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Research Article
Multi-genetic Analysis of *Colletotrichum* spp. Associated with Postharvest Disease of Fruits Anthracnose in Special Region of Yogyakarta, Indonesia

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Abstract

Background and Objective: Postharvest disease caused by *Colletotrichum* spp. caused major losses. The species of *Colletotrichum* are difficult to distinguish if only seen from their morphology. This study investigated *Colletotrichum* isolates associated with tropical fruits anthracnose using multi-genetic analysis and the cross-infection potency of each isolate among tropical fruits. **Materials and Methods:** The fruit samples were collected from markets in the Special Region of Yogyakarta, Indonesia and its surrounding area. The fruits affected by anthracnose subjected to isolation, resulting in 15 isolates. Morphology of colony and conidia then characterized and clustered with UPGMA. The seven representative isolates were selected for molecular identification. The multi-genetic analysis was used by combining ITS, Glyceraldehyde 3-phosphate dehydrogenase (gapdh) and tub2 sequence genes. A cross-infection test was conducted by using selected species from the multi-genetic analysis. **Results:** Multi-genetic analysis clustered the selected isolates into four species. Isolates from banana, avocado, papaya and citrus belonged to gloeosporioides species complex, including *C. siamense*, *C. asianum* and *C. gloeosporioides*. Isolates from apple, guava, mango and citrus belonged to acutatum species complex, including *C. sloanei*. The cross-infection test in this study showed that *C. siamense* could cause anthracnose on banana, apple, citrus and avocado, *C. asianum* on avocado, papaya, apple and citrus, *C. gloeosporioides* on citrus and apple, *C. sloanei* on apple, guava, citrus and papaya. **Conclusion:** The *C. siamense*, *C. asianum*, *C. gloeosporioides* and *C. sloanei* found associated with tropical fruits anthracnose. The potency of the cross-infection test revealed the board range in the pathogenicity of the *Colletotrichum* isolates.

Key words: *Colletotrichum* spp., fruit anthracnose, multi-genetic analysis, ITS, gapdh, tub2, postharvest diseases, disease management

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Fruits and vegetables are important commodities that affect the economy in developing countries such as Indonesia. The production of some popular tropical fruits has decreased by around 3% in a year. Postharvest product loss can reach 20-50% due to inappropriate handling of harvest and postharvest. In post-harvest, it is mostly caused by postharvest disease despite the use of modern storage facilities and technologies. Postharvest diseases reduce fruit quantity and quality, the fruits may not unsaleable but still reduce in value[1-3]. Well-known fungi caused major postharvest losses is *Colletotrichum*. *Colletotrichum* is the causal agent of anthracnose disease. It can affect many fruits such as chili, avocado, mango, banana, papaya, guava, citrus fruits, etc[4].

Colletotrichum has many species and the taxonomic of the genus *Colletotrichum* changes frequently. *Colletotrichum* spp. are difficult to distinguish if they are only seen from their morphology. The difference is difficult to see because there are overlapping morphological characters in the *Colletotrichum* spp. The plasticity of *Colletotrichum* morphological characters make molecular technique analyzes more reliable for their classification. Identification of species using molecular techniques analysis is necessary. This helps early detection and provides appropriate guidance for the next steps in disease management[5-7].

New reports of some novel *Colletotrichum* species in 2019 used multi-genetic analysis were described as *C. javanense*, *C. makassarense* and *C. tainanense* that associated with anthracnose of chili fruit in West Java (Indonesia), Makassar, South Sulawesi (Indonesia) and Tainan (Taiwan). *Colletotrichum siamense* and *C. fructicola* that included in 11 different *Colletotrichum* species identified in the same research was first reported causing anthracnose in chili in Indonesia, Sri Lanka, Thailand and Taiwan, although it has been reported as infecting many plant species before[8].

Based on that information, cross-infection of *Colletotrichum* spp. can occur on fruits. Fruits that always available in the field and market and mostly cultivated in mixed-cropping systems make the potential for cross-infection cause greater susceptibility to anthracnose disease[9]. Cross-infection potential tests between fruits were conducted to provide information to develop an integrated system for controlling postharvest loss due to anthracnose disease[10]. Lakshmi *et al*.[10] reported that *C. gloeosporioides* obtained from mango developed anthracnose symptoms on papaya, guava, acid lime, custard apple, pomegranate and cashew. The symptoms also developed on fruits when the isolates from six other fruits were inoculated on mango, with different susceptibility. Phoulivong *et al*.[11] also reported the potential cross-infection of *C. asianum*, *C. cordylinicola*, *C. fructicola*, *C. siamense* and *C. simmondsii* to infect a wide host range. Suparman *et al*.[9] reported *C. gloeosporioides* isolated from papaya and eggplant and *C. capsici* isolated from chili could infect all tested fruits (papaya, eggplant, chili) except common bean.

There is not much research studying the diversities of *Colletotrichum* species from tropical fruits and the relationship of each isolate, especially in Indonesia. Therefore, there is a need to renewing the data of *Colletotrichum* isolates from other tropical fruits with molecular data using multi-gene technologies, morphological character and pathogenicity to get updated information of the host-pathogen relationship. The purposes of this study were to investigate the phylogenetic relationship of *Colletotrichum* isolates associated with tropical fruits anthracnose and the cross-infection potency of each isolate among tropical fruits.

MATERIALS AND METHODS

Study area: The study was carried out at the Laboratory of Plant Disease Clinic, Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Special Region of Yogyakarta, Indonesia from August, 2019 until April, 2020.

Fruits sampling: Fruits samples with anthracnose symptoms were collected from markets in the Special Region of Yogyakarta, Indonesia and its surrounding area. There were 7 fruits collected from markets, i.e., avocado, guava, banana, papaya, apple, mango and citrus.

Isolation of *Colletotrichum* spp.: Fruits with anthracnose symptoms were disinfected by using 70% alcohol by rubbing the surface. The area between the healthy and sick part was cut 1 cm × 1 cm and cultured on Potato Dextrose Agar (PDA) medium on a Petri dish. The cultures were incubated for 4-7 days in room temperature. Isolates from various commodities were identified based on the morphology of *Colletotrichum* spp. following Weir *et al*.[12] and Damm *et al*.[13] Cultures of *Colletotrichum* sp. then were re-cultured in another Petri dish with PDA medium for subsequent tests.

