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Taxonomic and phylogenetic assessment of selected fungal pathogens associated with banana fruits in the local markets of northern Thailand

Binu C. Samarakoon · Milan C. Samarakoon · Dhanushka N. Wanasinghe · Ruvishika S. Jayawardena · Kevin D. Hyde · Putarak Chomnunti

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Abstract Bananas are susceptible to various post-harvest diseases caused by a diverse range of pathogens. The present study focused on the identification and characterization of two prevalent banana fruit diseases, anthracnose and speckle, in three provinces of northern Thailand. Symptomatic banana fruits were collected from local markets. Surface-sterilized, infected banana skin segments were used to isolate the associated fungi on potato dextrose agar. Morphologically distinct isolates of *Colletotrichum* spp. and *Corynespora* sp. were obtained from the anthracnose lesions and speckles, respectively. Fungal identification was based on morphology and phylogenetic analyses of the ITS, LSU, *act*, *cmdA*, *tub*2, *chs*-1, and *gapdh* sequences. *Colletotrichum musae* and *C. siamense* were identified as the causal agents of post-harvest anthracnose in the bananas Kluai Namwa (*Musa acuminata*×*M. balbisiana*; ABB genomic group; Pisang Awak) and Kluai Khai (*M. acuminata*; AA genomic group; Sucrier), respectively. *Corynespora torulosa* was found to cause speckles in Kluai Namwa fruits. Koch's postulates were successfully established by inoculating fresh and unripe banana fruits with the identified strains, and confirmed the pathogenicity. *Colletotrichum musae*, *C. siamense,* and *C. torulosa* were re-isolated from the inoculated fruits and justified with morpho-molecular data. To our knowledge, this is the first confirmed occurrence of *C. torulosa* causing banana fruit speckles in Kluai Namwa in Thailand. In addition, we document the presence of *C. siamense,* causing post-harvest anthracnose in Kluai Khai. This study contributes to a better understanding of post-harvest banana diseases and addresses the current challenges in the commercial banana industry in northern Thailand.

Keywords Anthracnose · *Colletotrichum siamense* · *Corynespora torulosa* · First report · *Musa* · Speckles

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B. C. Samarakoon · R. S. Jayawardena · K. D. Hyde · P. Chomnunti (✉)
School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand
e-mail: putarak.cho@mfu.ac.th

B. C. Samarakoon · R. S. Jayawardena · K. D. Hyde
Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand

M. C. Samarakoon
Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand

D. N. Wanasinghe
Centre for Mountain Futures (CMF), Kunming Institute of Botany, Chinese Academy of Sciences, Honghe, Yunnan 654400, China

Introduction

Bananas (*Musa* spp.) are traded worldwide due to their abundance of essential nutrients, such as antioxidants, fibers, vitamins, and minerals. These nutritional properties offer numerous health benefits to consumers (Kumar et al., 2012). However, bananas are susceptible to various diseases both before and after harvest, primarily caused by fungi (Anthony et al., 2004; Raut & Ranade, 2004; Supriya et al., 2009; Maswada, 2017; Jones, 2019; Xie et al., 2022). The post-harvest deterioration of bananas significantly reduces the shelf-life, market quality, and local preference, leading to substantial financial losses in the commercial banana industry (Amin & Hossain, 2012; Mohapatra et al., 2010).

More than 20 distinct fungal infections have been identified in both pre-harvest banana bunches and throughout the post-harvest handling chain (Alvindia et al., 2002; Jones, 2019). Anthracnose is a significant and destructive disease that has been reported in post-harvest bananas during transportation, storage, and marketing (Simmonds, 1941). Its symptoms are characterized by black sunken lesions with orange to salmon pink spore masses, or acervuli, on the fruit (Simmonds, 1941; Zakaria, 2021). The pathogen invasion occurs at the pre-harvest stage but remains dormant until ripening (Muirhead & Deverall, 1981; Simmonds, 1941; Swinburne & Brown, 1983). The effective penetration of the fungus into the fruit's epidermis is hindered by the accumulation of phytoalexins until the fruit ripens (Brown & Swinburne, 1981).

Several species of *Colletotrichum* (Glomerellaceae, Glomerellales, Sordariomycetes) (Wijayawardene et al., 2022), mostly *C. musae*, have been identified as the causative agents of the disease (Simmonds, 1941; Zhimo et al., 2017; Zakaria, 2021). In addition, *C. chrysophilum* (Mexico; Fuentes-Aragón et al., 2021), *C. gloeosporioides* (Ecuador, Malaysia, Pakistan, Alam et al., 2021; Intan-Sakinah et al., 2013; Riera et al., 2019), *C. karstii*, *C. paxtonii* (China; Huang et al., 2021), *C. scovillei* (China; Zhou et al., 2017), *C. siamense* (Turkey; Uysal & Kurt, 2020), *C. theobromicola* and *C. tropicale* (Brazil; Vieira et al., 2017) have also been reported as anthracnose pathogens in banana fruits. Among them, the pathogenicity of *C. theobromicola* and *C. tropicale* is yet to be confirmed (Vieira et al., 2017).

Fruit speckles on bananas are also known as *Deightoniella* spots, pin-spotting, salt and pepper spots, and swamp spots (Jones, 2019). This disease has been found on all cultivars of banana, with Lady-finger being notably more susceptible than others (Jones, 2019). The symptoms appear on the fruit skin at the pre-harvest stage as superficial, reddish to black minute spots, often with water-soaked margins (Pasberge-Gauhl, 2002; Vawdrey, 2008; Almenares & Pérez-Vicente, 2019; Jones, 2019). Several pathogens can cause fruit speckles, and the etiology of the disease has become complex (Jones, 2019; Pasberg-Gauhl, 2000; Vawdrey & Campagnolo, 2000). However, Meredith (1961) first described the symptoms caused by *Corynespora torulosa* (Corynesporascaceae, Pleosporales, Dothideomycetes) (Wijayawardene et al., 2022) in Jamaica. The pathogen was also subsequently reported in Australia (Vawdrey, 2008) and Cuba (Almenares & Pérez-Vicente, 2019). Some of the speckle symptoms on banana fruits were different from those caused by *C. torulosa* which onsets broader lesions (Pasberg-Gauhl, 2000; Vawdrey & Campagnolo, 2000). Therefore, identification of the true causative agents of banana fruit speckles is challenging, necessitating further investigations (Pasberg-Gauhl, 2000; Vawdrey & Campagnolo, 2000; Jones, 2019).

Thailand is the second-largest banana exporter in the ASEAN region (Suvittawa, 2014; Termpitipong, 2021), hosting approximately 50 varieties (Anupunt, 2002). Bananas are a significant part of local Thai cuisine and play a vital role as raw materials for various economic products (Bansiddhi, 2003). Three clones, namely Kluai Hom Thong (*Musa acuminata*; AAA genomic group; Gros Michel), Kluai Khai (*M. acuminata*; AA genomic group; Sucrier), and Kluai Namwa (*M. acuminata*×*M. balbisiana*; ABB genomic group; Pisang Awak), are commercially grown in northern Thailand for their high yield and adaptability (Anupunt, 2002; Bansiddhi, 2003). Many studies have investigated fungal pathogens in Hom Thon bananas (Gros Michel) due to their high availability in commercial markets. However, there is a lack of research on post-harvest fungal diseases in Kluai Namwa and Kluai Khai bananas in Thailand. This study aims to fill a research gap by investigating the fungal pathogens associated with anthracnose and speckles in Kluai Namwa and Kluai Khai fruits in the northern region.

Bananas were collected from local markets in Chiang Mai, Chiang Rai, and Nan provinces in northern Thailand from 2018 to 2022. A total of 56 fungal isolates were obtained during the study. The latter were categorized into three primary groups based on cultural characteristics and microscopic morphology. From these, nine were selected for DNA extraction based on the severity of the initial symptoms where the cultures were isolated. Phylogenetic relationships were established for the pathogens and morphological illustrations and descriptions were provided. This is the first report of *Corynespora torulosa* causing fruit speckles on Kluai Namwa in Thailand. We identified *Colletotrichum siamense* from the post-harvest anthracnose lesions on Kluai Khai with confirmed pathogenicity tests for the first time in Thailand. Additionally, *C. musae* was also recognized as causing anthracnose on Kluai Namwa fruits in northern Thailand.

Materials and methods

Sample collection and isolation of fungi

Two banana clones, viz., Kluai Khai (*Musa acuminata*; AA genomic group; Sucrier clone) and Kluai Namwa (*M. acuminata* × *M. balbisiana*; ABB genomic group; Pisang Awak clone), were selected for the study. Post-harvest banana bunches (peel colour: natural green to yellow with brown spots; 13 to 16 weeks old after the formation of the inflorescence) were randomly sampled. The collection sites were the local markets in Chiang Mai, Chiang Rai, and Nan provinces in northern Thailand. During 2018–2022, two main seasons in the region were selected for the study: the dry season (November–May) and the rainy season (June–October). Clones were chosen based on their availability, public preference, and continuous year-round fruit supply to local markets. Besides research significance, clone selection also incorporated input from cultivators and sellers, along with reliable information on fruit physiology and age.

