivasaprasad et al. (1996)
	ITS4	TCCTCCGCTTATTGATATGC		
<i>Cg</i> -specific primer	CgInt	GGCCTCCCGCCTCCGGGCGG	57.2	Mills et al. (1992)
	ITS4	TCCTCCGCTTATTGATATGC		

Table 2. Fungicide levels of the *Colletotrichum* species complex to carbendazim at different concentrations (0.1, 1, 10, 100, and 1,000 μ g/mL) and the recommended rate (500 μ g/mL)

Levels	Colony diameters	Resistant levels
-	6.00 mm	Highly sensitive (HS)
+	7.00 – 10.00 mm	Sensitive (S)
++	11.00 – 14.00 mm	Resistant (R)
+++	15.00 mm	Highly resistant (HR)

Table 3. Details of *Colletotrichum* species taxa used in the phylogenetic analysis and all isolates collected from mango anthracnose disease

Species	Isolate/Culture	GenBank accession numbers ¹				References
		ACT ²	CHS-1 ²	ITS ²	TUB ²	
<i>C. asianum</i>	ICMP18580, CBS130418	JX009584	JX009867	FJ972612	JX010406	Wei et al. (2012)
	IMI313839, ICMP18696	JX009576	JX009753	JX010192	JX010384	Wei et al. (2012)
	MAFF306627, ICMP18603	JX009579	JX009825	JX010195	...	Wei et al. (2012)
	HKUCC10862, ICMP18605	JX009465	JX009787	JX010194	...	Wei et al. (2012)
	RB001	LC616858	LC618309	MK215695	LC635528	This study
	RB005	LC616859	LC618310	MK215689	LC635529	This study
	RB007	LC618294	LC618311	MK215692	LC635530	This study
	RB010	LC618295	LC618312	MK215694	LC635531	This study
	RB011	LC618296	LC618313	MK215701	LC635532	This study
	RB012	LC618297	LC618314	MK215700	LC635533	This study
	PC001	LC618298	LC618315	MK212353	LC635534	This study
	PC003	LC618299	LC618316	MK212354	LC635535	This study
	PC004	LC618300	LC618317	MK215703	LC635536	This study
	PC005	LC618301	LC618318	MK215686	LC635537	This study
<i>C. gloeosporioides</i>	ICMP17821, CBS112999	JX009531	JX009818	JX010152	JX010445	Wei et al. (2012)
	ICMP18694	JX009481	JX009796	JX010155	...	Wei et al. (2012)
	ICMP12939	JX009462	JX009747	JX010149	...	Wei et al. (2012)
	ICMP18730	JX009548	JX009861	JX010157	...	Wei et al. (2012)
	CS001	...	...	MK128499	LC628900	This study
	CS002	...	...	MK212348	LC628901	This study
	CS003	...	...	MK212349	LC628902	This study
	CS004	...	...	MK212350	LC628903	This study

	CS005	...	...	MK212351	LC628904	This study
	CS006	...	...	MK215702	LC628905	This study
	CS007	...	...	MK215691	LC628906	This study
	CS008	LC618302	LC618321	MK212352	LC628907	This study
	CS009	...	...	MK215693	LC628908	This study
	CS010	...	...	MK215704	LC628909	This study
	RB002	...	...	MK212942	LC635519	This study
	RB004	LC618303	LC618322	MK215698	LC635520	This study
	RB008	...	...	MK212948	LC635521	This study
	RB009	...	...	MK212947	LC635522	This study
	RB013	...	...	MK215707	LC635523	This study
	RB015	...	...	MK215705	LC635524	This study
	PC008	...	...	MK215687	LC635525	This study
	PC009	LC618304	LC618323	MK215697	LC635526	This study
	PC010	...	...	MK215696	LC635527	This study
<i>C. siamense</i>	ICMP12567	JX009541	JX009761	JX010250	JX010387	Wei et al. (2012)
	ICMP18578, CBS130417	FJ907423	JX009865	JX010171	JX010404	Wei et al. (2012)
	HKUCC10884, ICMP18575	JX009455	JX009785	JX010256	...	Wei et al. (2012)
	HKUCC10881, ICMP18618	JX009512	JX009786	JX010257	...	Wei et al. (2012)
	RB003	LC618305	LC618319	MK212943	LC635538	This study
	RB006	...	...	MK215699	LC635539	This study
	PC002	LC618306	LC618320	MK212355	LC635540	This study
<i>C. acutatum</i>	CBS144.29	JQ949722	JQ949062	JQ948401	JQ950052	Damm et al. (2012a)
	CBS112996, STE-U5292	JQ005839	JQ005797	JQ005776	JQ005860	Damm et al. (2012a)
	CBS111993, STE-U3037	JQ949670	JQ949010	JQ948349	JQ950000	Damm et al. (2012a)
	CBS112760, STE-U4468	JQ949713	JQ949053	JQ948392	JQ950043	Damm et al. (2012a)

