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Research Article

Morphological and Molecular Identification of *Colletotrichum* spp. Associated with Chili Anthracnose Disease in Yogyakarta Region(*)

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ABSTRACT

Colletotrichum sp., the causal agent of anthracnose disease, is one of the important pathogenic fungi in chili which can cause considerable yield losses, especially during the rainy season. This study aimed to identify the species of Colletotrichum isolates obtained from chili cultivation area in The Special Region of Yogyakarta Province both morphologically and molecularly. As a comparison, a Colletotrichum isolate obtained from Magelang Regency, Central Java Province was used as comparison isolate. From the isolation result, it was obtained 14 isolates of Colletotrichum that generally had conidia that were fusiform to cylindrical with two pointed or slightly blunt ends, or crescent shapes with a various size range between 9.02-19.38 µm × 2.37–8.57 µm. Based on morphological observations using UPGMA analysis, these 14 isolates could be divided into 4 groups with 7 different types. Representative isolates of each type in different groups and a comparison isolate were identified molecularly by multi-gene analysis using the ITS1-4, *gapdh* and *tub*2 genes. The result showed that B1, G1, K2 and Mg isolates were closely related to *Colletotrichum scovillei*, J1 with *C. truncatum*; S1 and S2 with *C. siamense*; and J2 with *C. makassarii*. From the pathogenicity test on wounded chili, it showed that *C. scovillei* and *C. siamense* isolates had higher virulence than *C. truncatum* and *C. makassarii* isolates.

Keywords**:** anthracnose; chili; *Colletotrichum makassarii*; *C. scovillei*; *C. siamense*; *C. truncatum*

INTRODUCTION

Chili is one of the regional leading commodity planted in various agroecosystem. A suitable agro-climatic condition supports the chili production in Yogyakarta area (Sutardi & Wirasti, 2017). Therefore, chili production has also increased due to a program, called "Leading Horticulture in Special Region of Yogyakarta". In 2016–2017, production of *Capsicum annuum* increased 1.29%, while *C. frutescent* increased up to 6.20% (Central Bureau of Statistics, 2017). However, Riyandi (2017) reported that the production of red and green chilies in Sleman declined up to 60%. Those declined was caused by fungal pathogen showing anthracnose symptoms. Anthracnose caused by *Colletotrichum* spp. produces circular or angular sunken lesion with concentric rings of acervuli that are often wet and producing peach conidial mass. In humid condition, the lesions may coalesce. Conidial mass may occur scatteredly or in concentric rings on the lesion (Than *et al*., 2008).

Even though chili has economically and nutritional importance, information on anthracnose disease of chili is limited. Pathogens infecting chili in Special Region of Yogyakarta including the current study areas have not yet been characterized, and not fully documented although diseases affecting the crop have been reported. Identification of species is essential for effective disease management. Morphological characterization is a primarily identification, and this is usually not sufficient to differentiate species. Specific primers also could not identify *Colletotrichum* species (Prakoso *et al*., 2019). Thus, it needs further technique such as the implementation of multi-genes phylogenetic analyses for proper identification of these species (de Silva *et al*., 2017).

Colletotrichum acutatum, C. gloeosporioides and *C. capsici* are species mostly found on chili anthracnose in Indonesia (Andriani *et al*., 2017; Hartati *et al.*, 2019). Recent taxonomic studies of *Colletotrichum* revealed *C. acutatum* species complex from infected chili fruit in Thailand being re-identified as *C. scovillei* and from Indonesia as *C. nymphaeae* (Damm *et al*., 2012). Similarly in Gloeosporioides complex, *C. siamense, C. fruticola,* and *C. asianum* were newly

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re-identified species. These species cause chili anthracnose found in Thailand and India (Phoulivong *et al*., 2012; Sharma & Shenoy, 2014). Recognition of new species applying combination of morphological characteristics and molecular approaches using multi-gene analyses should be conducted. The objective of this study were to identify phylogenetic relationship of *Colletotrichum* isolates associated in chili anthracnose in Yogyakarta Special Region, based on morphology and phylogenetic analyses. The pathogenicity of the isolates was also assessed.

MATERIALS AND METHODS

Study Area

Collection of anthracnose disease symptoms on chilies was carried out in different agro-ecological zones in Special Region of Yogyakarta (Figure 1). The study area was located at 8°30′ N – 7°20′ N latitude and 109°40′ E – 111°0′ E longitude with different altitude. Purposive random sampling was selected to collect symptomatic plant organs found in five districts, namely Bantul, Sleman, Kulon Progo, Gunung Kidul and Yogyakarta. Diseased plant organs such as leaves, panicles and fruits were collected and brought to Laboratory of Plant Clinic in Universitas Gadjah Mada.

Morphological Identification of Pathogen

At the laboratory, portions of infected plant organs such as fruit and leaves were cut into small pieces and then dipped in sodium hypochlorite (NaOCl) for 30 seconds, then rinsed in sterile distilled water for three times. The pieces were placed on sterile paper, allowed to dry before plating onto Potato Dextrose Agar (PDA) and then they were incubated at room temperature. Isolated colonies were sub-cultured into fresh plates until pure cultures were obtained. Pure cultures were identified by visual examinations (macroscopic) and observed under light-microscope (microscopic). The pathogens were identified based on their cultural and morphological characters. A full of fungal culture grown on PDA plates were taken on a glass slide and observed with microscope for the presence of *Colletotrichum* spp. conidia. After confirming the conidia, the cultures were purified and stored in room temperature. The fungi were identified on the