Based on the Von Loesecke ripening scale (Companhia de Entrepostos e Armazéns Gerais de São Paulo, 2006), the ripening stages of bananas were divided into seven categories: Stage 1 = entirely green peel; Stage 2 = green peel with traces of yellow; Stage 3 = more green than yellow peel; Stage 4 = more yellow than green peel; Stage 5 = yellow peel with traces of green; Stage 6 = entirely yellow peel; Stage 7 = yellow peel with brown spots. Additionally, the maturity of the banana specimens was documented in collaboration with both the commercial seller and the cultivator. The days were counted in weeks since the formation of the banana inflorescence.

Anthracnose symptoms were characterized by black, sunken lesions on the fruit, often with spore masses or acervuli on the necrotic surface (Abayasekara et al., 2013). Twenty banana bunches showing the latter symptoms (10 from each clone) were collected. The specimens were next classified into three main categories based on the severity of disease symptoms, as described in Table 1 (supplementary materials). Subsequently, ten bunches of Kluai Namwa bananas showing speckles (superficial, minute black to brown spots with water-soaked margins; Vawdrey, 2008) were collected. The categorization and symptom assessment of the diseased fruits were conducted according to Table 2 (supplementary materials). All the specimens were taken in sealed polythene bags to the laboratory, after which the disease symptoms were recorded and photographed. The symptomatic fruits were separated from the bunches where necessary. The details of the post-harvest physiology of the specimens, collection sites, climatic conditions, and symptom evaluation are attached in the supplementary material section (Table 3).

Fungi were isolated from infected lesions on different banana specimens (Table 3). Tissue segments were separated from the advancing edges of anthracnose lesions (5×5 mm^2) and speckles (2×2 mm^2) on banana skins using a sterilized blade. Infected pieces were surface sterilized using 70% ethanol and 1% sodium hypochlorite (NaClO) for one to three minutes, followed by rinsing twice in sterile distilled water (Senanayake et al., 2020). The additional chemicals were removed by drying them on sterile filter paper. Tissue segments (four per plate) were transferred onto PDA plates boosted with 50 µg/mL^{-1} tetracycline to inhibit bacterial growth under aseptic conditions and incubated at 25 °C for seven days.

A total of 183 isolates were obtained from the initial plates. From these, 56 isolates were selected, transferred to new PDA plates, and then incubated for 10–14 days at 25 °C under light conditions. Pure cultures were grouped into three categories (CGA = *Colletotrichum*

group A, CGB = *Colletotrichum* group B, and CORGC = *Corynespora* group C) (Table 3), based on colony characters and microscopic features (i.e., the morphology of conidia and conidiophores). Subsequent identification revealed that CGA is *Colletotrichum musae*, CGB is *C. siamense*, and CORGC is *Corynespora torulosa*. Morphological identification (Table 3) followed comparisons with reference specimens: *C. musae* (epitype = CBS 116870; Su et al., 2011), *C. siamense* (holotype = MFLU 090230; Prihastuti et al., 2009), and *C. torulosa* (epitype = CBS H-21456; Crous et al., 2013).

The colony characteristics were examined after 21 days. To prepare herbarium specimens, cultures were grown in water agar with 1.5% glycerol and kept for sporulation under light conditions. The plates were placed on the aluminium tray with silica gel and kept in a desiccator. Dried cultures were stored in wax paper bags with silica gel and deposited in the Fungarium of Mae Fah Luang University (Herb. MFLU), Chiang Rai, Thailand. For further study, living cultures were submitted to the Culture Collection Unit of Mae Fah Luang University (MFLUCC).

DNA extraction, PCR amplification and sequencing

Out of the initial 56 isolates, nine fungal cultures were chosen for DNA extraction, with three coming from each group (CGA, CGB, and CORGC). These selections represented mild (1/5), moderate (2/6), and severe (3/7) disease categories (Fig. 1). The isolates that were selected to generate molecular data are shown in bold in Table 3. Mycelium scrapings from fourteen-day-old cultures were crushed using metal beads with TissueLyser LT (QIAGEN Sample and Assay Technologies; Miami, Florida, United States). The DNA extraction was performed using the column-based PureDireX Genomic DNA Isolation Kit (Blood/Cultured Cell/Fungus; New Taipei City, Taiwan) following the manufacturer's guidelines.

DNA sequence data was generated for eight gene regions: Internal transcribed spacer (ITS), partial 28S large ribosomal subunit (LSU), actin (*act*), beta-tubulin 2 (*tub2*), chitin synthase (*chs-1*), glyceraldehyde-3-Phosphate dehydrogenase (*gapdh*), the second largest subunit of the DNA-directed RNA polymerase II (*rpb2*), translation elongation factor 1-alpha gene (*tef1-α*). The sequencing methodology followed Jayawardena et al., (2021) for *Colletotrichum* and Voglmayr and Jaklitsch, (2017) for *Corynespora*.

Specific primers were used to amplify the gene regions, including ITS5/ITS4 (White et al., 1990), LR0R/LR5 (Vilgalys & Hester, 1990), ACT512F/ACT783R (Carbone & Kohn, 1999), Bt2a/Bt2b (Glass & Donaldson, 1995), CHS-79F/CHS345R (Carbone & Kohn, 1999) and GDF1/GDR1 (Templeton et al., 1992).

Polymerase chain reaction (PCR) was conducted following the protocol of Samarakoon et al., (2021), with a total of 40 cycles. The annealing temperatures for the different gene regions ranged from 53 °C (ITS and LSU), 54 °C (*act*), 56 °C (*chs-1*) and 58.5 °C (*gapdh* and *tub2*) with a 1 min annealing time. The amplified PCR fragments were sent to SolGent Co., Ltd. South Korea, for sequencing. The sequence data have been generated and deposited in GenBank for further analysis and reference.

Pathogenicity tests

Three fungal isolates (MFLUCC 23-0032, MFLUCC 23-0033, and MFLUCC 23-0027) were used for pathogenicity tests. A banana grower and a sanitized cultivation from the Rattana dormitory area (Nang Lae village, Chiang Rai) were selected for the fruit supply. The age of the banana inflorescences was monitored. Uniformly sized banana hands, specifically 12–13 weeks old (ripening stage 1 = entirely green peel; based on the Von Loesecke ripening scale), were selected for artificial inoculation. These banana hands were chosen from both Kluai Namwa and Kluai Khai clones. Fruits with blemishes and disease symptoms were excluded. The banana hands were washed with running water and surface-sterilised with 70% ethanol. Next, the fruits were rinsed with sterilized distilled water and air-dried for 3–6 h. Fingers were not separated from the hands, and the crown area was sealed with parafilm to reduce water loss and secondary pathogen invasion.

To prepare the conidial suspension, the mycelia were scraped, mixed with distilled water, and filtered through sterilized glass wool. The final concentration of conidia was set to 1×10^6 mL^{-1} using a hemocytometer (Neubauer Counting Chamber, Kyrios-Soter Scientific; made in Germany). Twenty microliters of the conidial suspension from each fungal isolate were applied to three evenly spaced sites along sterilized

