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Molecular Characterization of *Colletotrichum* Isolates Associated with Anthracnose of Mango Fruit

(Pencirian Molekul Pencilan *Colletotrichum* Berkait dengan Antraknos Buah Mangga)

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ABSTRACT

Colletotrichum *species are well-known causal agent of anthracnose. A study was conducted to determine the identity of* Colletotrichum *associated with anthracnose of mango (*Mangifera indica*) fruits. Thirty five* Colletotrichum *isolates were isolated from anthracnose lesion of two mango cultivars, Chokanan and Harum Manis. Based on the conidial morphology, two morphotypes (I and II) of* C. gleosporioides *were identified. Based on BLAST search of ITS regions and β-tubulin sequences, majority of the isolates showed 99-100% similarity with* Colletotrichum *sp. from mango and other hosts and three isolates, 100% similarity with* C. asianum. *From phylogenetic analysis using maximum likelihood method of combined datasets, the isolates from mango formed three clades, which corresponded to* C. gloeosporioides *sensu lato and* C. asianum. *Therefore, the present study showed that the isolates associated with anthracnose of mango belong to* C. gleosporioides *sensu lato and* C. asianum.

Keywords: Anthracnose; β-tubulin; Colletotrichum*; ITS regions; mango*

ABSTRAK

Spesies Colletotrichum *adalah agen penyebab utama antraknos. Suatu kajian telah dijalankan untuk menentukan identiti spesies* Colletotrichum *yang berkait dengan antraknos buah mangga (*Mangifera indica*). Tiga puluh lima pencilan* Colletotrichum *telah dipencilkan daripada lesi antraknos dua kultivar mangga, Chokanan dan Harum Manis. Berdasarkan morfologi konidium, dua morfotip (I dan II)* C. gleosporioides *telah dikenal pasti. Berdasarkan carian BLAST kawasan ITS dan gen β-tubulin, majoriti pencilan menunjukkan 99-100% kesamaan dengan* Colletotrichum *sp. daripada mangga dan perumah yang lain dan tiga pencilan, 100% kesamaan dengan* C. asianum. *Analisis filogenetik menggunakan kaedah kebolehjadian maksimum gabungan data, menunjukkan kesemua pencilan mangga membentuk tiga klad, yang sejajar dengan* C. gloeosporioides *sensu lato dan* C. asianum. *Oleh itu, kajian ini menunjukkan bahawa pencilan yang berkait dengan antraknos mangga adalah* C. gloeosporioides *sensu lato dan* C. asianum.

Kata kunci: Antraknos; β-tubulin; Colletotrichum; *kawasan ITS; mangga*

INTRODUCTION

Colletotrichum species are one of the most important pathogens causing anthracnose disease in a wide host range including legumes, vegetables and fruit crops especially in tropical region (Bailey & Jeger 1992). Symptoms of anthracnose begin as water soaked lesions that become soft, slightly sunken and containing conidia. Masses of conidia can be seen in the central region of the lesions on mature host. Infection often occurs in the farm, during storage, transportation and during marketing (Ilag et al. 1994).

Colletotrichum species infected a number of economically important tropical fruit crops including mango (*Mangifera indica*). Infection in the orchard can cause reduction in yields. Infection during storage can also cause losses as the fruits are not marketable. Blemished fruits do not meet the standard quality of the fruit for consumption and export.

Although there are reports of *Colletotrichum* species associated with anthracnose of mango in Malaysia, the identification is based mainly on morphological characters which could lead to inaccuracies as a number of broad, informal 'group species' within *Colletotrichun* have been recognized (Sutton 1992). Therefore, molecular method is often used to resolve the identity of *Colletotrichum* isolates associated with anthracnose. One of the most common methods used for identification of *Colletotrichum* species is DNA sequencing which includes but is not limited to internal transcribed spacer (ITS) sequence analysis and β-tubulin gene, a protein coding gene to resolve taxonomic status of closely related *Colletotrichum* species.

Species differentiation is important to formulate disease control strategies. In a mixed population of *Colletotrichum* species, sensitivity of one species to a certain fungicide as opposed to the other may cause a shift in population structure (Freeman et al. 1998). Thus, this study was undertaken to determine the identity of *Colletotrichum* isolated from anthracnose of mango fruit by using morphological characteristics and sequencing of ITS regions and β-tubulin gene.