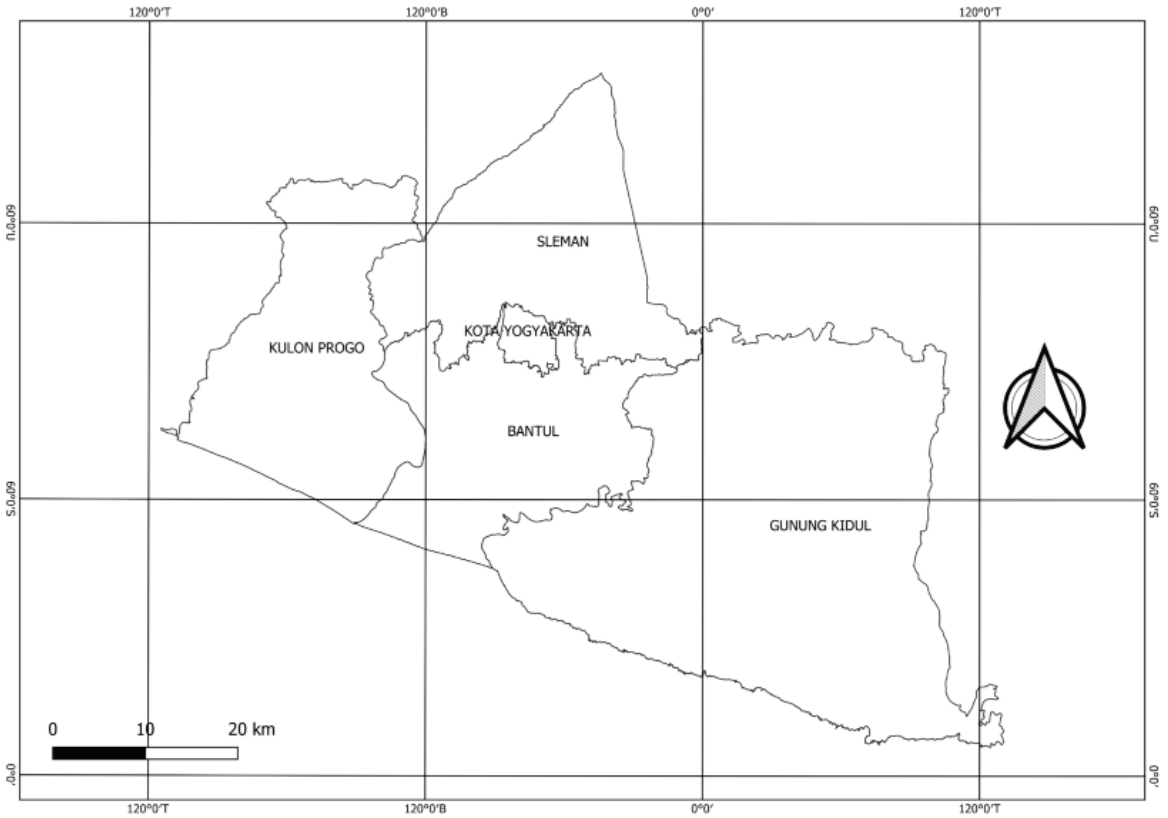

Figure 1. Location of chili anthracnose exploration in Special Region of Yogyakarta

basis of morphological characteristics as suggested by de Silva *et al*. (2017) and Eryna *et al*. (2017). For maintenance, the *Colletotrichum* spp. culture were sub-cultured on PDA slant and allowed to grow at room condition. These pure cultures were used for characterization.

Morphological characteristics were identified using pure culture of *Colletotrichum* spp. on 10 days PDA inoculation. Macroscopic observation included colony textures, colors, growth rate, and conidiomata. Microscopic observation included conidial shape, length and width, and presence of appressoria. Conidial length and width were measured for 30 randomly selected conidia for each isolates with 3 replicates, with range, mean, and standard deviation. Setae were noted on the 30 days inoculation. Then, Squared Euclidian distance between genotypes was calculated from the morphological standardized data matrix by Unweighted Pair Group Method using Arithmetic Averages (UPGMA) method. Clustering was conducted by Sequential Agglomerative Hierarchical Non-overlapping (SAHN) clustering using NTSYSpc.

DNA Extraction, PCR Amplification, and Sequencing

Representative isolates of different sites were selected from morphological characterization based on UPGMA result. Mycelial disk were excised from the margin of colonies and inoculated into PDA in at room temperature for 7 days. Cultures were harvested by scalpel. Extractions were done using protocol of Genomic DNA Mini Kit (plant). Genomic DNA was visualized in 1% (w/v) agarose gel after staining. DNA extraction were ready to use or stored at -20^{0}C.

DNA of the isolates was further analyzed with multi-genes sequences, namely ITS rDNA, β-tubulin (*tub2*), and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*). The genes were amplified and sequenced using respective primer pairs for each region: ITS1 + ITS4 (ITS; White *et al.,* 1990), TUB2T1 + TUB2T2 (*tub2*; Woudenberg *et al.,* 2009), GDF1 + GDR1 (*gapdh*: Guerber *et al.,* 2003). The PCR for each reaction was performed in Biorad thermal cycler in a total volume of 25 µl, comprised of 9.5 µl miliQ water sterile, 12.5 µl taq DNA polymerase (My Taq HS Red Mix; Bioline), 1 µl of each primer, and 1 µl template DNA. The Polymerase Chain Reaction (PCR) cycling conditions followed Standard MyTaq HS Red Mix Protocol, for annealing temperatures adjusted to 55^{0}C for ITS and *tub2*, and 60^{0}C for *gapdh.* The PCR products were assessed on 1% (w/v) agarose gel containing DNA staining, and run on electrophoresis machine (100 V for 25 minutes). The products were then visualized under UV transilluminator, and noted the size (bp). The DNA sequence analyses of the PCR products were carried out at either LPPT UGM or Genetika Science.

Phylogenetic Analyses

Gene sequences of each isolates were examined using MEGA 64X and aligned by CLUSTALW2 (Larkin *et al*., 2007). Selected reference or ex-type strains sequences were added in the analyses and trimmed all ends to obtain the same size sequence before constructing phylogenetic tree. Ex-type strains were selected based on chili as host with some additional other plants. The reference isolates in Table 1 were adopted from de Silva *et al*. (2017) and de Silva *et al*. (2019). Concatenated datasets comprised ITS, *tub2*, and *gapdh*. Phylogenetic tree was generated with a maximum-likelihood (ML) as implemented in MEGA 64X with 1000 bootstrap replicates.

Pathogenicity Assay

The representative isolates were inoculated into healthy chili (*C. annuum*) fruits to confirm the pathogenicity of the fungi. Healthy fruits were surface sterilized using alchohol 70% and rinsed by sterilized aquadest 3 times. Mycelial disks (5 mm diameter) from 10 days old of *Colletotrichum* culture was inoculated on wounded fruit, and then incubated in humid closed chambers at room temperature. Diameter of the symptom was measured on 10 days inoculation. Pathogenicity assay was designed using Completely Randomized Design (CRD) with five replicates. The experiment was repeated three times. The generated data were analyzed using SPSS. Mean values among treatments were compared $\alpha = 0.05\%$ level of significance.

RESULTS AND DISCUSSION

Field Observation

Explorations of *Colletotrichum* spp. were conducted at 14 sites in five regencies throughout Yogyakarta Special Region with various altitudes (Table 2).