Purification of *Colletotrichum* spp. with single-spore isolation: Suspension of *Colletotrichum* spp. was prepared by cutting 1×1 cm of 7 days culture on PDA medium and adding 500 µL sterile water in 1.5 mL tube. The suspension was homogenized by the vortex. The suspension obtained was scratched on a new PDA medium and incubated at room temperature for 12-24 hrs. The germinated conidium was taken under a microscope using a sterile needle and replaced to a new PDA medium. Incubation was conducted at room temperature.

Morphological analysis: Morphological characters were analyzed by characterizing the cultures include colony color, texture, conidiomata, growth rate, conidial dimension. The length and width of conidia were measured by choose 30 random conidia for each isolate after 10 days of incubation. Data of morphological character then was analyzed used NTsys 2.10 program and UPGMA method (Un-weighted Pair Groups with Arithmetical Averages). The *Colletotrichum* spp. isolates then were chosen as representatives according to their groups for further tests.

Molecular identification: Selected isolates represented each morphological group then were subjected to molecular identification. DNA of the isolates was extracted with Plant Mini Kit (Geneaid). The DNA obtained was amplified using the PCR technique using primers ITS1 and ITS4[14,15], primers tub2 (β-tubulin), T1 and T22[16], primers gapdh (Glyceraldehyde 3-phosphate dehydrogenase), GDF1 and GDR1[17]. The PCR was carried out in a total volume 25 µL, comprised of 9.5 µL miliQ sterile water, 12.5 µL DNA Taq polymerase (My Taq HS Red Mix; Bioline, London, United Kingdom), 1 µL forward primer, 1 µL reverse primer and 1 µL DNA template. Amplification was conducted using T100 Thermal Cycler (Biorad, California, United States). PCR program for ITS included an initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min and final cycle at 72°C for 5 min. The PCR program for gapdh and tub2 included an initial denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, 60°C for 45 sec, 72°C for 1 min and final cycle at 72°C for 10 min. PCR products were visualized with electrophoresis through 1% agarose gel at 50 V for 50 min and UV transilluminator with 1 kb DNA ladder (Thermo Fisher Scientific, Maltham, United States). DNA sequence analysis of the PCR product obtained was by sending it to the 1st Base, Malaysia.

Multi-gene phylogenetic analysis: The sequence of ITS, gapdh and tub2 genes of the chosen isolates were aligned by Clustal W to obtain a consensus sequence. Alignments were optimized manually in Bioedit. MEGA7.0. was used to build the phylogenetic tree with the maximum-likelihood (ML) algorithm. The phylogenetic tree was constructed with the combined ITS, gapdh and tub2 dataset. The sequences were compared with selected species (Table 1) and the NCBI sequence database was used the BLAST algorithm for approximate identification.

Cross-infection test: Selected species of *Colletotrichum* from multi-gene phylogenetic analysis then used for cross-infection tests. Host fruits were prepared by purchased healthy fruits which untreated, un-waxed, physiologically mature and unripe from the market. The surface of fruits was sterilized by rubbing the surface of the fruits using 70% alcohol. Sterilized fruits were placed in a plastic box with tissue paper then was sprayed with sterilized water to maintain at least 95% relative humidity. Fruits were inoculated using a wound and non-wound inoculation method.

The wound inoculation method was conducted by pin-pricked the fruits with a sterile needle in the middle portion of fruit, then mycelial disk from 10 days old C*olletotrichum* spp. was placed onto the wound. Non-wound inoculation method was without pin-pricked the same fruits, then mycelial disk from 10 days old *Colletotrichum* spp. was placed onto the surface of the fruits. The inoculated samples were incubated in the containers at 28-30°C in a 12 hrs light/dark cycle[10].

Fruits used in cross-infection tests were guava (*Psidium guajava*), citrus (*Citrus* sp.), apple (*Malus domestica*), banana (*Musa* sp.), papaya (*Carica papaya*) and avocado (*Persea americana*) with seven treatments (isolates) and three replicates per fruit. The infection was measured based on lesion development on the symptom of fruit. Scored according to Montri *et al.*[18]:

$$\text{Disease severity (\%)} = \frac{\text{Lesion length}}{\text{Fruit length}} \times 100$$

The data were analyzed used analysis of variance ($p<0.05$) with DMRT for multiple range tests using SAS V9.0.