<i>C. tropicale</i>	MAFF239933, ICMP18672	JX009480	JX009826	JX010275	JX010396	Wei et al. (2012)
	CBS124949, ICMP18653	JX009489	JX009870	JX010264	JX010407	Wei et al. (2012)
<i>C. fructicola</i>	CBS125395, ICMP18645	JX009543	JX009873	JX010172	JX010408	Wei et al. (2012)
	ICMP18581, CBS130416	JX009501	JX009866	JX010165	JX010405	Wei et al. (2012)
<i>C. nympheae</i>	CBS129926, CPC18719	JQ949537	JQ948877	JQ948216	JQ949867	Damm et al. (2012a)
	CBS515.78	JQ949518	JQ948858	JQ948197	JQ949848	Damm et al. (2012a)
<i>C. simmondsii</i>	CBS122122, BRIP28519	JQ949597	JQ948937	JQ948276	JQ949927	Damm et al. (2012a)
	IMI313840, CPC18875	JQ949605	JQ948945	JQ948284	JQ949935	Damm et al. (2012a)
<i>C. theobromicola</i>	ICMP18567	JX009457	JX009803	JX010287	JX010377	Wei et al. (2012)
	ICMP17895	JX009568	JX009828	JX010284	JX010382	Wei et al. (2012)
<i>C. boninense</i>	CBS128547, ICMP10338	JQ005507	JQ005333	JQ005159	JQ005593	Damm et al. (2012b)
	CBS123755, MAFF305972	JQ005501	JQ005327	JQ005153	JQ005588	Damm et al. (2012b)

¹BRIP: Plant Pathology Herbarium, Department of Employment, Economic, Development and Innovation, Queensland, Australia; CBS = CBS-KNAW, Fungal Biodiversity Centre, Utrecht, Netherlands; HKUCC: The University of Hong Kong Culture Collection, Hong Kong, China; ICMP = International Collection of Microorganisms from Plant, Landcare Research, Auckland, New Zealand; IMI = Culture collection of CABI Europe UK Centre, Egham, UK; MAFF: MAFF Genebank Project, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan; PD: Plantenziektenkundige Dienst Wageningen, the Netherlands; STE-U: Culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa.

²ACT = actin gene; CHS-1 = chitin synthase; ITS = rDNA internal transcribed spacer region; TUB2 = β -tubulin

Table 4. Incidence of infection on mango cv. Nam Dork Mai See Tong fruits and leaves by *Colletotrichum* various species.

Fungal species	Infection incidence (%)		Disease severity (cm)	
	Fruits	Leaves	Fruits	Leaves
<i>C. asianum</i>	75	100	3.93ab	2.45ab
<i>C. gloeosporioides</i>	100	100	4.28a	2.89a
<i>C. siamense</i>	60	100	3.48b	1.75b

Table 5. Level of carbendazim resistance and point mutations at different codons in the β -tubulin gene of 3 species of *Colletotrichum* causing mango anthracnose disease.