Table 1. Strain of *Colletotrichum* species used in the phylogenetic analysis with details of host and location, and GenBank accession numbers of the sequences

Species	Accession Number	Host	Country	GenBank Accession Number		
				ITS	*gapdh*	*tub2*
Acutatum complex						
C. brisbanense	CBS 292.67	*Capsicum annuum*	Australia	JQ48291	JQ948621	JQ949942
C. cairnense	CBS 140847	*Capsicum annuum*	Australia	KU923672	KU923704	KU923688
C. guajavae	IMI 350839	*Psidium guajava*	Netherland			
C. javanense	UOM 1115	*Capsicum annuum*	Indonesia	MH846574	MH846472	MH846574
C. scovillei	CBS 120708	*Capsicum annuum*	Thailand	JQ948269	JQ948599	JQ949920
	CBS 126529	*Capsicum* sp.	Indonesia	JQ948267	JQ948597	JQ949918
C. simmondsii	BRIP 63649	*Capsicum* sp.	Australia	KU199252	KU199254	KU199253
	BRIP 63650	*Capsicum* sp.	Australia	KU199258	KU199260	KU199259
C. nymphaeae	CBS 126528	*Capsicum* sp.	Indonesia	JQ948219	JQ948880	JQ949870
C. queenslandicum	BRIP 63699	*Capsicum annuum*	Australia	KU923681	-	KU923697
	BRIP 63700	*Capsicum annuum*	Australia	KU923682	-	KU923698
Boninense complex						
C. karsti	CAUOS1	*Capsicum* sp.	China	KP890103	KP890134	KP890110
	CBS 128545	*Capsicum annuum*	New Zealand	JQ005207	JQ005294	JQ005641
Gloeosporioides complex						
C. aeschynomenes	OBrC1	*Solanum melongena*	India	KU239115	KU239576	KU239241
	ICMP 17673	*Aeschynomene virginica*	USA	NR120133	JX009930	JX010392
C. alienum	CBS 112991	*Leucadendron* sp.	Portugal	KC297070	KC297001	KC297098
	CBS 133930	*Protea* sp.	Portugal	KC297076	KC297000	KC297096
C. endophyticum	UOM 1137	*Capsicum annuum*	Thailand	MH728809	MH707467	MH846566
C. fruticola	CPC 28644	*Capsicum annuum*	Thailand	MH728811	MH707465	MH846564
	CPC 30253	*Capsicum annuum*	Taiwan	MH728817	MH707463	MH846559
C. grossum	CAUG7	*Capsicum* sp.	China	KP890165	KP890159	KP890171
C. makassarii	CPC 28556	*Capsicum annuum*	Indonesia	MH728815	MH728821	MH846561
	CPC 28612	*Capsicum annuum*	Indonesia	MH728812	MH728820	MH846563
C. psidii	ICMP 19120	*Psidium* sp.	Italy			JX010443
C. siamense	CPC 30210	*Capsicum annuum*	Indonesia	MH707472	MH707453	MH846548
	CPC 30221	*Capsicum annuum*	Thailand	MH707475	MH707456	MH846551
C. tainanense	CPC 28607	*Capsicum annuum*	Taiwan	MH728818	MH728823	MH846558
	UOM 1290	*Capsicum annuum*	Taiwan	MH728805	MH728819	MH846570
C. tropicale	CPC 28607	*Capsicum annuum*	Indonesia	MH728814	MH707464	MH846562
	UOM 1002	*Capsicum annuum*	Indonesia	MH728807	MH707469	MH846568
C. viniferum	CAUG27	*Capsicum* sp.	China	KP145440	KP145412	KP145468
Orchiadearum complex						
C. plurivorum	CPC 28638	*Capsicum annuum (leaf)*	Thailand	MH805810	MH805816	MH805824
	UOMM2	*Capsicum annuum*	Malaysia	MH805815	MH805821	MH805827
Truncatum complex						
C. truncatum	CBS 151.35	*Phaseolus lunatus*	USA	GU227862	GU228254	GU228156
	CBS 120709	*Capsicum frutescens*	India	GU227877	GU228269	GU228171
Monilochaetes infuscans (Outgroup)	CBS 869.96	Unknown	Unknown	GU180626	JX546612	JQ005864

Table 2. Exploration Sites of *Colletotrichum* spp. associated with chili anthracnose

Regencies	Isolates	Location	Altitude (m a.s.l.)	Chili Species	Organ
Bantul	B1	Tridharma Garden, Faculty of Agriculture, UGM	103	*C. frutescens*	Leaf
	B2	Tridharma Garden, Faculty of Agriculture, UGM	103	*C. frutescens*	Fruit
	B3	Pantai Kuwaru St. 22, Sansen, Murtigading, Mayungan	13	*C. frutescens*	Leaf
Gunung Kidul	G1	Asem Gedhe St., Blimbing, Karangrejek, Wonosari	176	*C. frutescens*	Fruit
	G2	National III St., Patuk	136	*C. frutescens*	Fruit
	G3	Srikaya, Bleberan, Playen	158	*C. frutescens*	Fruit
Jogja	J1	Lowanu, Umbulharjo	88	*C. frutescens*	Fruit
	J2	Taman Sari, Patehan, Kraton	107	*C. frutescens*	Fruit
Kulon Progo	K1	Brosot, Sentolo	7	*C. frutescens*	Leaf
	K2	Pantai Trisik St., Kranggan, Galur	5	*C. frutescens*	Fruit
Sleman	S1	Pasir Luhur St., Palgading, Sinduharjo, Ngaglik	291	*C. annum*	Fruit
	S2	Margokaton, Seyegan	149	*C. frutescens*	Fruit
	S3	Wedomartani, Ngemplak	201	*C. frutescens*	Leaf
Magelang*)	Mg	Sarangan 1 St., Banyurojo, Mertoyudan	343	*C. annuum*	Fruit

Note: *)Magelang as comparative sample

Altitude was varied from 5 up to 343 m above sea level. The abundance of chili plantation triggers many living pathogens, including pathogenic fungi. *Colletotrichum* spp. is one of plant pathogenic fungi associated with anthracnose including chili.

Based on the observation during this study, it was found that anthracnose disease in red chili was characterized by initial symptoms of small spots that are slightly curved. Anthracnose symptoms were found not only on fruit, but also leaf tissue (Figure 2). On fruit, there were black and peach rot symptoms and forming concentric circles. On the leaves, the symptoms of anthracnose were sunken brown spots with dark margin. The leaves were dropped as the disease development. As it was reported by Rahmat *et al*. (2011), plant symptoms were initially expressed as water-soaked, slightly sunken, dark dot like lesions on leaf blade. Within 2 to 3 days the lesions increased rapidly and most of the leaves were infected and the infected plant started to die from the top. The leaves and flowers of infected plants became soft and dropped from the plants. Within 7 to 10 days the disease became very severe and infected plants will die.