Table 1: Isolates used for multi-genetic analysis in this study

Species	Isolates name	Host	GenBank accession number		
			tub2	gapdh	ITS
***Acutatum* species complex**					
C. abscissum	COAD 1877	*Citrus sinensis* cv. Pera	KP843135	KP843129	KP843126
C. acerbum	CBS 128530	*Malus domestica*	JQ950110	JQ948790	JQ948459
C. acutatum acutatum	CBS 112996	*Carica papaya*	JQ005860	JQ948677	JQ005776
C. citri	CBS 134233	*Citrus aurantifolia*	KC293661	KC293741	KC293581
C. fioriniae	CBS 125396	*Malus domestica*	JQ949950	JQ948629	JQ948299
	IMI 324996	*Malus pumila*	JQ949952	JQ948631	JQ948301
	CBS 129938	*Malus domestica*	JQ949947	JQ948626	JQ948296
	CBS 129930		JQ949955	JQ948634	JQ948304
	ATCC 28992	*Malus domestica*	JQ949948	JQ948627	JQ948297
C. guajavae	IMI 350839, CPC 18893	*Psidium guajava*	JQ949921	JQ948600	JQ948270
C. limetticola	CBS 114.14	*Citrus aurantifolia*	JQ949844	JQ948523	JQ948193
C. paranaense	CBS 134729	*Malus domestica*	KC205060	KC205026	KC204992
C. paxtonii	IMI 165753	*Musa* sp.	JQ949936	JQ948615	JQ948285
C. sloanei	BRIP48742	*Litchi chinensis*	KU221365	KU221342	KU498289
C. simmondsii	BRIP28519	*Carica papaya*	FJ 907443	FJ 972580	FJ 972601
	CBS294.67	*Carica papaya*	FJ 907444	FJ 972581	FJ 972610
	CBS 122122	*Carica papaya*	JQ949927	JQ948606	JQ948276
***Gloeosporioides* species complex**					
C. aenigma	ICMP 18608	*Persea americana*	JX010389	JX010044	JX010244
C. alienum	ICMP12071	*Malus domestica*	JX010411	JX010028	JX010251
C. asianum	IMI 313839, ICMP 18696	*Mangifera indica*	JX010384	JX009915	JX010192
C. gloeosporioides	CBS93597	*Citrus sinensis*	FJ 907445	FJ 972582	FJ 972609
	IMI 356878	*Citrus sinensis*	JX010445	JX010056	JX010152
C. hystricis	CBS 142411, CPC 28153	*Citrus hystrix*	KY856532	KY856274	KY856450
C. kahawae subsp. *ciggaro*	ICMP 12952	*Persea americana*	JX010426	JX009971	JX010214
C. musae	CBS 116870	*Musa* sp.	HQ596280	JX010050	JX010146
C. psidii	CBS145.29	*Psidium* sp.	JX010443	JX009967	JX010219
C. queenslandicum	ICMP1778	*Carica papaya*	JX010414	JX009934	JX010276
C. siamense	ICMP 12567	*Persea americana*	JX010387	JX009940	JX010250
	ICMP 17795	*Malus domestica*	JX010393	JX010051	JX010162
C. tropicale	CMM 4071	*Mangifera indica*	KC517258	KC517181	KC329785
	CMM 4243	*Musa* sp.	KU213604	KU213601	KU213603
Dematium species complex					
C. fructi	CBS 346.37	*Malus sylvestris*	GU228138	GU228236	GU227844
Boninense species complex					
C. citricola	CBS 134228	*Citrus unshiu*	KC293656	KC293736	KC293576
	CBS 134229	*Citrus unshiu*	KC293657	KC293737	KC293577
	CBS 134230	*Citrus unshiu*	KC293658	KC293738	KC293578
C. constrictum	CBS 128504, ICMP 12941	*Citrus limon*	JQ005672	JQ005325	JQ005238
C. karstii	CBS 113087	*Malus* sp.	JQ005615	JQ005268	JQ005181
	CBS 128524	*Citrullus lanatus*	JQ005615	JQ005282	JQ005195
	CBS 128551	*Citrus* sp.	JQ005642	JQ005295	JQ005208
	CBS 129832	*Musa* sp.	JQ005611	JQ005264	JQ005177
	CBS 129824	*Musa AAA*	JQ005649	JQ005302	JQ005215
Unidentified species complex					
C. musicola	CBS 132885	*Musa* sp.	MG601003	MG600798	MG600736
C. cliviae	CMM 3742	*Mangifera indica*	KC992327	KC702941	KC702980
C. plurivorum	MAFF 305790	*Musa* sp.	MG600993	MG600789	MG600726
C. tropicicola	BCC 38877	*Citrus maxima*	JN050246	JN050229	JN050240

RESULTS

Anthracnose symptoms on fruits sample: The symptoms of anthracnose disease associated with *Colletotrichum* spp. from varied tropical fruits in the Special Region of Yogyakarta market and its surrounding areas were slightly different on each fruit. The symptoms varied from brown to black spots (mango, banana) and light brown to dark lesion sunken areas (guava, citrus), some lesions had black or pink spore masses at the center part as it ages (apple, avocado, papaya) (Fig. 1).

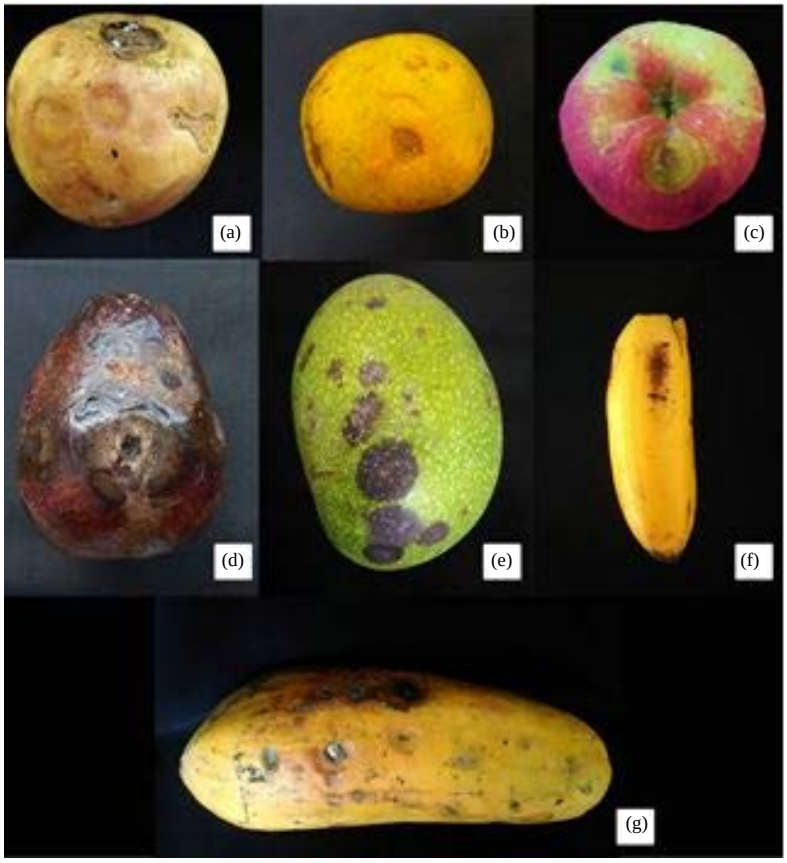

Fig. 1(a-g): Anthracnose symptoms in fruits caused by *Colletotrichum* spp.
(a) Guava, (b) Citrus, (c) Apple, (d) Avocado, (e) Mango, (f) Banana and (g) Papaya

Culture and conidia morphology: Total 15 *Colletotrichum* isolates were collected from varied tropical fruits. *Colletotrichum* isolates showed variations in cultural and morphological characteristics on PDA after 10 days of incubation under room temperature (Table 2). Most isolates were cottony and had the concentric ring. There was only one isolate that had a non-cottony texture and no concentric ring, it was APL-MLG collected from apple fruit. The color of the aerial view of all isolates was different such as brownish, greyish, white and greenish-grey. The color of the reverse view of all isolates was different such as brownish, greyish, white, pinkish and greenish-grey. The growth rate was also different among all isolates. PSG-JG isolate grew the fastest, APL-MLG isolate grew slower than all isolates.