Species	Isolate	MIC (μ g/L)	Resistance level	Position of Genotype (bp)			
				167	198	200	240
<i>C. asianum</i>	RB001	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	RB005	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	RB007	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	RB010	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	RB011	100	R	TTC (F)	GAG (E)	TAC (Y)	CTG (L)
	RB012	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	PC001	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	PC003	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	PC004	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	PC005	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
<i>C. gloeosporioides</i>	CS001	10	S	TTC (F)	GAG (E)	TTC (F)	CTG (L)
	CS002	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	CS003	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	CS004	100	R	TTC (F)	GAG (E)	TAC (Y)	CTG (L)
	CS005	100	R	TTC (F)	GAG (E)	TAC (Y)	CTG (L)
	CS006	10	S	TTC (F)	GAG (E)	TTC (F)	CTG (L)
	CS007	10	S	TTC (F)	GAG (E)	TTC (F)	CTG (L)
	CS008	10	S	TTC (F)	GAG (E)	TTC (F)	CTG (L)
	CS009	10	S	TTC (F)	GAG (E)	TTC (F)	CTG (L)
	CS010	100	R	TTC (F)	GAG (E)	TAC (Y)	CTG (L)
	RB002	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	RB004	10	S	TTC (F)	GAG (E)	TTC (F)	CTG (L)
	RB008	100	R	TTC (F)	GAG (E)	TAC (Y)	CTG (L)
	RB009	100	R	TTC (F)	GAG (E)	TAC (Y)	CTG (L)
	RB013	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	RB015	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	PC008	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	PC009	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	PC010	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
<i>C. siamense</i>	RB003	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	RB006	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
	PC002	>1,000	HR	TTC (F)	GCG (A)	TTC (F)	CTG (L)
U14138 ¹	-	-	S	TTC (F)	GAG (E)	TTC (F)	CTG (L)

¹Buhr and Dickman (1993)

Figure legends:

Fig. 1 Field symptoms of mango anthracnose caused by *Colletotrichum* spp. on the (a-b) inflorescences, (c-d) leaves and (e-f) fruits.

Fig. 2 Morphological characteristics of *Colletotrichum* from 3 plantation areas: (A-J) colony on PDA incubated at 25°C under a photoperiod of 12 hr light / 12 hr dark for 5 days, (K-T) cylindrical shape of *Colletotrichum* conidia, and (U-AD) several appressoria (Fig. S1) under a compound microscope at 400X (scale bar = 20 μm).

Fig. 3 A maximum likelihood phylogenetic tree of representative 15 isolates of the *Colletotrichum* species complex using the partial sequence data of the ACT gene, CHS-1 gene, ITS region and TUB gene (red asterisks). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches at black arrows, and the evolutionary distances were computed using the Kimura 2-parameter model combined with a gamma distribution (G). All analyses were conducted in MEGA version X

Fig. 4 Anthracnose symptoms on mango after inoculation with 3 species of *Colletotrichum* using unwounded technique on the (a) fruits and (b) leaves.

Fig. 5 Molecular detection of carbendazim fungicide resistance. (a) Partial β -tubulin sequences of 9 isolates amplified with TB2L/TB2R and compared with the reference sequence U14138 (*C. gloeosporioides* f. sp. *aeschynomene*), (b) Partial protein sequences of 9 isolates aligned with the reference sequence U14138 (colored according to the protein setting in MEGA version X), (c) PCR products subjected to 1.2% agarose gel electrophoresis, (d) Simulated digested products produced via *in silico* restriction digestion using by pDRAW3.2 with Bsh1236I, (e) Bsh1236I-digested product that distinguished between S and R strains, and (f) Secondary structure of β -tubulin; the arrows indicate codons 198 and 200.

Fig. 6 Virtual RFLP gel electrophoresis pattern derived from *in silico* digestion of partial the β -tubulin gene sequence using pDRAW32 software. In the computer-simulated digestions, the full set of eighteen representative enzymes: *ApaI*, *BamHI*, *BstUI*, *Fnu4HI*, *HaeIII*, *HindIII*, *HinfI*, *HpaI*, *HpaII*, *HpyAV*, *MaeIII*, *MboI*, *MseI*, *PstI*, *RsaI*, *SmaI*, *TaqI* and *XmaI* were used. MW is Fermentas 100 bp Plus DNA Ladder. (a) *C. gloeosporioides* – S strain, (b) *C. gloeosporioides* – R strain, (c) *C. gloeosporioides* – HR strain, (d) *C. siamense* – HR strain, (e) *C. asianum* – R strain, and (f) *C. asianum* – HR strain.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementaryinformation.pdf](#)