Colletotrichum isolates were collected from infected fruits and leaves of chili plants from five regencies in Yogyakarta Special Region, namely Bantul, Gunung Kidul, Kulon Progo, Sleman, and Yogyakarta (Jogja). In addition, comparative isolate was collected from Magelang, Central Java. Anthracnose symptoms were found on leaves tissue (28.57%) and fruits (71.43%) of total isolates. These results are in line with Ranathunge *et al.* (2012) that also found mostly, *C. capsici* had major damage at ripe fruit stage.

All isolates from different location producing conidia referred to *Colletotrichum* spp. (Figure 3). *Colletorichum* isolates were distributed on different altitudes. The isolates were mostly found on moderate altitude. It was due to 65.65% area of Yogyakarta lies at 100–499 m above sea level. Based on exploration, *Colletotrichum* spp. associated with chili anthracnose could be found on both of low to moderate altitudes ranged from 5 to 343 m asl. They were mostly found on 103–176 m asl. It was in line with Masanto *et al*. (2009) who reported that *Colletotrichum* spp. could be found on low to moderate altitudes ranging from 3.96 to 146.91 m asl. Beside it, National Land Board of Yogyakarta (2013) divided the province into 4 altitudes, mentioned: low (<100 m asl); moderate (100–499 m asl); high (500–999 m asl); and very high (>1000 m asl). Since Special Region of Yogyakarta lies on moderate altitude, approximately 82.67%, the disease incidence was largely detected on moderate altitude. The moderate altitudes were dominated by Sleman and Gunung Kidul regencies.

Morphological Characteristic

Macroscopic and microscopic observation resulted in various morphological characters (Table 3, Table 4). Figure 4 showed aerial and reverse view on each isolates. Those isolates were then clustered to

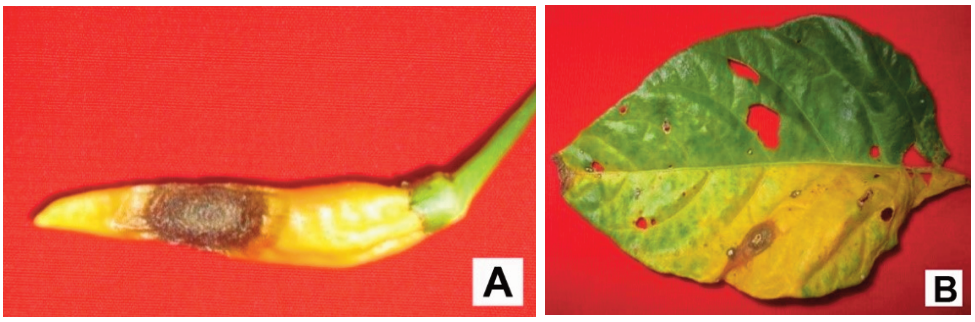

Figure 2. Anthracnose symptoms on chili: (A) *Colletotrichum frutescens* on fruit; (B) *C. frutescens* on leaf

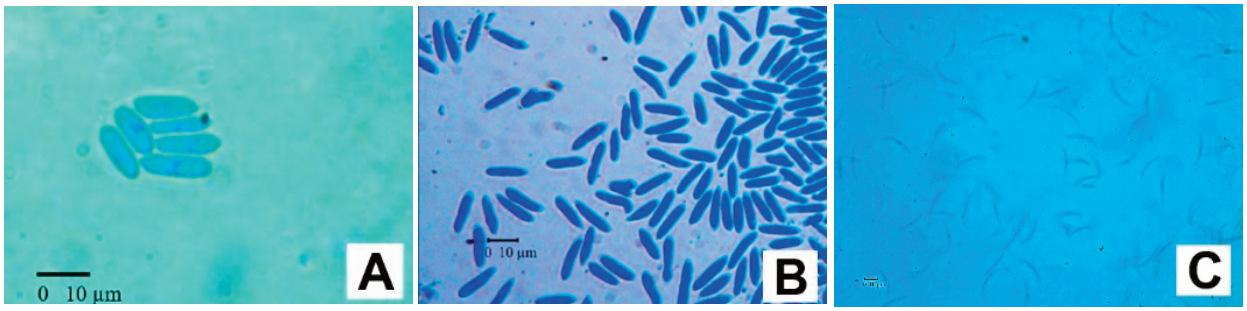

Figure 3. Conidial shapes of *Colletotrichum* spp. found in Special Region of Yogyakarta; A: fusiform (B1, B2, B3, J2, K1, K2, S1, S2, S3, Mg); B: Cylindrical (G1, G2, G3); C: falcate (J1)

distinguish morphological types using UPGMA method (Figure 5). The method revealed 14 isolates were classified into two clusters at a level of 0.64 in the coefficient scale. Cluster I comprised of 10 isolates and clusters 2 comprised of 4 isolates. Cluster I was represented into 2 groups obtained from 5 regencies, namely Bantul, Kulon Progo, Sleman, and Magelang. In comparison, cluster II were obtained from Gunung Kidul regency. Isolates obtained from Jogja were spread out in cluster 1 (J2) and cluster 2 (J1). The J1 generated type VII because it had contrast feature. Isolates in group 1 were obtained from Bantul (Type I) and Sleman (Type II and III). B1 was distinct from B2 and B3 at level of 0.91. The isolates from Group II were obtained from Kulon Progo, Magelang, and Jogja – Taman Sari (J2). This group had two types, one was J2 (Type IV) based on similarity 0.80-1 and the others were Kulon Progo and Magelang (Type V). Kulon Progo (K1, K2) isolates were distinct with Magelang (Mg) isolate at level of 0.91. Those types were concatenated data sets comprising macroscopic and microscopic observations.

Cluster I had colony with aerial view of white with orange pigment. The colony color from aerial view was almost similar for every isolate, only Mg, K1, and K2 had different colors. The Mg, K1, and K2 isolates were clustered here because they produced conidiomata. Based on microscopic characters, cluster 1 had the same conidial shape, fusiform, with various size. Conidial size ranged from 12.70 to 14.45 µm length and 3.67 to 5.38 µm width. Group I showed similarity of radial growth rate. Colony B1, B2, B3 and S1 were able to fill petri-dish within 10 days inoculation, whereas S2 and S3 were able to fill partially. At 0.84-1 similarity, S1 was distinct with Bantul isolates due to conidial shape. Bantul isolates had longer and wider conidial size than S1. All parameter was same except colony texture occurred between S2 and S3. They were distinct at 0.96. S2 had cottony while S3 did not.