Conidia produced by *Colletotrichum* isolates were fusiform and cylindrical with two ends acute or one end slightly obtuse (Table 2). There were only two isolates produced fusiform conidia, JRK-SMO and JRK-SMP isolate, collected from citrus fruits. The conidia size of all isolates were categorized by three groups, small for 8-11 µm conidia, medium for 11-14 µm conidia and big for conidia more than 14 µm conidia. APL-MLG, MGG-JG and JMB-GW4 isolate had small conidia. ALP-DMGO, JRK-SMO, JRK-KR5, JRK-KRM, PPY-GDN2 and PSG-JG isolate had medium conidia. ALP-DMGA, ALP-DRS, JRK-SMP, JRK-DRS, JRK-SL and MGG-SM2 isolate had big conidia.

Morphological character grouping was shown in Fig. 2, the first group had a small size of conidia and brownish mycelium color. The second group had a fast growth rate colony. The third group produced a white colony color and a fast growth rate. The fourth group was the only isolates that had a pinkish reverse side colony color. The fifth group produced greyish colony color, big size and cylindrical shape of conidia. The sixth group had a greenish-grey reverse side, medium-size and cylindrical shape of conidia and medium growth rate. The seventh group had the cylindrical shape of conidia, concentric ring and medium growth rate. APL-MLG, PSG-JG, JRK-SMO, PPY-GDN2, ALP-DMGA, JRK-KR5 and JMB-GW4 then were selected from each group as representative isolates. Figure 3 showed the aerial view, reverse view and conidia of 7 representative isolates of *Colletotrichum* spp. APL-MLG isolated from apple had

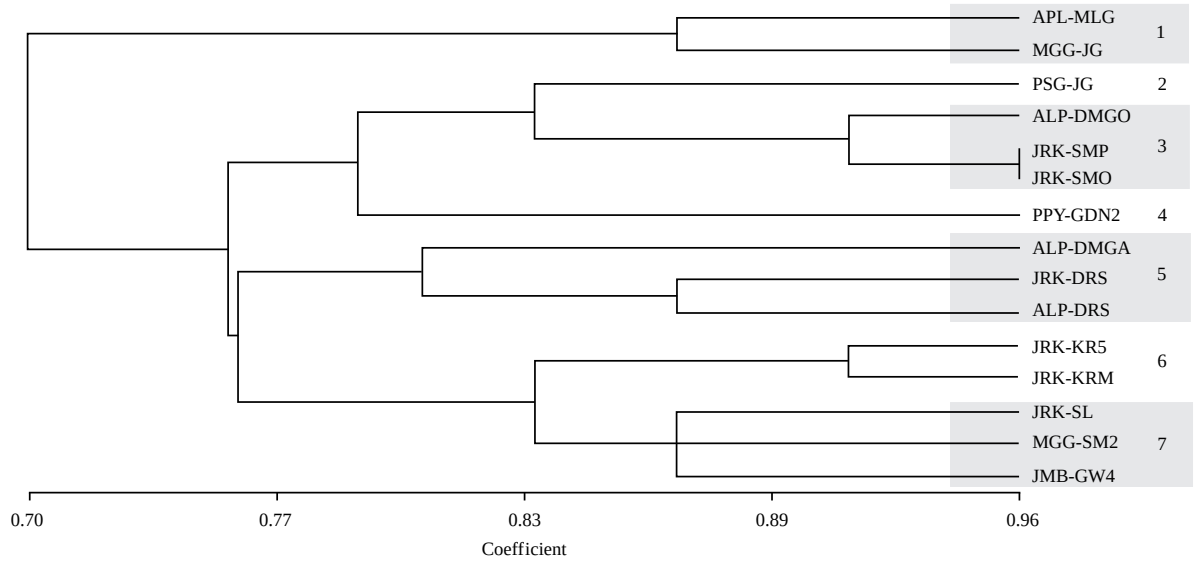

Fig. 2: UPGMA dendrogram showing 7 groups of *Colletotrichum* spp. isolates

The dendrogram was built based on the similarity of the morphological character of *Colletotrichum* spp. isolates from tropical fruits. The coefficient of similarity of each group is above 75%

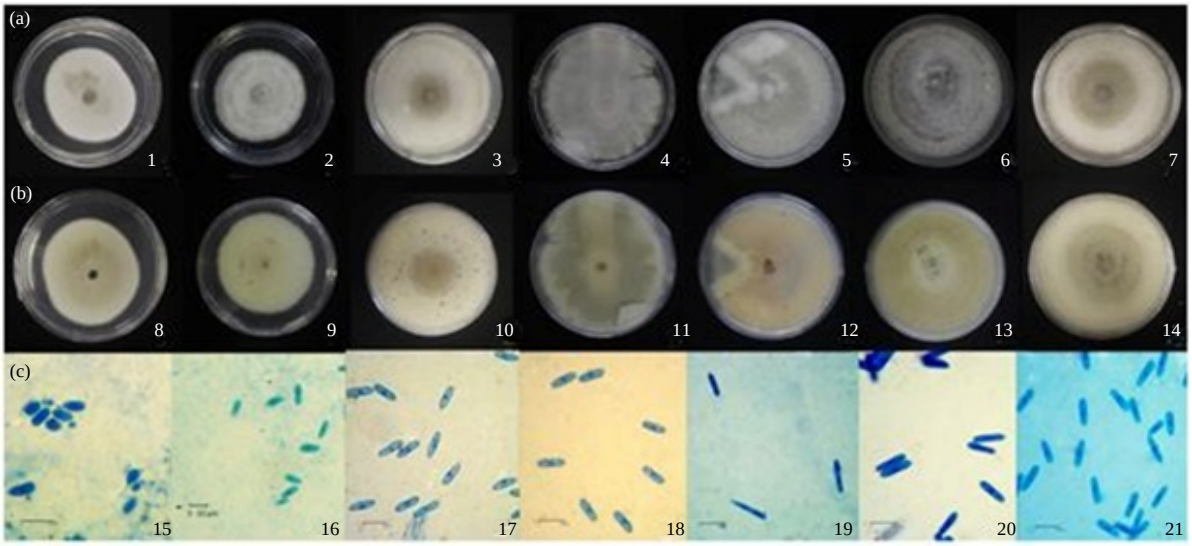

Fig. 3(a-c): Representative isolates of *Colletorichum* spp.