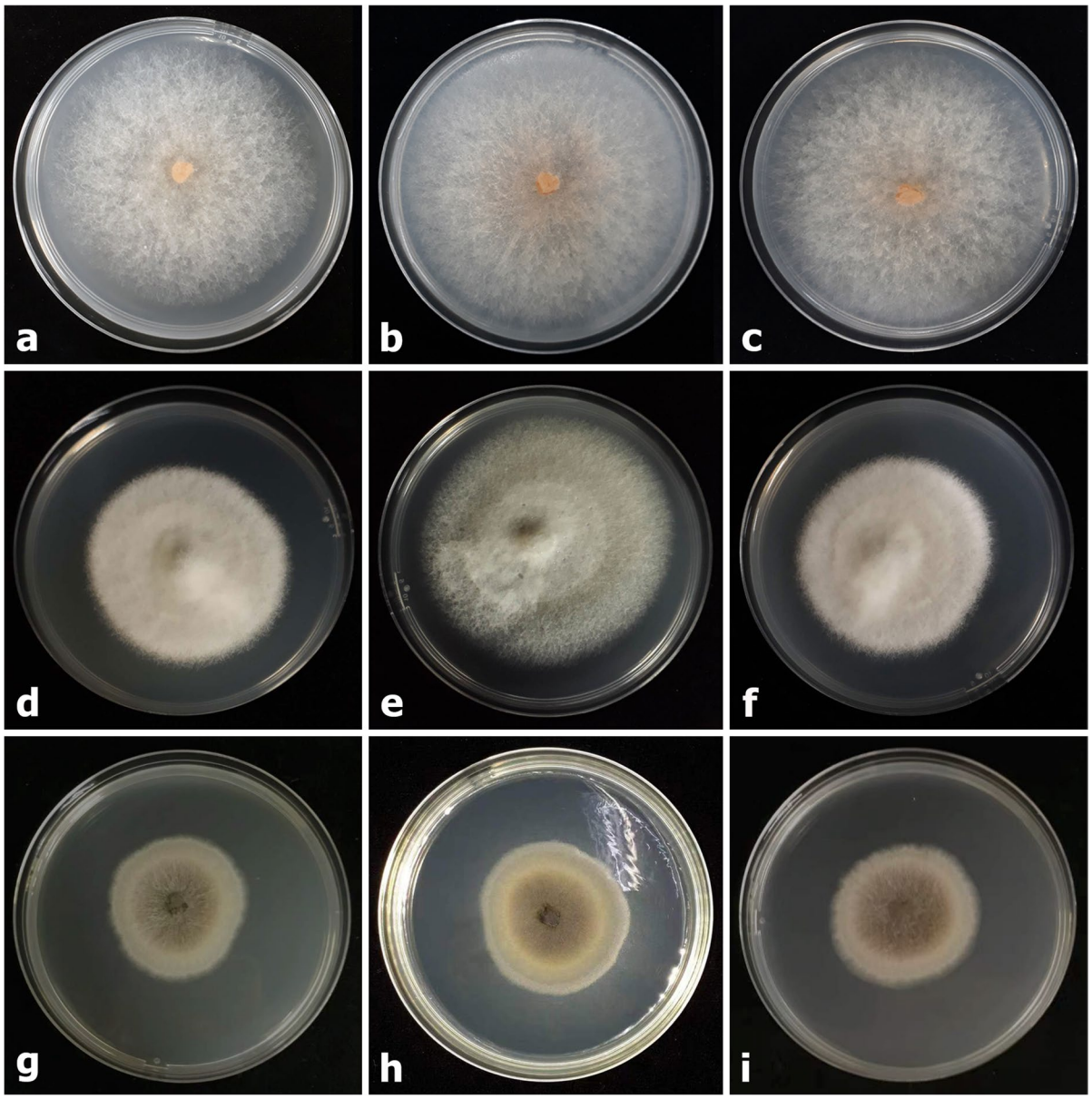


Fig. 1 Fungal colonies grow on the PDA after 7 days,.' **a–c** *Colletotrichum musae* (a = MFLUCC 23-0032; b = MFLUCC 23-0219; c = MFLUCC 23-0220); **d–f** *Colletotrichum siamense* (d = MFLUCC 23-0033; e = MFLUCC 23-0034; f = MFLUCC 23-0222); **g–i** *Corynespora torulosa* (g = MFLUCC 23-0027; h = MFLUCC 23-0028; i = MFLUCC 23-0029)

non-wounded and wounded fruit skins. The suspension was applied from the stem end to the blossom end of the fruits (Fig. 8). Nine replicates of fruits were used for each fungal isolate from the respective cultivar. Twenty microliters of sterilized distilled water were inoculated on fruits as a control treatment. Artificially inoculated fruits were incubated in separate moisture chambers at 28–30 °C. The treatments were examined daily, and the onset of disease symptoms was recorded and compared with those of the original diseased fruits. Fungal pathogens were re-isolated from the diseased symptoms of the fruit skins on PDA. Cultural characteristics and conidial morphology were compared with those of the original

isolates used during the challenged inoculation. The originality has been further confirmed with molecular data. Two parallel sets of experiments were conducted. The results of the experiment are attached herein as Fig. 8.

Sequence alignment and phylogenetic analyses

The DNA sequences generated in the study were checked with a BLAST search in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This revealed that our isolates belong to the *Colletotrichum gloeosporioides* species complex (Jayawardena et al., 2021; Weir et al., 2012) and Corynesporascaceae (Voglmayr & Jaklitsch, 2017). Additional sequences used in the analyses were obtained from GenBank based on recent publications (Talhinhas & Baroncelli, 2021; Voglmayr & Jaklitsch, 2017) (Table 1 and 2 in the supplementary material section). Single- and multigene alignments were generated, and phylogenetic analysis (Maximum likelihood trees and Bayesian analysis) was conducted following the parameters outlined by Samarakoon et al. (2021). Branches with Bayesian posterior probabilities (PP) equal to or greater than 0.95 are indicated above each node of the phylogenetic trees (Figs. 5 and 7).

Results

Isolation summary

Fungi were isolated from infected lesions; 58 isolates from anthracnose symptoms and 40 isolates from speckles symptoms in different banana specimens (Table 3). A total of 56 fungal isolates were selected for further investigation (Table 3, Column 17). Among these, 24 cultures were obtained from the anthracnose symptoms of Kluai Namwa. The latter were identified as *Colletotrichum musae* and categorized as group CGA (Table 3). Out of these, 21 were identified based solely on their morphology, while three (MFLUCC 23-0032; MFLUCC 23-0219; MFLUCC 23-0220) were confirmed using both morphology and phylogeny (Figs. 1, 3 and 5). Sixteen isolates were identified as *C. siamense*, with 13 confirmed only from morphology and three (MFLUCC 23-0033; MFLUCC 23-0034; MFLUCC 23-0222) identified using both morpho-molecular data (Figs. 1, 4 and 5). These isolates were obtained from the anthracnose symptoms of Kluai Khai and represented group CGB (Table 3). A total of 14 *Corenespora torulosa* isolates were selected from speckles in the Kluai Namwa. Among them, three (MFLUCC 23-0027; MFLUCC 23-0028; MFLUCC 23-0029) were identified based on morphology (Figs. 1 and 6) coupled with molecular data (Fig. 7), and the remaining 11 were identified solely through morphology. These isolates formed the CORGC group in Table 3.

Post-harvest anthracnose in banana fruits

Pathogenicity test

Two *Colletotrichum* species (viz., MFLUCC 23-0032 = *C. musae* and MFLUCC 23-0033 = *C. siamense*) were able to cause typical anthracnose lesions on the non-wounded fruit surface after 4–6 days of the artificial inoculation. At first, the symptoms appeared as minute reddish-brown pinholes and later turned into black sunken necrotic patches (Fig. 8h–r) on the fruits of ripening index 3 and 4. Seven days after inoculation, the anthracnose symptoms were notably identical to the original symptoms from which the latter *Colletotrichum* species were first isolated (Fig. 2a–f). The control fruits did not develop any disease symptoms. Fungi were re-isolated from the disease areas to establish Koch's postulates. The conidial and colony morphologies of the re-isolated pathogens were similar to those of the initial isolates used for inoculation. On the wounded peel, symptoms appeared after 2–4 days of artificial inoculation, similar to those observed on the non-wounded method. *Colletotrichum musae* (MFLUCC 23-0032) provoked symptoms on the wounded fruit surface after 2 days in ripening stages 1 and 2 (entirely green peel and green with traces of yellow peel). In addition, *C. siamense* (MFLUCC 23-0033) initiated symptoms after 3 days on more yellow than green (ripening stage 4) wounded fruits.

Symptom development observed on the collected fruits during the study

The anthracnose infection caused by *Colletotrichum musae* initially appeared as brown to black spots of 0.5–0.8 cm in diameter on the skin (ripening stage 6 and 7) (Fig. 2a). The lesions were initially rounded

Fig. 2 Anthracnose and speckle symptoms that have been reported in the study. **a** Initiation of anthracnose symptoms caused by *Colletotrichum musae* (fruits of Namwa banana); **b**, **c** Diamond-shaped necrotic patches and severe symptoms with salmon orange spore masses (in the fruits of Namwa banana) caused by *C. musae*; **d**, **e**, **f** Necrotic lesions and the severe symptoms in the skin with round necrotic patches, concentric rings and salmon pink spore masses caused by *C. siamense* in Khai banana fruits; **g**–**j** Namwa banana fruits displaying speckle symptoms; **h**, **i** Necrotic spots surrounded by water-soaked margins. The notable symptoms are indicated by an arrow

or irregular in shape and sometimes took on a diamond shape (Fig. 2b) at the curved fruit shoulders. The size of the lesions increased to 2–3 cm. The fruit tissue has sunken and appears black upon ripening, however, the pulp remained unaffected. Sporulation (Fig. 2b, c) and white mycelial exudates were detected after 2 to 4 days of the first symptom onset on the skin. The spores were orange to salmon pink and located as slimy, sticky droplets or masses on the mature necrotic tissue. When overripe (16 weeks old), the sunken lesions, together with conidial droplets, started fusing with the adjacent diseased areas