Group II was represented into 4 isolates, namely J2, Mg, K1, and K2. Isolate J2 was distinct with K1, K2, and Mg isolates in several characteristics at 0.80-1, such as colony color in aerial and reverse view, conidial size, and mycelial texture. The difference among K1, K2, and Mg were on colony color in aerial and reverse view and growth rate. Mg growth rate was slower than K1 and K2, and it had green color in aerial view. Cluster II belonged to

Table 3. Macroscopic morphology of *Colletotrichum* spp.

Species	Isolates	Growth rate	Texture	Aerial View	Reverse View	Conidiomata
C. annum	S1	Fast	Cottony	White with orange pigment	Concentric orange	Present
	S3	Slow	Not Cottony	Greyish	Orange – green	Present
C. frustescens	B1	Fast	Cottony	White with orange pigment	Orange – green	Present
	B2	Fast	Cottony	White with orange pigment	Concentric dark	Present
	B3	Fast	Cottony	White with orange pigment	Orange – green	Present
	G1	Fast	Cottony	Greyish	Black spot	Absent
	G2	Fast	Cottony	Greyish	Black spot	Absent
	G3	Fast	Cottony	Greyish	Black spot	Absent
	J1	Slow	Cottony	Greyish	Yellowish	Present
	J2	Fast	Cottony	White with orange pigment	Concentric yellow	Present
	K1	Fast	Not Cottony	Brown	Concentric ring	Present
	K2	Fast	Not Cottony	Brown	Concentric ring	Present
	S2	Slow	Cottony	Greyish	Orange - green	Present
	Mg	Slow	Not Cottony	Green	Concentric ring	Present

Table 4. Microscopic morphology of *Colletotrichum* spp.

Isolates	Conidial Shape	Conidia Size (µm)				Appressoria	Setae
		Length	Range	Width	Range		
B1	Fusiform	14.30±0.96	10.02–17.94	5.36±0.52	3.45–7.68	Present	Absent
B2	Fusiform	14.29±0.91	10.87–17.87	5.35±0.50	3.24–7.29	Present	Absent
B3	Fusiform	14.24±0.88	10.05–17.67	5.34±0.48	3.56–7.16	Present	Absent
G1	Cylindrical with two ends acute or one end slightly obtuse	13.02±1.08	09.25–18.60	3.68±0.36	2.37–5.20	Present	Absent
G2	Cylindrical with two ends acute or one end slightly obtuse	12.60±1.44	09.75–19.22	4.45±0.45	3.15–5.83	Present	Absent
G3	Cylindrical with two ends acute or one end slightly obtuse	12.48±1.11	09.05–18.84	4.48±0.43	3.33–5.80	Present	Absent
J1	Falcate	13.01±1.03	09.79–17.98	3.68±0.32	2.62–5.13	Present	Present
J2	Fusiform	12.70±0.85	10.02–17.67	3.67±0.32	2.62–4.83	Present	Absent
K1	Fusiform	12.73±0.76	09.13–17.50	3.72±0.29	2.73–4.73	Present	Absent
K2	Fusiform	12.82±0.74	09.75–17.60	3.70±0.28	2.73–4.70	Present	Absent
S1	Fusiform	12.75±0.76	10.03–17.28	5.35±0.54	2.63–7.65	Present	Present
S2	Fusiform	14.45±1.10	10.47–19.94	5.28±0.64	2.03–8.57	Present	Present
S3	Fusiform	14.33±1.00	10.03–19.38	5.38±0.58	2.47–8.50	Present	Absent
Mg	Fusiform	12.94±0.91	10.02–16.89	3.69±0.26	2.76–4.65	Present	Absent

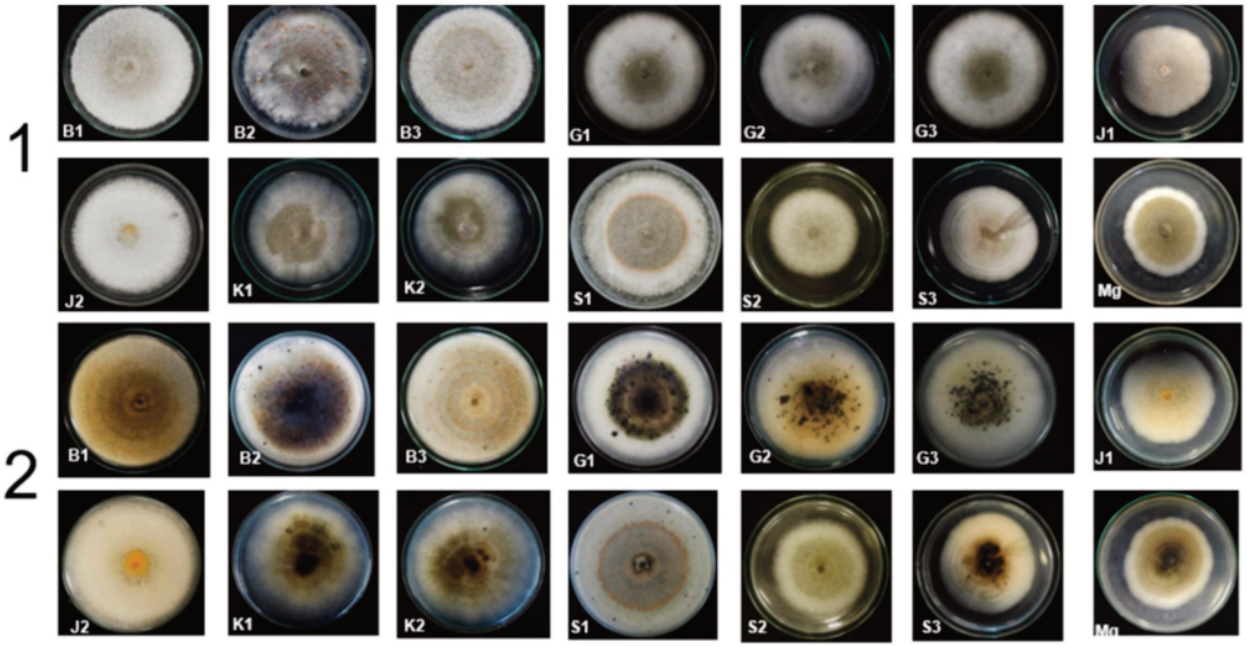

Figure 4. Aerial View (1) and Reverse View (2) of *Colletotrichum* spp. colony isolated from *Capsicum annuum* (S1, S3), *Capsicum frutescens* (B1- Banguntapan1, B2 - Banguntapan2, B3 - Murtigading, G1- Karangrejek, G2 - Patuk, G3 - Bleberan, J1 - Lowanu, J2 - Ngupasan, K1 - Brosot, K2 - Galur, S2 - Seyegan, Mg - Mertoyudan) on day-10 cultured on PDA in 12 hours light and 12 hours dark condition