(a) Aerial view, (b) Reverse view and (c) Conidia *Colletotrichum sloanei* from (1,8,15) apple/APL-MLG, (2,9,16) Guava/JMB-GW4, *C. gloeosporioides* from (3,10,17) citrus/JRK-SMO, (4,11,18) citrus/JRK-KR5, *C. asianum* from (5,12,19) papaya/PPY-GDN2, (6,13,20) avocado/ALP-DMGA and *C. siamense* from (7,14,21) banana/PSG-JG

brownish mycelium color {Fig. 3 (1, 8)} and small cylindrical conidia {Fig. 3 (15)}. PSG-JG isolated from banana had a greyish colony color {Fig. 3 (7, 14)} and medium cylindrical conidia {Fig. 3 (21)}. JRK-SMO isolated from citrus had white mycelium color {Fig. 3 (3, 10)} and medium fusiform conidia {Fig. 3 (17)}. PPY-GDN2 isolated from papaya had a greyish aerial view, pinkish reverse view colony color{Fig. 3 (5, 12)} and medium cylindrical conidia {Fig. 3 (19)}. ALP-DMGA isolated from avocado had greyish mycelium color {Fig. 3 (6, 13)} and big cylindrical conidia {Fig. 3 (20)}. JRK-KR5 isolated from citrus had greenish-grey mycelium color {Fig. 3 (4, 11)} and medium cylindrical conidia {Fig. 3 (18)}. JMB-GW4 isolated from guava had greyish mycelium color, concentric ring {Fig. 3 (2, 9)} and small cylindrical conidia {Fig. 3 (16)}. The isolates then were subjected to molecular identification.

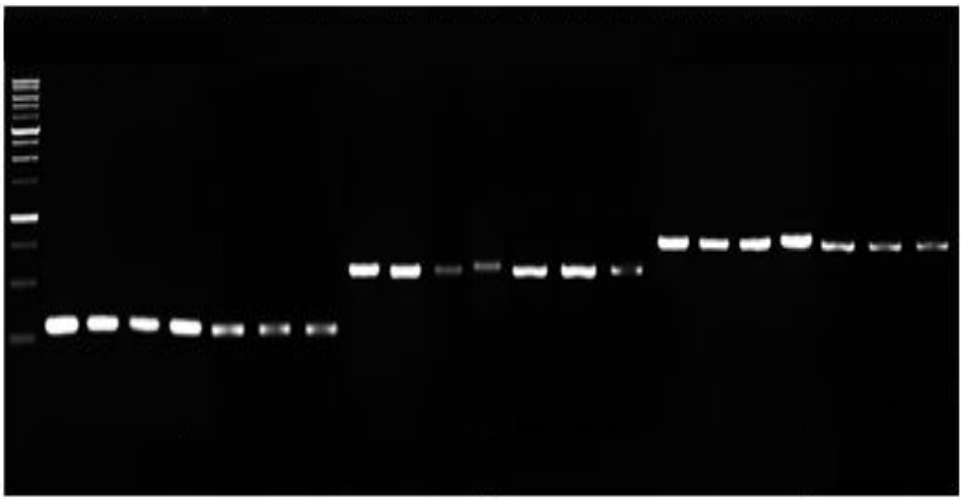

Fig. 4(a-c): PCR results for *Colletotrichum* spp. from varied tropical fruits
GeneRuler 1kb bp DNA ladder is shown in lane M, (a) Gapdh, (b) ITS and (c) Tub2 band position of all isolates from group representation (1,8,15) APL-MLG, (2,9,16) JRK-SMO, (3,10,17) ALP-DMGA, (4,11,18) JMB-GW-4, (5,12,19) PSG-JG, (6,13,20) PPY-GDN2 and (7,14,21) JRK-KR5

Molecular identification: Molecular identification of 7 representative isolates of *Colletotrichum* spp. performed using 3 primer pairs, i.e., ITS, gapdh and tub2. Figure 4 showed the PCR results of 7 representative isolates of *Colletotrichum* spp. (APL-MLG, JRK-SMO, ALP-DMGA, JMB-GW4, PSG-JG, PPY-GDN2, JRK-KR5). PCR amplified approximately 280 bp fragment from gapdh gene {Fig. 4A, (1-7)}, 550-600 bp fragment from ITS {Fig. 4B, (8-14)} and 750 bp from tub2 {Fig. 4C, (15-21)}. All selected isolates produced the expected amplicon sizes.

Multi-gene phylogenetic analysis: The phylogenetic tree was built by combining ITS, tub2 and gapdh sequence alignment of the selected isolates, BLAST algorithm and the reference species (Table 1). From 7 selected isolates, 5 isolates and 24 reference species were belong to gloeosporioides species complex, those 5 selected isolates were PSG-JG, PPY-GDN2, ALP-DMGA. JRK-KR5 and JRK-SMO (Fig. 5a). The other 2 selected isolates and 32 reference species belonged to acutatum species complex, those 2 isolates were APL-MLG and JMB-GW4 (Fig. 5a).

The analysis of gloeosporioides species complex using ITS, gapdh and tub2 sequence alignment comprised of 29 isolates with *C. fructi* CBS 346.37 (Fig. 5b) as the outgroup species clustered citrus isolates with *C. gloeosporioides*, papaya and avocado isolate with *C. asianum* and banana isolates with *C. siamense*. The analysis of acutatum species complex comprised of 34 isolates (Fig. 5c) with *C. gloeosporioides* ICMP 17821 as outgroup species clustered the apple and guava isolate with *C. sloanei*.

Cross-infection test: The percentage of fruits length in the cross-infection test of representative *Colletotrichum* isolates on tropical fruits, some isolates could infect non-host fruits but in different pathogenicity (Table 3). On wound inoculation, *Colletotrichum sloanei* from apple infected guava, citrus and papaya as non-host fruits with the percentage of infections 23.11, 6.19 and 3.62%, respectively. *Colletotrichum sloanei* from guava infected apple and citrus as non-host fruits with the percentage of infection 11.33 and 9.44%, respectively. *Colletotrichum siamense* from banana infected apple, citrus and avocado as non-host fruits with the percentage of infection 2.00, 9.74 and 8.33%, respectively. *Colletotrichum gloeosporioides* from *Citrus reticulata* (JRK-SMO) infected apple (6.67%) whereas isolates from *Citrus sinensis* (JRK-KR5) could not infect another fruit except the original host in the same genus, which was *Citrus reticulata*. *Colletotrichum asianum* from avocado infected apple (1.21%) and citrus (10.00%), whereas the same species isolated from papaya only infected citrus as non-host fruits with the percentage of infection 8.89%. On non-wound inoculation in this study, there were three isolates infected the original host, *C. sloanei* from guava (2.92%), *C. asianum* from papaya (2.98%) and avocado (1.00%). One isolates had the potency of cross-infection to other host, *C. sloanei* from guava infected apple as non-host fruit with the percentage of infection 5.33%. Table 4 showed a summary of potential cross-infection of representative *Colletotrichum* isolates among tropical fruits in this study, some isolates of *Colletotrichum* infected the other host and original host from they were isolated.