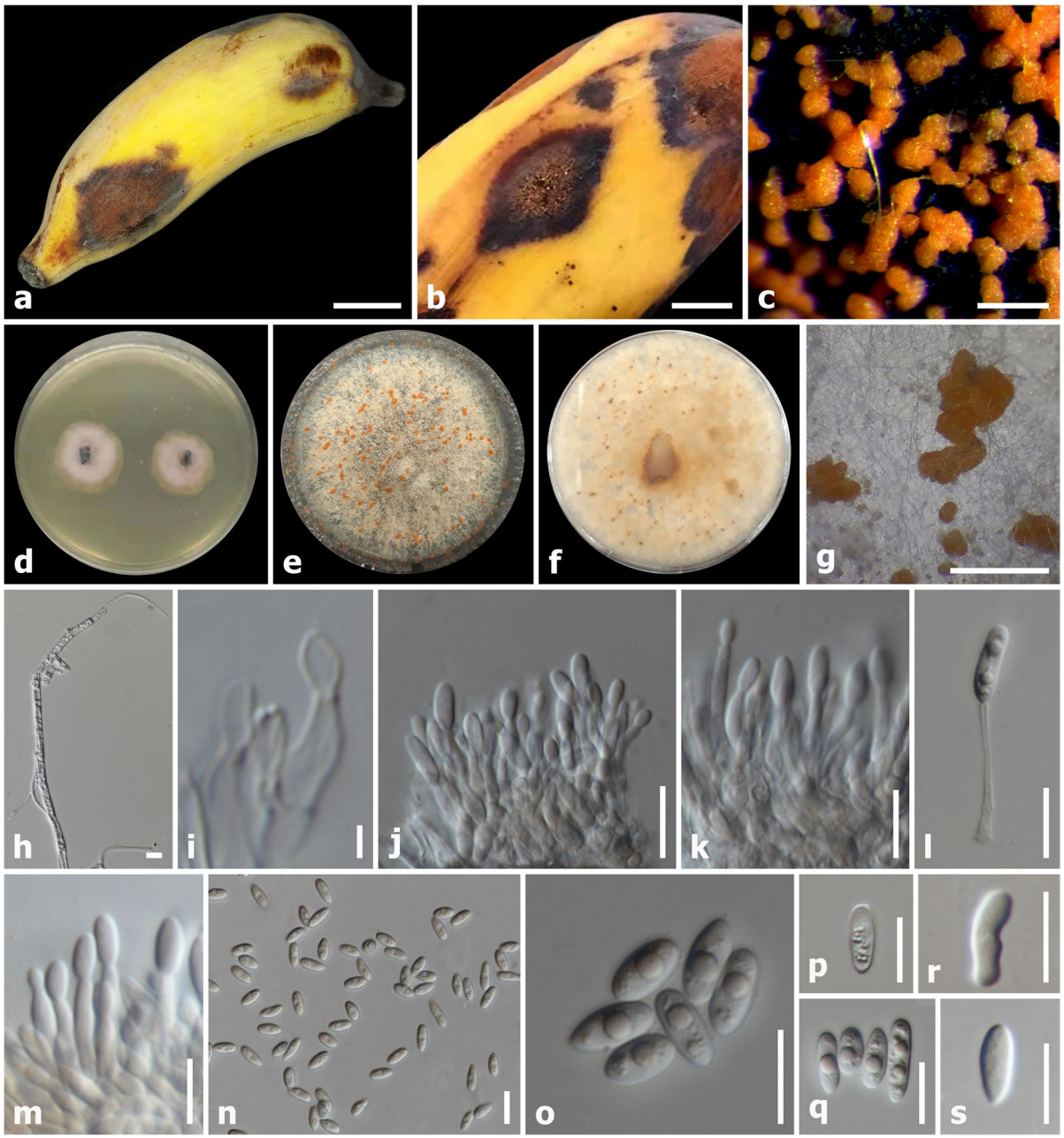


Fig. 3 *Colletotrichum musae* (MFLU 23-0279). **a**, **b** Anthracnose lesions on ripened banana skin; **c** Salmon orange conidial masses; **d** Colony on PDA formed from isolated diseased tissues after 3 days'; **e**, **f** Colonies on PDA after 10 days'; **g** Conidial masses on PDA; **h** Fungal hyphae **i**–**m** Attachments of conidiophores and conidia; **n**–**s** Conidia. Scale bars: 10 mm (**a**, **b**); 200 µm (**c**, **g**); 20 µm (**j**, **k**); 15 µm (**l**–**s**); 5 µm (**h**, **i**)

(Fig. 2c). In addition, the infection was spreading through the peripheral tissues of the fruit pulp. Multiple infections were observed after ripening stage 7 (16 to 17 weeks old) from different angles of the fruit. The decay had started, and the skin tissues were completely deteriorated. The infection had invaded the entire fruit after 8–10 days of ripening stage 7. Additionally, the fruit was completely covered

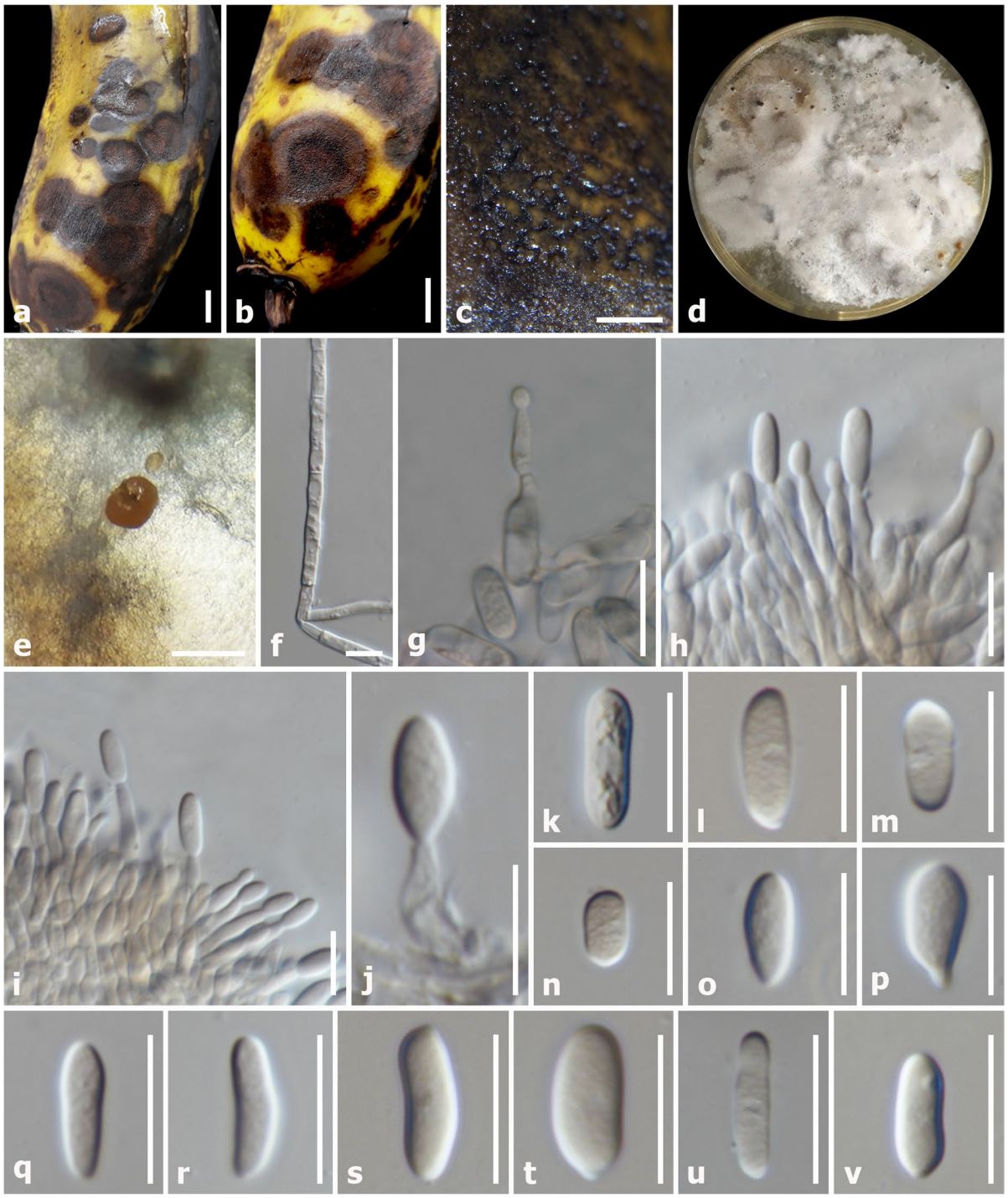


Fig. 4 *Colletotrichum siamense* (MFLU 23-0282). **a**, **b** Anthracnose lesions on ripened banana skin; **c** Salmon orange conidial masses; **d** Colony on PDA 14 days'; **e** Conidial masses on PDA **f** Fungal hyphae; **g–j** Attachments of conidia; **k–v** Conidial morphology. Scale bars: 1 cm (**a**, **b**); 500 µm (**c**, **e**); 15 µm (**g–v**); 5 µm (**f**)