Materials and Methods

Fungal isolates and morphological identification

Thirty five *Colletotrichum* isolates were recovered from anthracnose lesion of two mango cultivars Chokanan and Harum Manis. Mango fruits with anthracnose symptoms were obtained from several fruits stalls, markets and hypermarkets in Penang Island and state of Kedah, Peninsular Malaysia. Isolation was carried out using direct isolation in which a small amount of orange or black masses of conidia on the surface of the lesions were directly transferred onto PDA and incubated at 27±1ºC until visible growth of mycelia were observed which was about 3 - 4 days.

Cultures from single spore isolation using spore suspension method were used for species identification. For morphological identification, isolates of *Colletotrichum* were identified based on the descriptions by Mordue (1971). The shapes and sizes of conidia, setae occurrence, appressoria formation and colony colour were observed. Observations of appressoria were made by using spore suspension technique, whereby 1 cm^2 of a *Colletotrichum* culture on PDA were placed in sterilized distilled water. A drop of spore suspension was placed into a concave slide and cover with a cover slip. After 24 h, the appressoria formation was observed under a microscope (Olympus CX-41). For pigmentation, colour of the conidial masses and zonation were recorded.

Molecular identification and phylogenetic analysis

For DNA extraction, *Colletotrichum* isolates were cultured on PDA and incubated at 25±2ºC for 7 days. The mycelia were grounded into fine powder with liquid nitrogen. Genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen) according to the manufacturers' instruction.

The ITS regions were amplified using ITS4 and ITS5 primers as described by White et al. (1990) and ß -tubulin, using Bt2a and Bt2b primers (Glass & Donaldson 1995). For both ITS regions and ß –tubulin, PCR reactions were performed in a total volume of 25 µL reaction containing 1.5 mM MgCl, 10 mM Tris HCl, 0.2 mM dNTPs (Promega), 0.2 mM each primer, 2 unit *Taq* polymerase (Promega) and 100 ng genomic DNA.

PCR amplification was performed in DNA Engine™ Peltier Thermal Cycler Model PTC – 100 with an initial denaturation at 95ºC for 1 min followed by 34 cycles of denaturation at 94ºC for 1 min, annealing at 52ºC for 30 s and extension at 72ºC for 1 min, followed by final extension for 10 min at 72ºC. Negative controls were used to test for the presence of nonspecific reaction. The PCR product was detected in 1% agarose gel electrophoresis, run in Tris Borate EDTA (TBE) buffer at 110 min, 90 V and 400 mA. The gel was stained with ethidium bromide and visualized under UV transalluminator. The size of the amplified ITS regions and ß -tubulin bands was estimated by comparison to 1kb marker (Fermentas).

The PCR product was purified using PCR Purification kit (Qiagen) according to the manufacturers' instruction and sequenced by a service provider. After sequencing, the consensus sequence was BLAST against sequences in the GenBank database. The identity of each isolate was based on the closest match of the BLAST results.

Phylogenetic analysis was conducted based on combined datasets of ITS regions and ß–tubulin to generate maximum likelihood tree using MEGA5 (Tamura et al. 2011). For phylogenetic analysis, *Colletotrichum* spp. from mango that are available in the Genbank were also included in the analysis. *Colletotrichum acutatum* served as an outgroup.

Results and Discussion

Thirty five isolates of *Colletotrichum* were recovered from anthracnose lesion of Chokanan and Harum Manis mango varieties. Based on the conidial characteristics, the *Colletotrichum* isolates can be divided into two groups, morphotypes 1 and 2. Table 1 shows the morphological characteristics of both morphotypes. Morphotype 1 isolates produced cylindrical with rounded ends conidia and present of setae (Figure 1) whereas for morphotype 2 isolates, a mixture of cylindrical with rounded end conidia and conidia with narrow and slightly truncated base were observed (Figure 2). Appressoria of morphotype 1 formed from the mycelia whereas appressoria of morphotype 2

TABLE 1. Morphological characteristics of *Colletotrichum* isolated from anthracnose of mango fruits

Species	Morphological characteristics						
	Conidia			Appressoria		Setae	Pigmentation
	Length (µm)	Width (µm)	Shape (µm)	Length (µm)	Width (µm)		
Morphotype 1	14 – 15	3.0 – 4.0	Cylindrical, round apex and base	7.0 – 11.0	5.0 – 7.0	Present	Pale grey to black zonated colonies with abundant orange conidial masses
Morphotype 2	10 – 16	2.5 – 4.0	Cylindrical, round apex and truncated base	7.0 – 11.0	4.5 – 7.5	Absent	Orange-coloured conidial and white to grey colony with thin mycelium

formed from germinated conidia (Figure 1). The colony characteristics of morphotype 1 was pale to black colony with zonation of orange conidial masses and colony of morphotype 2 isolates was white to grey and orange conidial masses (Figures 1 and 2). The morphological characteristics of both morphotypes fall within the description of *C. gloeosporioides* by Mordue (1971). The results indicated the complexity of *C. gloeosporioides* which is not surprising as *C. gloeosporioides* has a wide host range and is regarded as a species complex