Table 2: Colony and conidia characteristic of each isolates *Colletotrichum* spp. from varied tropical fruits in Special Region of Yogyakarta

Isolate name	Colony color		Colony texture	Concentric ring	Conidiomata	Black spot	Conidial shape	Conidia size (µm)		Growth rate (mm day^{-1})
	Aerial view	Reverse view						Length	Width	
APL-MLG	Brownish	Brownish	Non-cottony	-	-	-	Cylindrical	8.40±4.06	4.02±1.12	5.68
ALP-DMGA	Greyish	Greyish	Cottony	v	v	v	Cylindrical	15.17±1.47	3.41±0.87	10.75
ALP-DMGO	White orange	White orange	Cottony	v	v	-	Cylindrical	12.13±4.59	3.50±1.55	11.15
ALP-DRS	White greyish	Greyish	Cottony	v	-	v	Cylindrical	16.16±4.99	2.99±0.65	11.28
JRK-SMO	White	White	Cottony	v	v	v	Fusiform	12.74±1.36	4.56±0.97	11.87
JRK-SMP	White	White	Cottony	v	v	v	Fusiform	14.66±1.64	4.42±0.91	11.45
JRK-DRS	Greyish	Greyish	Cottony	v	v	v	Cylindrical	14.87±1.53	4.78±0.67	11.37
JMB-GW4	White greyish	Greyish	Cottony	v	-	-	Cylindrical	9.55±2.74	3.67±0.72	7.12
JRK-KR5	White greyish	Greenish grey	Cottony	v	v	-	Cylindrical	11.40±1.47	3.58±0.88	8.20
JRK-KRM	Greenish grey	Greenish grey	Cottony	v	v	-	Cylindrical	12.36±1.78	3.43±0.68	9.67
JRK-SL	Greyish	Greyish	Cottony	v	-	-	Cylindrical	15.41±2.92	3.76±0.91	8.67
MGG-JG	Brownish grey	Brownish	Cottony	v	-	-	Cylindrical	8.38±2.48	4.39±1.07	10.08
MGG-SM2	White	White	Cottony	v	-	-	Cylindrical	14.19±2.15	3.43±0.69	9.67
PPY-GDN2	Greyish	Pinkish	Cottony	v	v	v	Cylindrical	13.65±3.66	3.44±0.79	11.32
PSG-JG	White greyish	Greyish	Cottony	v	v	-	Cylindrical	13.78±1.91	4.67±0.92	12.57

*v: Isolate had the characteristic of morphology, -: Isolate did not have the characteristic of morphology

Table 3: Percentage of fruits length infected in cross-infection test of *Colletotrichum* species on tropical fruits

Colletotrichum species	Isolates name	Host	Fruit length infected (%)											
			Apple		Guava		Banana		Citrus		Papaya		Avocado	
			w	nw	w	nw	w	nw	w	nw	w	nw	w	nw
C. sloanei	APL-MLG	Apple	3.64^{a}	0.00^{a}	23.11^{a}	0.00^{a}	0.00^{b}	0.00^{a}	6.19^{a}	0.00^{a}	3.62^{a}	0.00^{a}	0.00^{a}	0.00^{a}
	JMB-GW4	Guava	11.33^{a}	5.33^{a}	20.00^{b}	2.92^{a}	0.00^{b}	0.00^{a}	9.44^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
C. siamense	PSG-JG	Banana	2.00^{a}	0.00^{a}	0.00^{c}	0.00^{a}	9.33^{a}	0.00^{a}	9.74^{a}	0.00^{a}	0.00^{a}	0.00^{a}	8.33^{a}	0.00^{a}
C. gloeosporioides	JRK-SMO	Citrus	6.67^{a}	0.00^{a}	0.00^{c}	0.00^{a}	0.00^{b}	0.00^{a}	5.56^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
	JRK-KR5	Citrus	0.00^{a}	0.00^{a}	0.00^{c}	0.00^{a}	0.00^{b}	0.00^{a}	15.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
C. asianum	PPY-GDN2	Papaya	0.00^{a}	0.00^{a}	0.00^{c}	0.00^{a}	0.00^{b}	0.00^{a}	8.89^{a}	0.00^{a}	2.98^{a}	2.98^{a}	0.00^{a}	0.00^{a}
	ALP-DMGA	Avocado	1.21^{a}	0.00^{a}	0.00^{c}	0.00^{a}	0.00^{b}	0.00^{a}	10.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	9.00^{a}	1.00^{a}

*Means with the same letter in each column are not significantly different from each other based on DMRT, w: Wound, nw: Non-wound