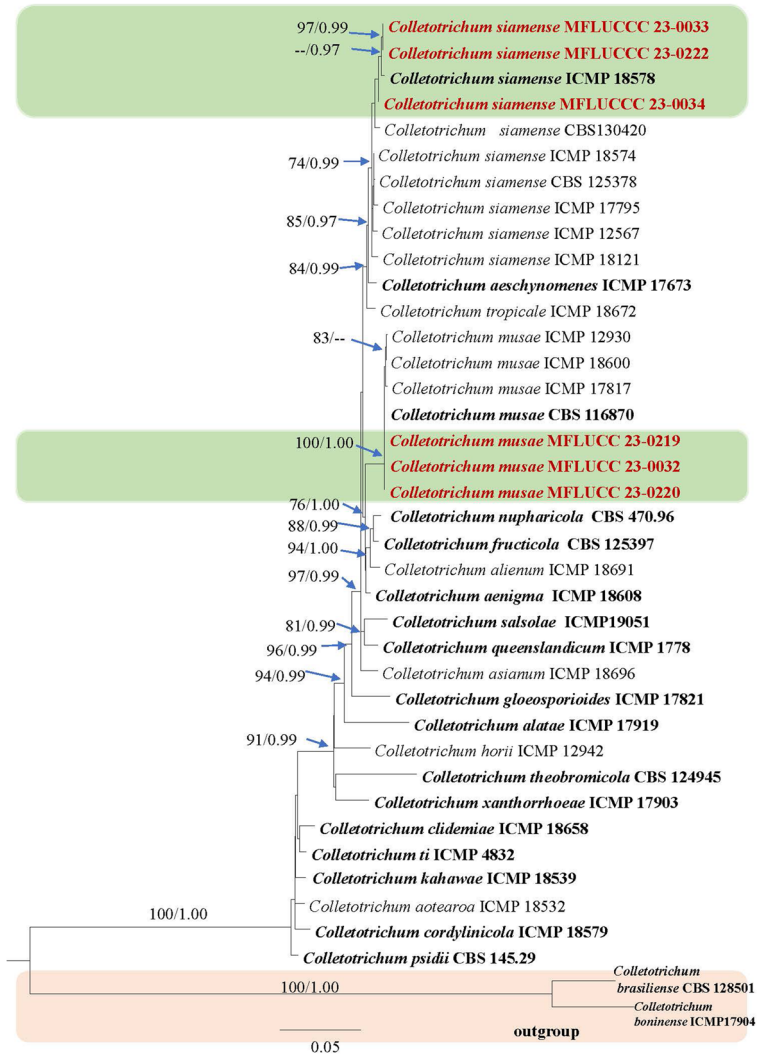


Fig. 5 Maximum likelihood tree generated by RAxML analyses of ITS, *gapdh*, *cmdA*, *act*, and *chs-1* sequences for the selected taxa of the *Colletotrichum gloeosporioides* complex, revealing the phylogenetic position of *C. musae* (MFLUCC 23-0032, MFLUCC 23-0219, MFLUCC 23-0220) and *C. siamense* (MFLUCC 23-0033, MFLUCC 23-0034, MFLUCC 23-0222). Bootstrap supports (≥70%) and posterior probabilities (≥0.95 PP) are displayed above the nodes. The roots of the phylogenetic tree are *C. boninense* (ICMP 17904) and *C. brasiliense* (CBS 128501). Strains generated in this study are bold and indicated in red. Ex-types, ex-epitypes and reference strains are bold and black-indicated. The scale bar shows the expected number of nucleotide substitutions per site. The best scoring RAxML tree is presented herein. The final ML optimization likelihood value of the tree is -7786.36. The matrix had 523 distinct alignment patterns and 10.61% of undetermined characters or gaps. Estimated base frequencies; A = 0.221514, C = 0.296385, G = 0.257678, T = 0.224423; substitution rates: AC = 1.007186, AG = 3.208328, AT = 0.791816, CG = 0.636968, CT = 5.586597, GT = 1.0; the proportion of invariable sites: I = 0.459373; the gamma distribution shape parameter: α = 1.567405

with conidial masses with a salmon-pink to orange appearance.

Colletotrichum siamense infection also causes black, sunken anthracnose lesions that are often rounded and merged with adjacent diseased tissues (Fig. 2d, e, f). The symptoms were recorded on the fruit skins of Kluai Khai at the post-harvest stage. Invasion and symptom onset were comparatively slow with respect to *C. musae*. The appearance of the first symptom on the skin was noted after 7–8

days of complete ripening (stage 7) with a diameter of 0.3–0.4 cm. The lesions expanded upon aging to 1–1.5 cm and coalesced later. Sporulation started on the lesion after 8–10 days of complete ripening. Slimy and sticky spore masses were visible in blackish-salmon pink and were sparse compared to the *C. musae* infection. When the fruit has overripened (15.5 to 16.5 weeks old), the sunken lesions on Kluai Khai fruit skins display a concentric ring pattern (Fig. 2f), dividing the necrotic area into 2–3 distinct zones. The invasion of *C. siamense* was comparatively slow when compared to *C. musae*. The decay of the fruits was notably slow and started after 9–11 days of ripening stage 7. When the necrotic regions began to merge at the final stage (16 to 17 weeks old), the fruit pulp had already been infected (Fig. 2f).

Taxonomy of Colletotrichum

Colletotrichum musae **(Berk. & M.A. Curtis) Arx**

Index Fungorum: IF295348, Facesoffungi Number: FoF121412 Fig. 3

Pathogenic on ripened fruits Kluai Namwa (*M. acuminata* x *M. balbisiana*; ABB genomic group; Pisang Awak). *Colonies* on PDA at 25 °C, light conditions, reach 5 cm diameter in 4 days, initially greyish white, irregular or entire or wavy margin. *Mycelium* initially flat and dense in the middle, sparse at periphery, when mature entire culture is sparse or abundant, fluffy, aerial *Fungal hyphae* 1.5–4.5 µm ($\bar{x}$ = 2.7 µm) in width, septate, smooth to rough, inflated, often hyaline at immaturity, branched, orange exudates present in mature mycelia. *Sporulation* occurs from the centre after 6 to 7 days and speeded to periphery under 25 °C, salmon pink or orange, slimy, sticky, well-developed conidial masses observed at the entire colony after 8 to 9 days, reverse pinkish orange, *Sclerotia* absent. *Setae* absent. *Conidiophores*, 10–30 µm long × 2–5 µm diam. ($\bar{x}$ = 20.5 × 3.8 µm, *n* = 30) micronematous, hyaline, cylindrical, tapered toward the apex, erect and straight, sometimes slightly flexuous, unbranched, aseptate, arising from the mycelium, base swollen and globose, irregularly shaped, apex pointed. *Conidiogenous cells* 2–4 × 1–3 µm ($\bar{x}$ = 3.7 × 1.8 µm, *n* = 20), hyaline, monoblastic, terminal, cylindrical, having rounded apices, no collarette. *Conidia* 10–15 × 7–4 µm ($\bar{x}$ = 13.4 × 5.33 µm, *n* = 40), abundant, hyaline, aseptate, guttulate, oval, elliptical or cylindrical, sometimes elongated and flexuous, often with acute base, rounded apex. Sexual morph: Not observed.

Materials examined: Thailand, Chiang Rai, Nang Lae, Fah Thai local market, associated with an anthracnose lesion of ripened fruit skin of Kluai Namwa, 15 October 2021, Binu C. Samarakoon, SM30 (MFLU 23-0279), living culture MFLUCC 23-0032. Thailand, Chiang Rai, Ban Du municipality market, on anthracnose lesion of a fruit bunch of Kluai Namwa, 23 November 2021, Binu C. Samarakoon, SM31 (MFLU 23-0280), living culture MFLUCC 23-0219. Thailand, Chiang Mai, Saraphi, associated with anthracnose lesions of Kluai Namwa, 16 April 2022, Binu C. Samarakoon, SM33 (MFLU 23-0281), living culture MFLUCC 23-0220.

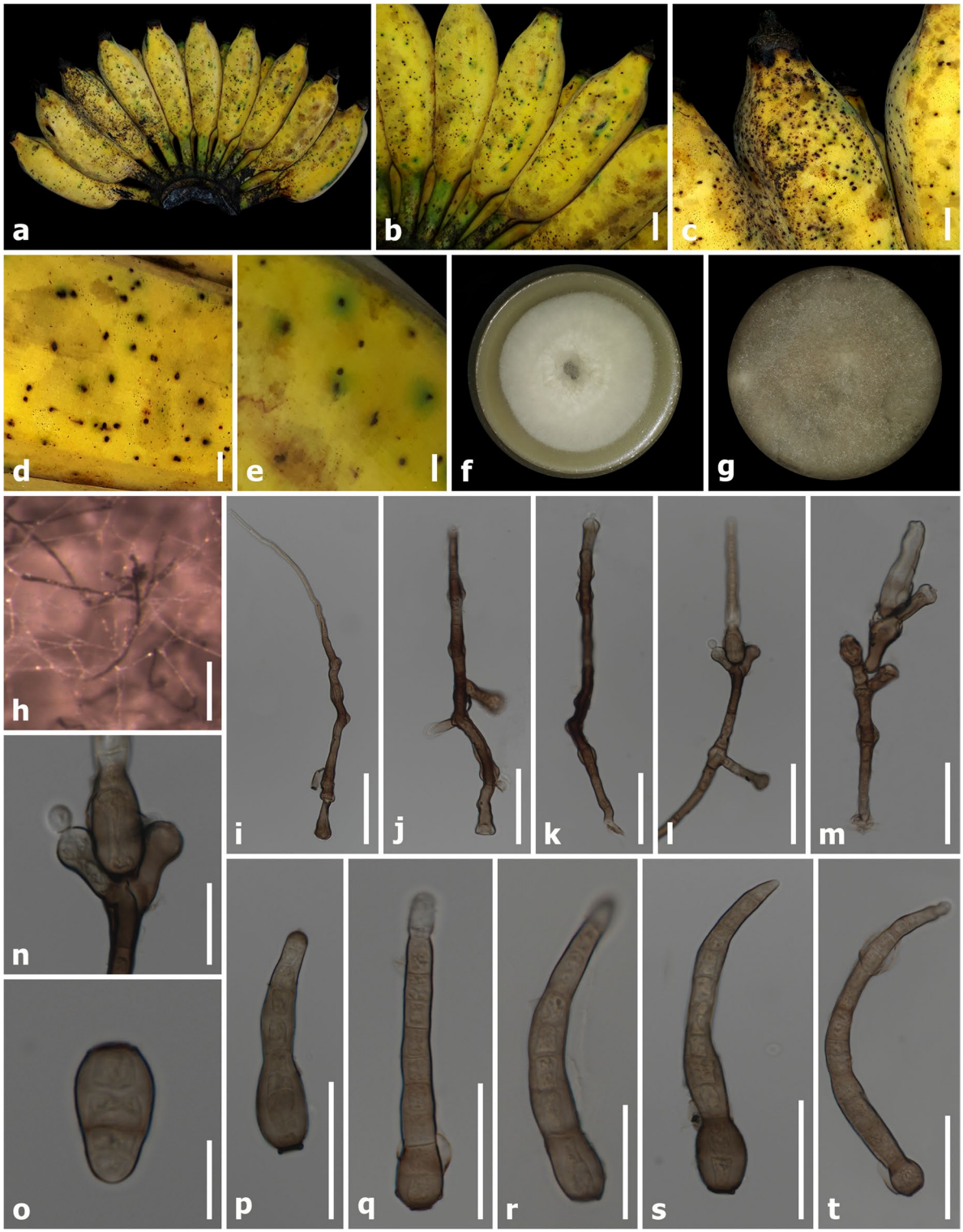
GenBank accession numbers: MFLUCC 23-0032: ITS = OR186193, *act* = OR209335, *chs*-1 = OR192988, *gapdh* = OR192994; MFLUCC 23-0219: ITS = OR186194, *act* = OR209336, *chs*-1 = OR192989, *gapdh* = OR192995; MFLUCC 23-0220: ITS = OR186195, *act* = OR209337, *chs*-1 = OR192990, *gapdh* = OR192996.