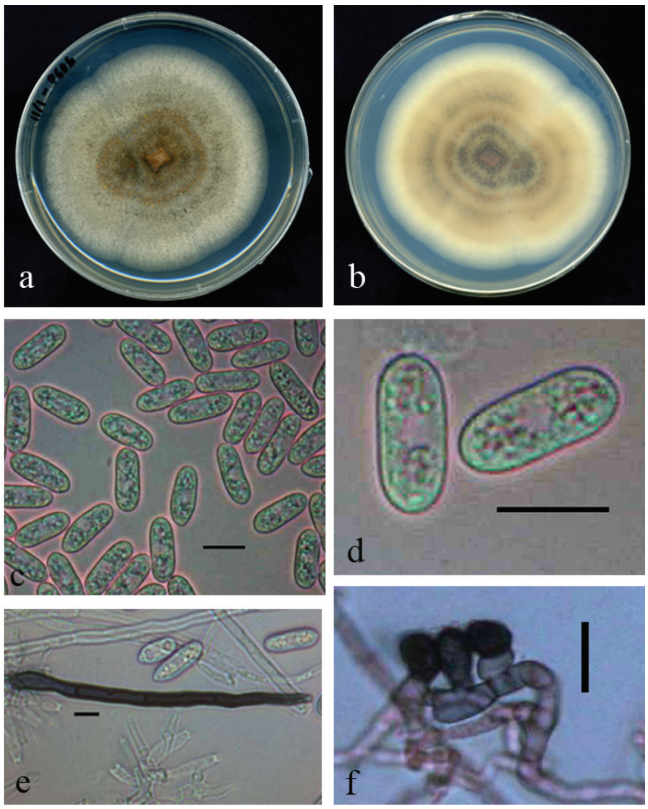

FIGURE 1. Morphological characteristics of *C. gloeosporioides sensu lato* morphotype 1 (a) colony on PDA (upper surface), (b) colony and PDA (lower surface), (c and d) conidia, (e) setae and (f) appressoria. Bars = 10 µm

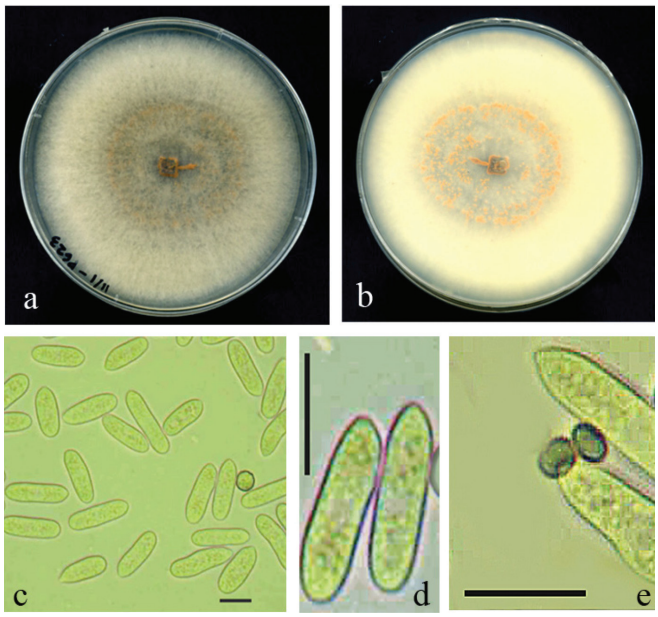

FIGURE 2. Morphological characteristics of *C. gloeosporioides sensu lato* morphotype 2 (a) colony on PDA (upper surface), (b) colony on PDA (lower surface), (c and d) conidia and (e) appressoria. Bars = 10 µm