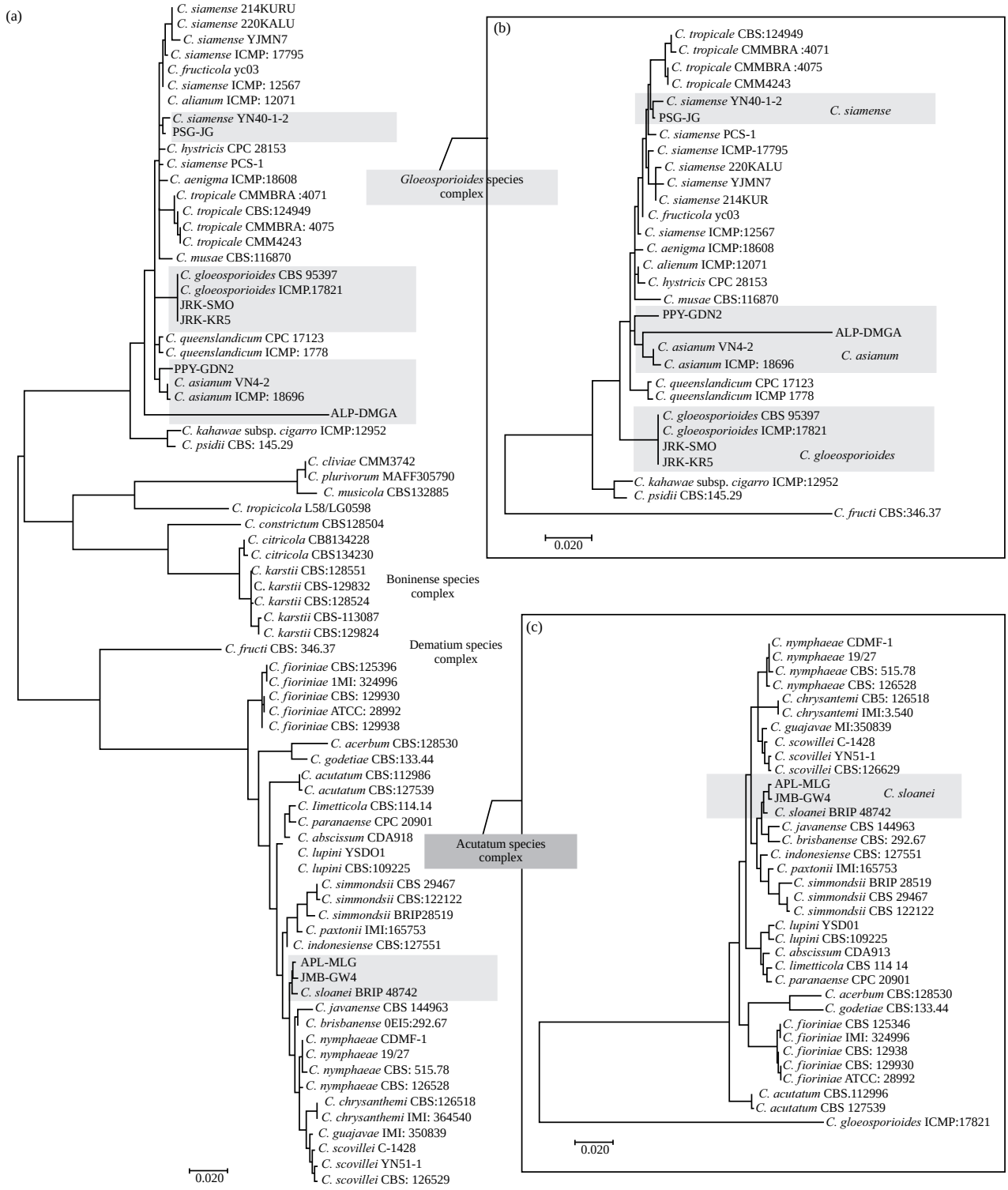

Fig. 5(a-c): Phylogenetic analysis using Maximum Likelihood (ML) algorithm combined gene of ITS, gapdh and tub2

(a) Sequence alignment showing the separation of *Colletotrichum* isolates into gloeosporioides species complex, boninense species complex, dematium species complex and acutatum species complex, the four clades containing tropical fruits isolates are indicated by blocks, (b) The analysis of gloeosporioides species complex was used *C. fructi* as outgroup species and (c) The analysis of acutatum species complex was used *C. gloeosporioides* as outgroup species

Table 4: Cross infection potency of *Colletotrichum* species among tropical fruits

			Cross infection potency					
Colletotrichum species	Isolates name	Host	Apple	Guava	Banana	Citrus	Papaya	Avocado
C. sloanei	APL-MLG	Apple	+	+	-	+	+	-
	JMB-GW4	Guava	+	+	-	+	-	-
C. siamense	PSG-JG	Banana	+	-	+	+	-	+
C. gloeosporioides	JRK-SMO	Citrus	+	-	-	+	-	-
	JRK-KR5	Citrus	-	-	-	+	-	-
C. asianum	PPY-GDN2	Papaya	-	-	-	+	+	-
	ALP-DMGA	Avocado	+	-	-	+	-	+

*+: Fruits infected by *Colletotrichum* isolates, -: Fruits not infected by *Colletotrichum* isolates

DISCUSSION

The results indicated that *Colletotrichum* species isolated from tropical fruits in the Special Region of Yogyakarta, apple, banana, citrus, avocado, papaya and guava isolates showed variations in cultural and morphological characters (Fig. 3). The morphological features including the cultural characteristics, size and shape of the conidia, present of conidiomata and colony growth rate than were investigated into the phylogenetic analysis (Table 2). This approach was explained by Cai *et al*.[5] that morphological characteristics and molecular data were needed to be linked as a polyphasic approach.

The *C. gloeosporioides* had a large range of colony color and growth rate, also very common on *Citrus* sp.[12]. Both isolate from citrus (JRK-SMO and JRK-KR5) had a similar colony color of *C. gloeosporioides* but different in size and shape of conidia compared with the description from Weir *et al*.[12], Prihastuti *et al*.[19], Aiello *et al*.[20] and Ramos *et al*.[21]. The other isolates from the same group in UPGMA dendrogram (Fig. 2), JRK-SMP, ALP-DMGO and JRK-KRM, had the characterization that similar to *C. gloeosporioides*. Fusiform conidia commonly found in *C. acutatum*, but two of the isolates (JRK-SMO and JRK-SMP) of *C. gloeosporioides* in this study were found to have a fusiform conidia {Fig. 3 (17)}.

The isolates derived from citrus (JRK-SMO, JRK-SMP, JRK-KR5, JRK-KRM) in this study were similar to *C. gloeosporioides* described by Ramos *et al*.[21] and Rhaim and Taylor[22] who conducted the same study using multi-gene analysis in the detection of *Colletotrichum* in citrus fruits. Obtained *C. gloeosporioides* isolates also had various colony colors and conidial sizes. Moges *et al*.[23] also explained that *C. gloeosporioides* was a common species associated with anthracnose in citrus. Avocado isolate (ALP-DMGO) which was in a group with group 3 (Fig. 2), had morphological characteristics similar to those explained by Sharma *et al*.[24], *C. gloeosporioides* obtained from avocados had orange colony colors and cylindrical conidial shapes and measuring between 12.0-17.0×3.5-6.5 µm.