Notes: According to the BLASTn search results of ITS, *gapdh, act,* and *chs-1* sequences, MFLUCC 23-0032, MFLUCC 23-0219 and MFLUCC 23-0220 showed a 100% similarity to *Colletotrichum musae* (ICMP12930). In the multigene phylogeny, our strains were grouped with *C. musae* with strong statistical support (100% ML; 1.00 BYPP) (Fig. 5). Based on morpho-molecular support, we report the occurrence of *Colletotrichum musae* on the anthracnose lesions of Kluai Namwa in Thailand.

Colletotrichum siamense **Prihastuti, L. Cai & K.D. Hyde**

Index Fungorum number: IF515410, Facesoffungi Number: FoF 03599 Fig. 4

Pathogenic on ripened fruits of Kluai Khai. *Colonies* on PDA at 25 °C, light conditions, reach 5 cm diameter in 4 days, initially white, mouse grey or greyish white or pinkish-white or brownish, irregular or lobate margin. *Mycelium* initially cottony and flat, when mature sparse, aerial, 3–6 µm ($\bar{x}$ = 3.5 µm) wide, septate, branched, rough, inflated, often hyaline at immature stage, brown exudates present at maturity. *Sporulation* occurs from the inoculated point after 14 to 28 days, few conidial masses were visible 25 °C, salmon pink or orange, slimy, sticky, reverse mouse gray or greyish white, *Sclerotia* present in some isolates. *Setae* not observed.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◂**Fig. 6** *Corynespora torulosa* (MFLU 23-0321). **a**, **b**, **c** Speckle symptoms on ripened banana skin; **d**, **e** Minute halos with water-soaked margins; **f** Colony on PDA after 14 days'; **g** Colony on PDA after 21 days'; **h** Sporulation on mycelium; **i**–**k** Conidiophores; **l**–**n** Attachment of conidia; **o**–**t** Conidia. Scale bars: 0.5 cm (**b**, **c**, **d**, **e**); 100 µm (**q**, **t**); 50 µm (**i**, **j**, **k**–**m**, **o**, **p**); 5 µm (**f**)

Conidiophores 10–25– (40)– (55) µm long × 2–7 µm diam. ($\bar{x}$ = 15.4 × 4.7 µm, *n* = 30) micronematous, hyaline, cylindrical, tapered toward the apex, erect and straight, sometimes slightly flexuous, unbranched, smooth, aseptate, arising from the mycelium, swollen at the base, pointed at apex. *Conidiogenous cells* 1.5–2 × 1–2 µm ($\bar{x}$ = 1.8 × 1.4 µm, *n* = 30), hyaline, monoblastic, terminal, cylindrical, having rounded apices, no collarette. *Conidia* 10–18 × 3–5 µm ($\bar{x}$ = 15.4 × 3.8 µm, *n* = 40), abundant, smooth, hyaline, aseptate, oval, elliptical or cylindrical, sometimes elongated and flexuous, varying in shape, often with a flat base, rounded apex. Sexual morph: Not observed.

Material examined: Thailand, Chiang Rai, Nang Lae, Fah Thai local market, associated with an anthracnose lesion of ripened fruit skin of Kluai Khai, 22 June 2021, Ruvishika S. Jayawardena, SM35 (MFLU 23-0282), living culture MFLUCC 23-0033. Thailand, Chiang Rai, Mae Chan municipality market, on anthracnose lesion of fruit bunch of Kluai Khai, 4 December 2021, Binu C. Samarakoon, SM42 (MFLU 23-0283), living culture MFLUCC 23-0034. Thailand, Chiang Rai, Ban Du municipality market, associated with anthracnose lesions of Kluai Khai fruit, 19 June 2022, Binu C. Samarakoon, SM36 (MFLU 23-0284), living culture MFLUCC 23-0222.

GenBank accession numbers: MFLUCC 23-0033: ITS = OR186196, *act* = OR209338, *chs*-1 = OR192991, *gapdh* = OR192997; MFLUCC 23-0034: ITS = OR186197, *act* = OR209339; *chs*-1 = OR192992; *gapdh* = OR192998; MFLUCC 23-0222: ITS = OR186198; *act* = OR209340; *chs*-1 = OR192993; *gapdh* = OR192999.

Notes: The BLASTn search results of ITS, *gapdh, act*, and *chs-1* sequences suggest that our stains (MFLUCC 23-0033; MFLUCC 23-0034; MFLUCC 23-0222) have a high similarity (ITS = 99.62%, 99.62%, 99.81%; *gapdh* = 100%, 100%, 99.56%, *act* = 100%, 100%, 100%, and *chs-1* = 100%, 100%, 100%), excluding gaps to *C. siamense* (ICMP 12567, ICMP 18574, ICMP 18121) with query covers of 99% to 100%. In the multigene phylogeny, our strains (MFLUCC 23-0033; MFLUCC 23-0034; MFLUCC 23-0222) clustered with *C. siamense* with strong statistical support (85% ML, 0.99 BYPP) (Fig. 5). Morphologically, our collection is similar to the illustrations of Prihastuti et al., (2009) and Weir et al., (2012) in conidial morphology and conidiophores. Based on morpho-molecular support, we document *C. siamense* causing anthracnose in Kluai Khai in Thailand. During the artificial inoculation of *C. siamense*, noticeable black blemish development was observed. This was significant when the fruit reached ripening stage 7. The latter spots were identified as a post-harvest physiological disorder on the fruit peel. This is due to chlorophyll degradation and stomatal cell death in ripening skin (Pongprasert et al., 2021).

Speckle in banana fruits

Pathogenicity test

The isolates of MFLUCC 23-0027, MFLUCC 23-0028 and MFLUCC 23-0029 were identified as *Corynespora torulosa* based on phylogeny and morphology (Figs. 6 and 7). After 3 days of artificial inoculations, *C. torulosa* strains caused minute necrotic halos in the inoculated areas on the non-wounded fruit peel at ripening stage 2 (peel colour: entirely green with yellow traces) (Fig. 8a). After 6 days of inoculation, the infection and the halos expanded the diameter of the necrotic patch to 1.5 cm. The fruits were at ripening stages 3, 4, and 5. At the same time, the fungal mycelium has been released from the diseased tissue with conidial proliferation. When the fruits reached ripening stage 6 (entirely yellow peel), the necrotic patches became more distinct, and the decay of the peel was initiated. Disease symptoms were not observed in control fruits. The challenge-inoculated fruits display identical disease symptoms with the original Kluai Namwa specimens in which the fungus has been isolated (Fig. 2g–j). To establish Koch's postulates, the pathogen was re-isolated from the infected areas and confirmed with morphology and DNA. On the wounded peel, symptoms appeared after 2 days of inoculation, similar to those observed on the non-wounded peel.