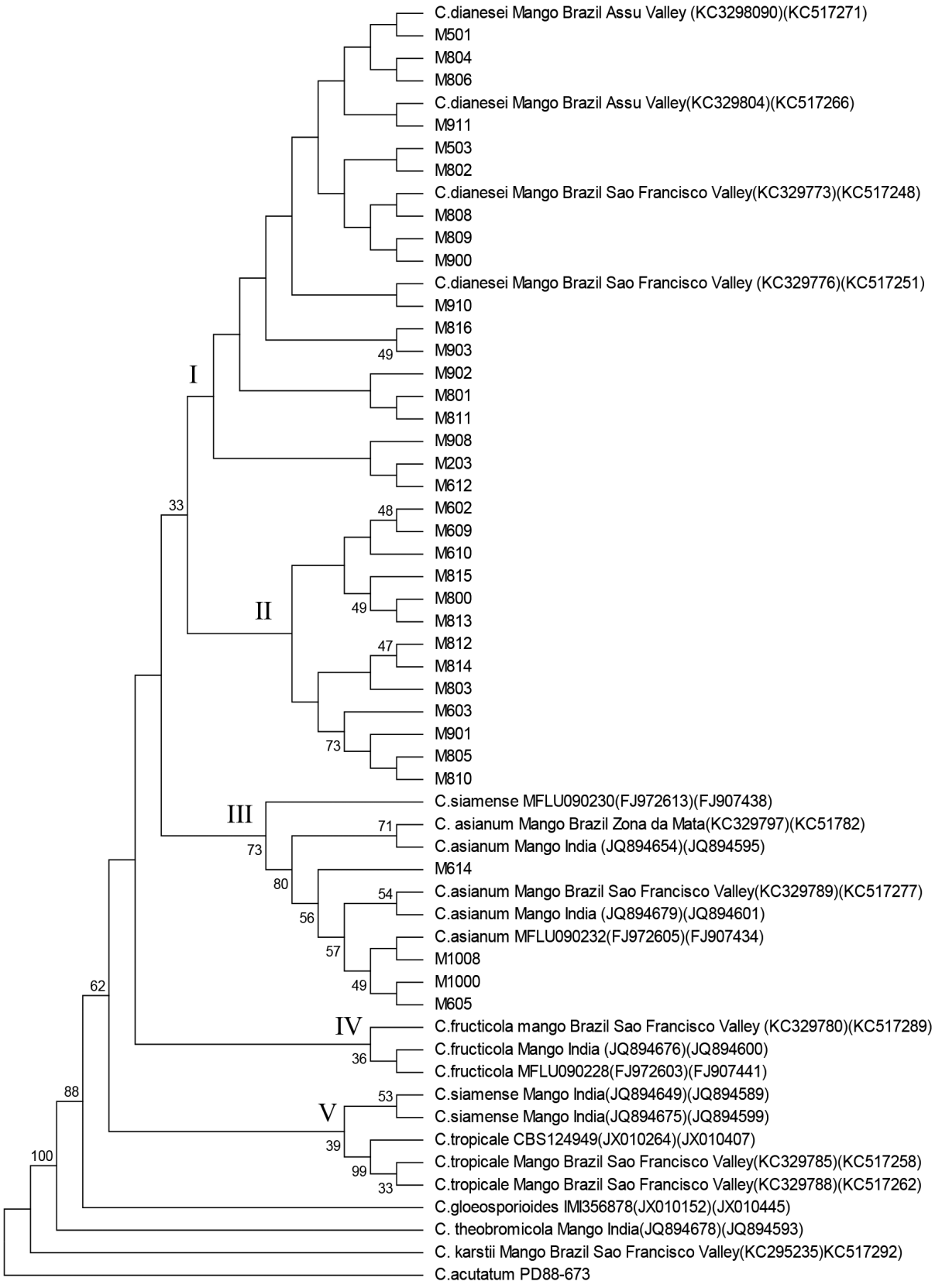

FIGURE 3. Phylogenetic tree inferred from maximum likelihood method showing the grouping of Colletotrichum isolates from anthracnose of mango fruits

with diverse genetic and biological characters (Damn et al. 2010) . Thus, *C. gloeosporioides* species complex comprised many morphologically similar species but are genetically different which have also been reported by Cai et al. (2009) and Johnston et al. (2008).