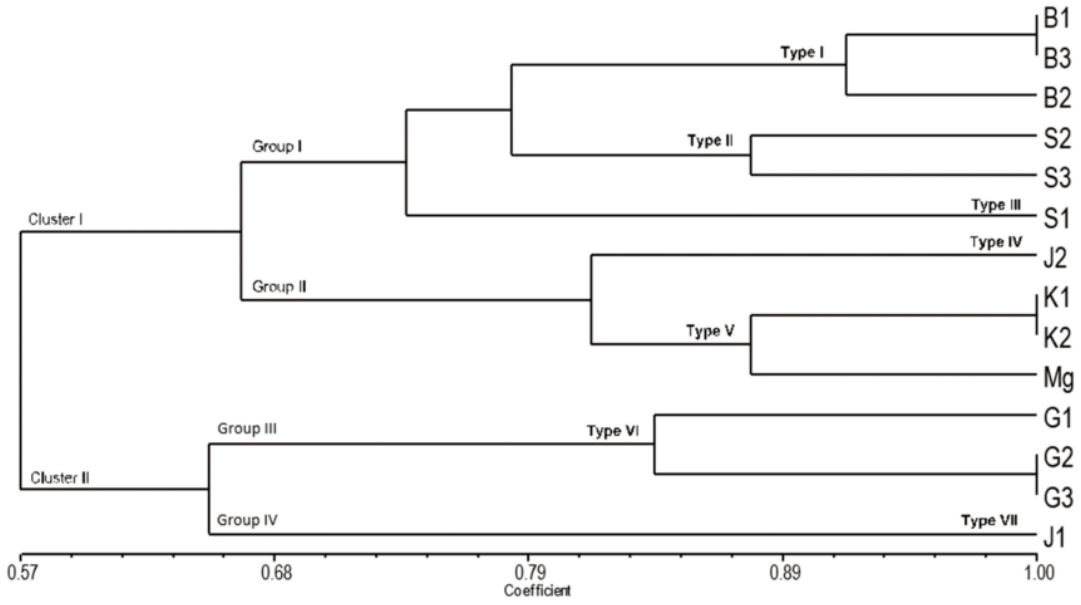

Figure 5. Dendogram of *Colletotrichum* isolates based on morphological characteristics using UPGMA

Gunung Kidul (G1, G2, G3) group and an isolate namely J1. Colony colors were not enough to differentiate among Gunung Kidul isolates. However, additional data such as conidial shape and size, growth rate, and setae appearance could be supporting data to differentiate isolates. Gunung Kidul isolates had cylindrical with two ends acute or one end slightly obtuse. J1 was the only isolate grouped into group IV. It had falcate conidial shape where 71.43% was fusiform and 21.43% was cylindrical with two ends acute or one end slightly obtuse. J1 was also the only isolate producing acervuli as a fruiting body, when the others were conidiomata (Figure 6).

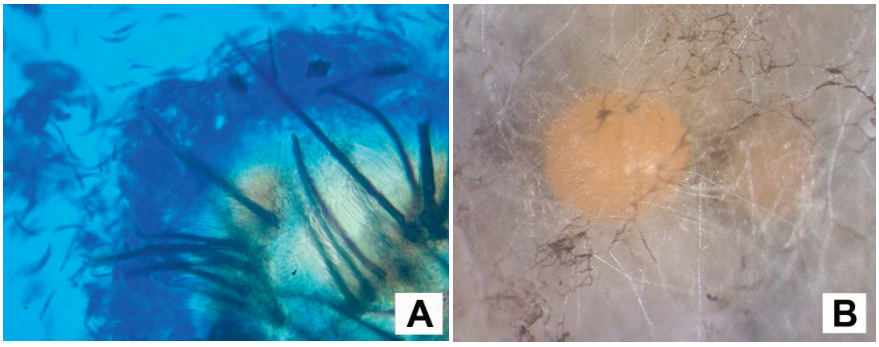

Figure 6. Fruiting bodies observed on PDA, A: acervulus (J1) on day 20, B: conidiomata (S1) on day 8 culture

Phylogenetic Tree using Multi-genes

Phylogenetic analysis using ITS, *gapdh* and *tub2* gene sequences delineated gloeosporioides complex, acutatum complex, and truncatum complex (Figure 7). Isolates of B1, G1, and K2 were generated in *C. scovillei*, S1 and S2 were *C. siamense*, and J1 was *C. truncatum*. In addition, isolates of Jogja 2 (J2) and Magelang (Mg) were distinct from chili *Colletotrichum* ex-type or references adopted. The species of *Colleotrichum* found in this study were as follows:

Colletotrichum siamense

The S1 and S2 isolates belong to *C. siamense* clade which belongs to gloeosporioides complex. Colonies on PDA were 9 mm diameter within 10 days. White with orange pigment, grey aerial view colonies with orange acervular conidiomata at the center; concentric orange and orange-green zone reverse view. Setae was absent, while appressoria were observed. Conidia were hyaline, aseptate, smooth-walled, fusiform with both ends bluntly rounded, 10.03–19.94 × 2.03–8.57 (µm) width.

Colletotrichum makassarii

The J2 isolate showed close relationship with *C. makassarii.* Colony was white with orange pigment on aerial view, in the other hand, reverse view was concentric yellow. Conidial shape was fusiform measured 12.70±0.85 µm length ranged 9.79–17.98 µm and 3.68±0.32 µm width ranged 2.62–4.83 µm. Its fruiting bodies were conidiomata scattered abundantly on PDA but setae was not observed on PDA.