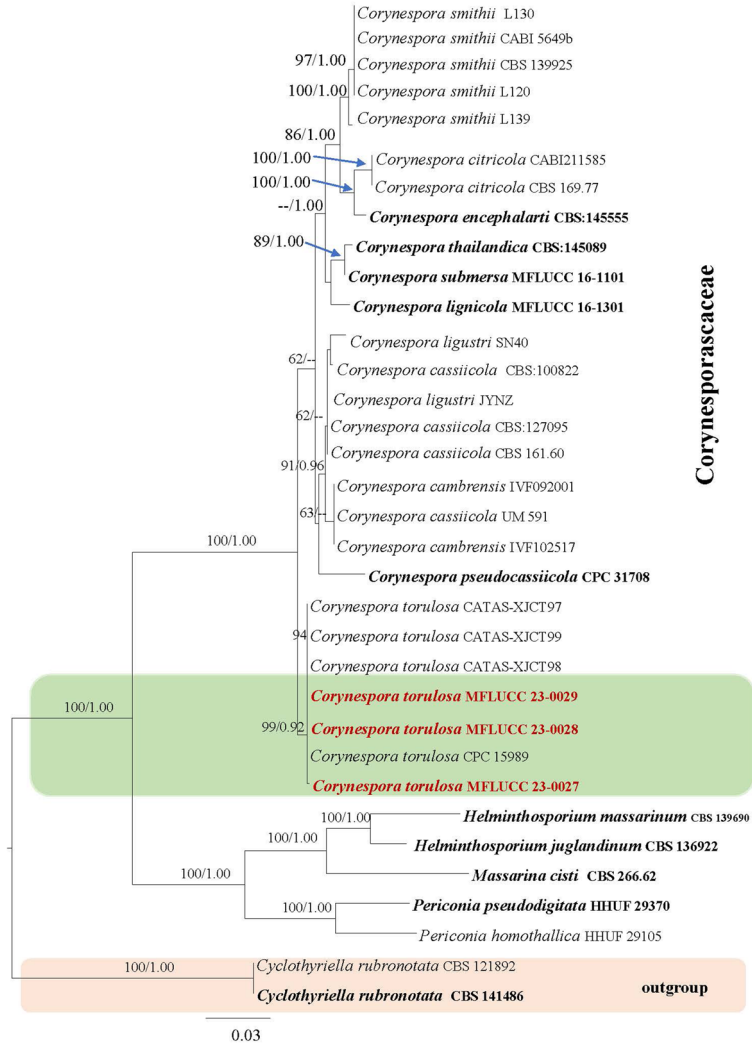

Fig. 7 Maximum likelihood tree constructed by RAxML analyses of LSU and ITS, sequence data for selected taxa in Pleosporales, showing the phylogenetic placement of *Corynespora torulosa* (MFLUCC 23-0027, MFLUCC 23-0028, MFLUCC 23-0029). The Bootstrap supports (≥60% ML) and the posterior probabilities (≥0.95 PP) are indicated on the nodes. *Cyclothyriella rubronotata* (CBS 141486 and CBS 121892) is used to root the tree. Newly generated isolates are in bold blue. Ex-type cultures are indicated in bold black. The scale bar represents the expected number of nucleotide substitutions per site. The combined LSU and ITS gene alignment is comprised of 34 sequences of selected taxa in Pleosporales. The best scoring RAxML has a final ML optimization likelihood value of -4840.65. This sequence matrix had 344 distinct alignment patterns, with 21.40% of undetermined characters or gaps. The estimated base frequencies were as follows: A = 0.238317, C = 0.247411, G = 0.290022, T = 0.224249; substitution rates; AC = 3.340170, AG = 3.161593, AT = 1.586743, CG = 1.699826, CT = 10.049079, GT = 1.0; proportion of invariable sites: I = 0.415725; gamma distribution shape parameter: α = 0.560028. All of the trees (ML and BI) produced by the multi-gene alignment had the same topology and did not significantly deviate from Voglmayr and Jaklitsch (2017)

Symptom development of the collected fruits

Speckles caused by *Corynespora torulosa* were recorded and collected from green banana bunches (ripening stage 1) and ripened fruits (stage 3 to 6) of Kluai Namwa (Fig. 2g–j and Fig. 6a–e). Symptoms are comprised of tiny reddish-brown to black spots (0.5 mm to 1 mm) in diameter on banana skin. The speckles were scattered throughout the fruit surface, but they were sometimes dense and aggregated at

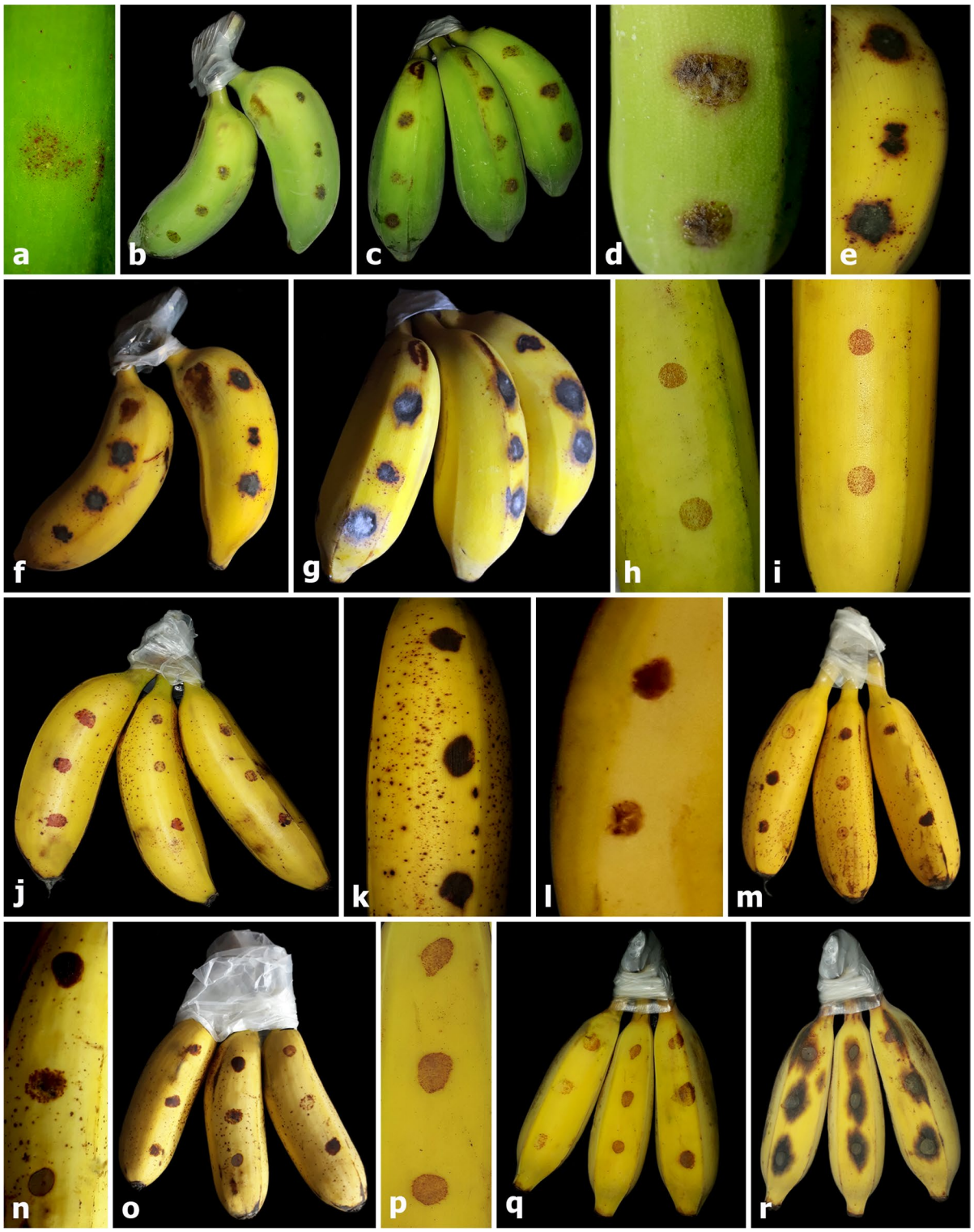

Fig. 8 Results of the pathogenicity test. **a**–**f** Development of diseased symptoms caused by *Corynespora torulosa* in Namwa banana; **h**–**o** Anthracnose symptoms after the inoculation of *Colletotrichum siamense* in Khai bananas; **p**–**r** Artificial inoculation of *C. musae* on Namwa bananas

the stalk end area and blossom end. The spots in the green banana bunches were surrounded by a water-soaked margin (Fig. 2h, i). The spots did not expand at the post-harvest stage. When the ripening started, the speckles became more distinct as tiny necrotic patches on a yellow background (Fig. 6a–e). Banana skin has only been affected by the pathogen and the infection has not penetrated the fruit pulp.