From PCR amplification of ITS regions, 600 bp band was produced from all the isolates, and from ß – tubulin, 500 bp band. Based on ITS regions sequences, majority of the isolates showed 99-100% similarity with *Colletotrichum* sp. from mango and *Colletotrichum* sp. from other hosts. Only three isolates (M1008, M1000 and M605) showed similarity with *C. asianum* (100% similarity). Similar with ITS regions, ß–tubulin sequences also showed 99-100% similarity with *Colletotrichum* sp. from mango and *Colletotrichum* sp. from other hosts, but four isolates (M614, M1008, M1000 and M605) showed 100% similarity with *C. asianum*. The isolates which showed similarity with *Colletotrichum* sp. are referred to as *C. gloeosporioides* species complex or *C. gloeosporioides sensu lato* as based on morphological characteristics these isolates were similar with species description of *C. gloeosporioides* by Mordue (1971). In a study by Weir et al. (2012), all the taxa that were included in *C. gloeosporioides* species complex fit the traditional morphological species concept of *C. gloeosporioides* described by von Arx (1970), Mordue (1971) and Sutton (1992). Therefore, in this study, the same concept was applied.

From maximum likelihood tree (Figure 3), *Colletotrichum* isolates from anthracnose of mango can be divided into three clades (I, II and III). Isolates in clade I are related to *C. dianesei* and clade III to *C. asianum*. Isolates in clade II comprises only mango isolates. The isolates were not grouped according to the morphotypes. None of the isolates in the present study were grouped with *C. gloesporioides* epitype strain (IMI356878) as well as with other *Colletotrichum* species except *C. dianesei* and *C. asianum* which have been reported from anthracnose of mango (Clades IV, V and other individual clades) such as *C. fructicola*, *C. siamense*, *C. tropicale*, *C. theobromicola* and *C. karstii*.

Although based on morphological characteristics, the isolates were similar with *C. gloeosporioides*, phylogenetic analysis showed that none of the isolates were *C. gloesporioides* as the isolates were phylogenetically distinct from the epitype strain (IMI356878). *Colletotrichum gloesporioides* was previously reported as the causal pathogen of mango anthracnose in Malaysia (Kwee & Chong 1994). Kamle et al. (2013) reported that *C. gloeosporioides* was the causal pathogen of mango anthracnose in eastern Uttar Pradesh.

The results of the present study was similar with studies by Sharma et al. (2013) and Lima et al. (2013) which also showed that none of the *Colletotrichum* isolates recovered from mango anthracnose were *C. gloeosporioides*. Based on a study by Phoulivong et al. (2013), *C. gloeosporioides* is not a common pathogen on tropical fruits.

Phylogenetic analysis of the present study indicated that 31 isolates from anthracnose of mango are related to *C. dianesei* as these isolates were grouped in the same main clade with *C. dianesei* isolates. However, based on BLAST search, these isolates showed 99-100% similarity with *Colletotrichum* sp. from mango and other hosts. *Colletotrichum dianesei* was reported by Lima et al. (2013) as one of the *Colletotrichum* species causing anthracnose of mango in northeastern of Brazil and also the most frequently isolated species from anthracnose lesion of mango. Based on the results of the present study, the 31 isolates cannot be identified as *C. dianesei* because neither the morphological characteristics nor molecular data of the mango isolates were similar with *C. dianesei* descriptions and sequences of ITS regions and β-tubulin, respectively. The mango isolates may represent a new species in which the sequences data are not yet available for phylogenetic analysis. Thus, these isolates are referred to as *C. gloeosporioides sensu lato*. One of the methods that can be used to clarify the identity of these 32 isolates is using multi-gene phylogeny of several markers listed by Hyde et al. (2009). But the application of these genes is neither very efficient nor economical (Cai et al. 2009).

Only three isolates (M1008, M1000 and M605) could be assigned to known species i.e. *C. asianum* based on BLAST search of ITS regions and β-tubulin sequences and phylogenetic analysis. One isolate (M614) is closely related to *C. asianum*. These four isolates were grouped in the same group as the other *C. asianum* from mango. *Colletotrichum asianum* was isolated and originally described by Prihastuti et al. (2009) from coffee berries (*Coffea arabica*) in northern Thailand. *Colletotrichum asianum* was the second most prevalent species isolated from anthracnose of mango in northeastern of Brazil reported by Lima et al. (2013). To the best of our knowledge, this is the first report of *C. asianum* associated with anthracnose of mango fruits in Malaysia. However, *C. asianum* have been reported from mango in Australia, Colombia, Japan, Panama and the Philippines (Weir et al. 2012) as well as in South Africa (Sharma et al. 2013) and Sri Lanka (Krishnapillai & Wilson Wijeratnam 2014).

The results of the present study suggested, *C. gloeosporioides sensu lato* which are related to *C. dianesei* and *C. asianum* were associated with anthracnose of mango fruits.

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