Colletotrichum scovillei

The B1, K2, G1, and Mg isolates belong to acutatum complex. Colonies on PDA varied each isolates. The cultures had cottony and compact textures. Colors were white with orange pigment, greyish, and brown. Colonies on PDA were 8.5 mm diameter in 10 days. Acutatum complex have fusiform to cylindrical with two ends acute or one end slightly obtuse. Conidial length ranged 9.05–19.22 µm, and conidial width ranged 2.37–7.68 µm. All isolates produced conidiomata, except Gunung Kidul isolates. Conidiomata were abundant on B1, and lack on K2. In contrast, G1 did not produce conidiomata. Setae were absent on those isolates. Appressoria were present on all isolates. As a comparative isolate, Mg isolate belonged to acutatum complex. The isolate produced conidiomata with fusiform conidia. Conidial size was 12.94±0.91 µm length ranged 10.02–16.89 µm and 3.69±0.26 µm width ranged 2.76–4.65 µm. Appressoria were present. In contrast, setae was absent.

Colletotrichum truncatum

The J1 isolate belongs to truncatum complex. Previously, *C. truncatum* was named as *C. capsici.* It produced gray colony on PDA. This is a slowly grow isolate, 0.1 mm day^{-1} on PDA. The isolate produced conidia detached from abundant acervuli, the asexual fruiting bodies. Average conidial length was 13.01±1.03 µm ranged 9.79–17.98 µm. On the other hand, the measurement of conidial width was 3.68±0.32 µm ranged 2.62–5.15 µm. Conidia were falcate (truncate both ends), aseptate, hyaline, and uninucleate. The acervuli also produced abundant setae. The setae were pointed, elongate, strait or slightly curved, aseptate, smooth and dark brown. The setal length was 50.56–104.78 µm range, whereas setal width was 20.76–25.97 µm range.

Pathogenicity Assay

Anthracnose symptoms were observed on healthy chili inoculated by *Colletotrichum* isolates by wounding method.

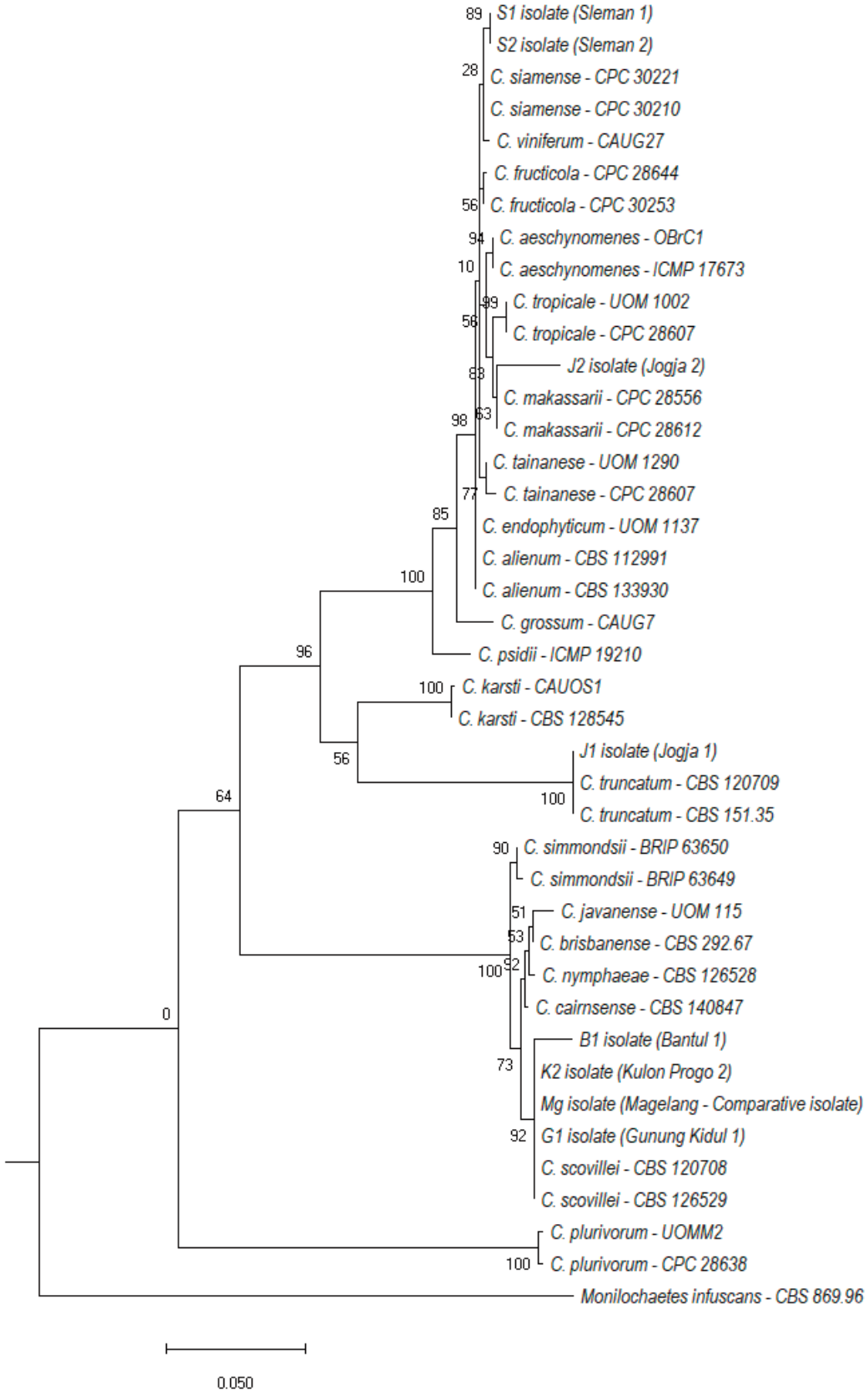

Figure 7. Maximum-Likelihood (ML) consensus tree of the combined genes analysis of ITS, *tub2*, and *gapdh* sequence alignment showing separation of *Colletotrichum* isolates into *C. siamense, C. scovillei* and *C. truncatum*. *Colletotrichum* J2 and Mg could not be separated using these three genes; the scale bar shows the number of substitutions per nucleotide position; *Monilochaetes infuscans* CBS 869.96 is used as outgroup

Figure 8. Pathogenicity assay treated on chili fruits (*Capsicum annuum*) and the symptoms caused by *Colletotrichum* isolates;14 days after inoculation by a wounding method

Table 5. Disease score on a 0–9 scale of chili fruits (*Capsicum annuum*) for different *Colletotrichum* species inoculated by wounding method

Isolates	Species Name	Score Range Mean	Category
S1	*C. siamense*	7	Virulent
S2	*C. siamense*	7	Virulent
J2	*C. makassarense*	5	Moderately virulent
B1	*C. scovillei*	7	Virulent
G1	*C. scovillei*	7	Virulent
K2	*C. scovillei*	7	Virulent
Mg	*C. scovillei*	9	Highly virulent
J1	*C. truncatum*	5	Moderately virulent

The *C. scovillei* isolate was the most highly virulent isolate followed by *C. siamense*. Those isolates showed the highest disease severity producing large lesions with disease scores in the range 7–9 (Figure 8; Table 5).