Taxonomy of *Corynespora*

***Corynespora torulosa* (Syd. & P. Syd.) Crous**

Index Fungorum number: IF805829, Facesoffungi Number: FoF 35566 Fig. 6

Pathogenic on fruits of Kluai Namwa. *Colonies* on PDA at 25 °C, light conditions, reach 5 cm diameter in 6 days, at immature stage, greyish-white, mouse grey or white and later becomes brownish-black at maturity. Filamentous, or rhizoid form, raised. Margin entire, lobate or ciliate, *Stroma* none. *Mycelium* aerial 4.5–7.5 µm ($\bar{x}$ = 6 µm) in width, septate, branched, subhyaline and smooth at an immature stage, rough and pale to dark brown at a mature stage, swollen at septa where branches initiated. *Sporulation* initiated after 10 days on PDA, long single or 3–4 conidial clusters arise from mycelium, and showing brown to black appearance on colony; reverse completely black after sporulation. *Conidiophores* erect from the mycelium, 110–370 µm × 1–14 µm ($\bar{x}$ = 290.5 × 9 µm, *n* = 40) tapering towards apex, 9–12 µm wide at the base, 1–3 µm near the apex, arising singly or more often in dense tufts from superficial hypha, simple, torsive, straight or flexuous, branched or un-branched, pale brown to dark brown, septate, 4–5 thin flange of tissue parts appearing as wings on the surface, having corn shaped or clavate base and a rounded or flat apex. *Conidiogenous cells* monoblastic, integrated, terminal, percurrent, doliiform, collarette, producing hyaline spherical immature conidia at terminal end. *Conidia* (50)–150–200– (300) µm × 2–17 µm ($\bar{x}$ = 170.55 × 12.5 µm, *n* = 50) with blackish-brown scar at the base, formed singly, cylindrical, straight or notably curved, tapering towards rounded apex, mature conidia have a globose, or doliform shaped swollen distinct base, in 10–13 µm × 8–9 µm ($\bar{x}$ = 12.5 × 8.3 µm, *n* = 20), pale brown or golden brown, smooth to verruculose, with 0, 1, 2, 3 or more transverse pseudosepta, thin flange of tissue part attached to the surface of conidia like a wing.

Materials examined: Thailand, Chiang Rai, Nang Lae, Fatay local market, associated with speckle spots of ripened fruit skin of Kluai Namwa, 17 January 2022, Binu C. Samarakoon, B01 (MFLU 23-0321), living culture MFLUCC 23-0027. Thailand, Nan, local banana stall at Nan city area, on black spots of ripened Kluai Namwa, 4 December 2018, Binu C. Samarakoon, Nan2018 (MFLU 23-0322), living culture: MFLUCC 23-0028. Thailand, Chiang Mai, Chiang Dao, speckles of unripen Kluai Namwa bunch, 10 May 2021, Binu C. Samarakoon, B02 (MFLU 23-0323), living culture MFLUCC 23-0029.

GenBank accession numbers: MFLUCC 23-0027: ITS: OR198902, LSU:OR198899; MFLUCC 23-0028: ITS OR198903, LSU:OR198900; MFLUCC 23-0029: ITS: OR198904, LSU: OR198901.

Notes: *Corynespora torulosa* was previously known as *Deightoniella torulosa* (= *Brachysporium torulosum*). In the multigene phylogeny (LSU and ITS Fig. 7), our isolates were grouped with *C. torulosa* isolates with strong statistical support. The morphology of our strains is similar to the illustrations of Crous et al. (2013) and Elis (1971) in conidia and conidiophores. Hence, on the basis of morphology and phylogeny, we document the first occurrence of *C. torulosa* causing the speckles of Kluai Namwa fruits in Thailand.

Discussion

Banana fruits can be infected by anthracnose pathogens during the pre-harvest stage in the field throughout the growing season (Jones, 2019). The spores are produced on the decaying or senescing banana tissue (i.e., leaves, discharged fruits, stem parts) and dislocate via water (Simmonds & Mitchell, 1940). The accumulated conidia in plant debris reach the unripe fruits by rain splash or irrigation water until they reach the packing house. Under optimal temperature conditions, the conidia attach to the fruit surface and germination is initiated (Jones, 2019). Signal molecules induce defensive responses in the plants and restrict the successful penetration of the fungus into the fruit skin (Abayasekara et al., 2013). However, the physiological changes in the host that occur during

ripening (i.e., reduction of phytoalexins and inducible defense responses) help the fungal pathogens switch to aggressive growth as necrotrophs (Alkan & Fortes, 2015). This results in deteriorated fruit tissue and a lesion (Prusky et al., 2013). In addition to the field conditions, fungal penetration can occur through natural openings and wounds in the banana fruits during the post-harvest handling chain (Jones, 2019).

Anthracnose affects fruits in all dessert banana cultivars (Jones, 2019). Gros Michel (AAA) is less susceptible to green fruit-wound anthracnose than Cavendish (AAA) (Stover, 1972). Plantain (AAB) fruits are also known to be resistant to anthracnose pathogens (Stover, 1972). *Colletotrichum siamense* was identified in anthracnose lesions in bananas in Brazil (Vieira et al., 2017) and Turkey (Uysal & Kurt, 2020). The pathogen has also been found in leaf spots of bananas in China (Huang et al., 2021) and India (Kumar et al., 2017). Udayanga et al. (2013) documented the fungus associated with anthracnose lesions in bananas in Thailand, but the pathogenicity of the isolates was not confirmed.

Pre-harvested banana fruit bunches in the field exhibit speckle symptoms due to various abiotic and biotic stresses. Fungicides and agrochemicals, including leaf fertilizers, result in speckle-like symptoms on damaged fruits (Jones, 2019). Unharvested fruits are susceptible to insect feeding, egg deposition and fungal penetration, which can induce physiological reactions and cause necrotic flecks (Jones, 2019). However, *Corynespora* speckles can be distinguished from flower thrip damage by the absence of raised bumps on necrotic patches (Jones, 2019).

Previous studies identified 16 fungal species in different genera associated with pre-harvest banana speckles (i.e., *Cephalosporium, Colletotrichum, Corynespora, Cylindrocarpon, Fusarium, Penicillium,* and *Trichoderma*) (Jones, 2019). Artificial inoculation revealed that only a few taxa (*Colletotrichum musae, Corynespora torulosa, Cephalosporium* spp., *Fusarium semitectum* and *F. tricinctum*) produced the speckle symptoms. Specifically, *C. musae* causes larger necrotic patches, forming sunken black areas with salmon-pink spores (Ocfemia, 1927; Jones, 2019). The affected fruits ripened prematurely, leading to rot and eventual wrinkling (Ocfemia, 1927; Jones, 2019). All the banana cultivars are susceptible to speckle disease at the pre-harvest stage (Jones, 2019). In addition, *C. torulosa* has also been isolated from black leaf spots on banana leaves in India, Jamaica and Thailand (Jones, 2019; Koné et al., 2008; Meredith, 1961; Tongsri et al., 2017; Vardhana, 2017).

Infection by *Corynespora torulosa* begins with the dead plant leaves, and the inoculum is generated by the rain or dew (Meredith, 1961). Conidial discharge occurs when the humidity drops and conidia become airborne. Conidia germinate on the fruit skin in moist conditions, producing appressoria. The penetration is indicated by a reddish-brown discoloration (Fig. 8a) on the green fruit skin and produces speckles after three days (Meredith, 1961). We have isolated *C. torulosa* strains from the speckled symptoms of ripened banana fruits available in the municipal markets at the post-harvest stage. However, we believe that the pathogen infected the fruits at the pre-harvest stage, and symptomatic unripe fruits might have been transferred to the local sellers. Additionally, we isolated *C. torulosa* strains from green banana bunches at the same local counters, further confirming our findings.

During the dry season in northern Thailand, we could not locate many *Corynespora torulosa*-associated speckle symptoms. However, after heavy rainfall in the wet season, the pathogen was successfully isolated from freshly harvested banana bunches. Many post-harvest green banana bunches in the Nang Lae area (Fah Thai local market, Chiang Rai) displayed *C. torulosa* speckles during this wet period. However, the fungus disappeared from the fruits when the environment dried up. Further research is required to confirm the etiology of *C. torulosa*-associated speckle disease and the seasonal changes. The endophytic and saprobic lifestyles of the isolated pathogens will be presented in upcoming publications.

Bananas are a major crop in Thailand, economically significant and deeply rooted in Thai culture. They are a staple dessert and cooking fruit, widely available in local markets. Research on post-harvest fungal diseases in the fruit is crucial for enhancing disease management in Thailand, benefiting agriculture, food security, public health, and quarantine policies.

Conclusion

Colletotrichum musae was identified as a post-harvest pathogen causing anthracnose in Kluai Namwa. The

pathogenicity of *C. siamense* that causes post-harvest anthracnose of Kluai Khai was confirmed in Thailand for the first time. *Corynespora torulosa* was identified as causing fruit speckles in Kluai Namwa, the first report of this in Thailand. The morphological and phylogenetic studies of the ITS, LSU, *act, cmdA, tub2, chs-1*, and *gapdh* sequences were used to identify the fungi. Fresh and unripe banana fruits were infected with the identified strains to prove Koch's postulates, which confirmed their pathogenicity.

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Data Availability Dried cultures deposited in the Fungarium of Mae Fah Luang University (Herb. MFLU; https://cefrfungarium.mfu.ac.th/), Chiang Rai, Thailand. living cultures were submitted to the Culture Collection of Mae Fah Luang University (MFLUCC; https://fungalcenter.mfu.ac.th/services/culture-collection/deposit-forms.html). The DNA sequence data have been deposited in GenBank (https://www.ncbi.nlm.nih.gov/) for further reference. Morphological illustrations and related information were submitted to GMS MICROFUNGI (https://gmsmicrofungi.org) and the Faces of Fungi (FoF) database (https://www.facesoffungi.org/).

Declarations

The authors declare no conflicts of interest and confirm no ethical issues associated with the publication. All authors have carefully reviewed and consented to its submission to the European Journal of Plant Pathology. The manuscript is original and has not been published elsewhere.

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