Isolate from banana (PSG-JG) clustered with *C. siamense* YN40-1-2 isolated from mango fruits had the same colony color and shape of conidia[25]. A study by Uysal and Kurt[26] explained the morphological character of *C. siamense* causing anthracnose on banana fruits had white to grey colony color and concentric ring, these were similar to the isolates from this study, but the conidia shape was different. *C. siamense* previously reported associated with coffee berries[21], mango and avocado[27].

Isolate from papaya (PPY-GDN2) and avocado (ALP-DMGA) clustered with *C. asianum* VN4-2 and *C. asianum* ICMP 18696 isolated from mango fruits and had the same colony color, the shape of conidia, also in the range of conidia size and mycelium growth rate[28]. Isolates from the same group in UPGMA dendrogram, JRK-DRS and ALP-DRS also had the same morphology character as *C. asianum*. *C. asianum* commonly found from mango, but Giblin *et al*.[27] study found one isolate of *C. asianum* isolated from mango was pathogenic to avocado. Phoulivoung *et al*.[29] studied the *Colletotrichum* species associated with tropical fruits found that *C. asianum* was obtained from mangoes, whereas the *Colletotrichum* species obtained from papaya was *C. fructicola* which also in the gloeosporioides species complex. Previous studies conducted by Prihastuti *et al*.[21] reported that *C. asianum* was one of *Colletotrichum* species associated with coffee berries in northern Thailand.

Apple isolates (APL-MLG) and guava isolate (JMB-GW4) clustered with *C. sloanei* BRIP 48742. The conidia size and shape were quite similar compared to Damm *et al*.[13]. Isolates from mango (MGG-JG, MGG-SM2) and isolate from citrus (JRK-SL) and the other isolates from the same group in UPGMA dendrogram also had a similar morphological characterization with *C. sloanei*. Conidia of the representative *Colletotrichum* isolates were cylindrical with one end round and one end acute {Fig. 3 (15,16)}. This is the first report of *C. sloanei* on apple, guava, mango and citrus. The host range and pathogenicity of *C. sloanei* is still little known. *C. sloanei* belongs to acutatum species complex and best distinguished from other species in acutatum species complex with gapdh or tub sequences[30].

In this study, colony and conidia morphology alone could not be distinguished between *Colletotrichum* subspecies, nor did they differentiate between acutatum and gloeosporioides species complex. Therefore to identify *Colletotrichum* to species level, molecular analysis was needed, especially with multi-genetic analysis. Previous studies have suggested that acutatum species complex and gloeosporioides species complex were the most confusing species complex to distinguish from their morphological parameters[12,13]. It was also mentioned that *Colletotrichum* species had very large conidia size range so that overlap could occur in morphological observations. *Colletotrichum* species from different hosts would form different colonies and conidia, apart from the host this could also be influenced by environmental conditions of isolate growth (growing media, temperature, light, etc.)[12,31,32].

Cross-infection test results in this study showed that there was the potency of cross-infection among tropical fruits. Isolates evaluated were able to produce symptoms in their original hosts, but not always on another (Table 4). Previous studies[10,33] reported similar results but with different isolates. *Colletotrichum sloanei* from guava had the highest percentage of infection on apple (11.33%), although it was not statistically different (Table 3) and interestingly the isolate of *C. sloanei* from apple had the highest percentage of infection on guava fruits (23.11%). Lakshmi *et al.*[10] also reported that the isolate of *Colletotrichum* could be more aggressive to other tested fruits than the original host. This was different from Hayden *et al.*[31] previously reported isolates of *Colletotrichum* were more aggressive in infecting the host from which they were originally isolated.

All isolates in this study could infect citrus fruits, but isolate *C. gloeosporioides* from citrus could not infect all non-host fruits. JRK-KR5 isolate only found infected citrus, this could be the sign this isolate is host-specific. Phoulivong *et al.*[11] cross-infection study showed that *Colletotrichum* strains could infect more than one host and one host also could be infected with many *Colletotrichum* species. The same species isolated from different hosts had different cross-infection ability and this should be considered when establishing new species. The different capabilities of *Colletotrichum* spp. to infect various kinds of fruits could be attributed to variations in the compositions of each kind of fruits[10]. Fruits susceptibility to *Colletotrichum* infection was connected to the degree of antifungal inhibitor present in these fruits[34].

Many isolates also caused lesions on fruits in wounded inoculation but not in unwounded inoculations. This has also happened in the previous study. This situation related to quiescent infection of the species, which was the lifestyle of *Colletotrichum* spp., where infection occurred at un-ripen fruit then developing fruit rot as the fruit ripens or the condition and environmental was supportive[6,35].

In a previous study, *C. sloanei* was reported to cause anthracnose on *Litchi chinensis* and the host range was little known[12,30]. *Colletotrichum siamense* was also previously reported caused anthracnose on peach, pear, coffee berries, citrus, guava, mango, chili, papaya[11,21,35-37]. *Colletotrichum gloeosporioides* previously was reported to associate with citrus, pear, avocado, mango, dragon fruit, olive, papaya[35,38-41]. *Colletotrichum asianum* previously was reported to associate with coffee berries, mango, chili, rose apple[11,21,42-45].

The current finding in this study showed *Colletotrichum* species were obtained from apple, citrus, guava, banana, mango, avocado and papaya. Cross-infection test results showed different pathogenicity of each isolate. This data can be used to see host-pathogen interactions of *Colletotrichum*. However, it is still necessary to identify the species complex of *Colletotrichum* and study the potency of cross-infection on different tropical fruits to add the data for this pathogen.

CONCLUSION

Multi-genetic analysis and morphological identification in this study revealed *C. siamense*, *C. asianum*, *C. gloeosporioides* and *C. sloanei* associated with tropical fruits anthracnose in Special Region of Yogyakarta, Indonesia. According to the author's knowledge, this is the first report of *C. sloanei* on apple, guava, mango and citrus in Indonesia. All isolates in this study had the potency of cross-infection, although each isolate varied in degrees of pathogenicity.

SIGNIFICANCE STATEMENT

This study discovered the *Colletotrichum* species associated with tropical fruits anthracnose by using multi-gene analysis and the cross-infection potency of the species that can be beneficial for the management of anthracnose caused by *Colletotrichum* on tropical fruits. This study will help the researchers to uncover the critical areas of the identification species complex of *Colletotrichum* in the areas that many researchers were not able to explore. Thus, a new theory on identifying species of *Colletotrichum* may be arrived at.

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