Discussion

The *Colletotrichum* species can be identified by their morphological or molecular characteristics. Some morphological characteristics, such as culture colony appearances, conidial morphology, growth rate, appressorial morphology, and the existence of septation, are important characteristics which help to distinguish the *Colletotrichum* species. Morphological characterization is primarily identification to group *Colletotrichum* isolates with the help of UPGMA method. Nevertheless, this method is not sufficient to determine among *Colletotrichum* species. In acutatum complex, conidia strains were sub-cylindrical, fusiform, ellipsoid, oblong, and blunt pointed ends (Sato *et al*., 2013). The diverse variability could not separate among species. In *C. gloeosporioides,* there were ± 600 isolates in morphological similarity. Thus, grouping based on morphological characteristics and host specificity was insufficient to solve *C. gloeosporioides* classification (Damm *et al*., 2012). Thus, at level of 0.85 similarity, eight isolates were selected as representative isolates including one comparative isolate. They were further analyzed in molecular level.

Generating multi-genes phylogenetic tree using ITS1-4, *tub2*, and *gapdh* showed more accurate on *Colletotrichum* isolates associated in chili anthracnose. High support value could be obtained to identify novel *Colletotrichum* species in this method. A multi-genes phylogenetic analysis is required for the accurate identification of species within species complex, for instance in acutatum species complex, *C. acutatum* is a species complex which is being re-identified as *C. scovillei* (Thailand) and *C. nymphaeae* (Indonesia) on chili fruits hosts (Damm *et al*., 2012).

Similarly in gloeosporioides complex reported in Thailand and India, the species complex comprises *C. siamense, C. fruticola,* and *C. asianum* (Phoulivong *et al*., 2012: Sharma & Shenoy, 2014). Phylogenetic analysis delineated B1, G1, and K2 as *C. scovillei*; J1 as *C. truncatum*; S1 and S2 as *C. Siamense* and J2 as *C. makassarense.* Magelang (Mg) isolate was not clustered within chili *Colletotrichum* ex-type or references adopted. This only provides complex species where Mg isolate belongs. Distinguishing among species needs more loci to be analyzed. Damm *et al*. (2012) added *gs* gene to separate *C. aenigma* from *C. alienum* and some *C. siamense* isolates.

In this study, it is recognized that the dominant anthracnose pathogens in Yogyakarta Special Region was acutatum complex. Previous studies reported that *C. scovillei* infected chilies in several countries like Korea, China, and Brazil (Oo *et al*., 2017; Liu *et al*., 2016; Caires *et al*., 2014). Recently, *C. scovillei* was reported infecting chili in Bali, Indonesia (Khalimi *et al*., 2019). The amount of 25% of the total isolates were *C. siamense* (gloeosporioides complex) causing anthracnose of chili fruit in Yogyakarta Special Region. *Colletotrichum siamense* has been reported to infect chili in Asia, including Indonesia (de Silva *et al*., 2019). Ability of *C. siamense* in cross infection results in proposing broad host range (Phoulivong *et al*., 2012). Its host range is herbaceous to woody plants (Meng *et al*., 2019; Liu *et al*., 2018). *C. siamense* isolates from different sites showed different morphological characteristics with various growth rates and cultures. Variability in morphological characteristics indicated that the species has high intra-specific diversity. Morphological characteristics of *C. siamense* isolates varied from different countries. Representative conidial measurements for isolates representing different sub-clades in phylogenetic tress (Sri Lanka) (Prihastuti *et al*., 2009). Isolate J2 as *C. makassarense* is a novel species found in Java Island. Previously, it was found in Sulawesi Island, Indonesia (de Silva *et al*., 2019). *C. truncatum,* truncatum species complex, was less species found in this study. Information regarding *C. truncatum* species complex is less frequently found. Identification of *C. truncatum* (J1 isolate) using UPGMA correlated to multi-gene analysis. Because, it is contrast to others in producing falcate conidia which means two conidial ends are sharpened (Sutton, 1992). It is also called as truncate, apical acute and basal truncate (Damm *et al*., 2009). Conidia were produced from black acervuli. Similarly to Sakhivel *et al.* (2018), the mycelium was white during the initial stages and gradually turned greyish orange, and producing black setae from acervuli *C. truncatum* was regarded polyphagous. It can infect several plants like *Glycine max* and jasmine leaf (Backman *et al.*, 1982; Wikee *et al*., 2011). *Colletotrichum truncatum* firstly found in Indonesia in papaya (Rangkuti *et al*., 2017). This is a novel that *C. truncatum* infects chili in Indonesia.

All isolates found in this study were pathogenic on wounded chili fruits. Various degree of severity resulted in different *Colletotrichum* isolates. The higher score of infection severity was a result of higher aggressiveness of the isolates. Previous study by Mongkolporn *et al*. (2010) comparing *Colletotrichum* isolates from *C. acutatum, C. gloeosporioides* and *C. truncatum* complexes showed that *C. acutatum* complex were the most aggressive pathogen having more than score six. Similar result was obtained that *C. scovillei* and isolates Mg as *C. acutatum* complex had 7–9 score. *Colletotrichum gloeosporioides* complex (*C. siamense* and *C. makassarense*) had lower severity score than *C. acutatum* complex. However, it had higher score than *C. truncatum.* Ranathunge *et al*. (2012) reported a quiescent (latent) stage in *C. truncatum* after initial infection on *C. annuum* with asymptomatic result until a week after inoculation. In comparison, *C. siamense* that was isolated and identified by Sharma & Shenoy (2014), has necrotrophic life style on *C. annuum.*

CONCLUSION

Colletotrichum species causing chili anthracnose showed genetic diversity. *Colletotrichum siamense* and *C. scovillei* were previously reported in Indonesia, yet this was the first reported in Yogyakarta Special Region. Although *C. truncatum* has been reported infecting other plant species before, this was the first report of *C. truncatum* infecting chili in Indonesia. *Colletotrichum makassarense* was also a novel species found in Java. More loci should be added in analyses to distinguish isolate Mg to confirm the species. Moreover, pathogenicity assay on chili fruit showed that Mg isolate had the highest virulence on wounded fruit, compared to all other isolates.

The existence of highly virulence species such as Mg isolate threatened biosecurity of the country.

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