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. frutescens* 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

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 tub2 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*
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°30ʹ N – 7°20ʹ N latitude and 109°40ʹ E – 111°0ʹ 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°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°C for ITS and tub2, and 60°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 alcohol 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 Complete 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 α = 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).
| Species          | Accession Number | Host                  | Country      | GenBank Accession Numbers |
|------------------|------------------|-----------------------|--------------|---------------------------|
| **Species Accession Number** | **Host**    | **Country** | **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    | JQ48269     | JQ948599 | JQ949920 |
| *C. simmondsii* | BRIP 63649       | *Capsicum sp.*       | Australia   | KU199252    | KU199254 | KU199253 |
| *C. simmondsii* | BRIP 63650       | *Capsicum sp.*       | Australia   | KU199258    | KU199260 | KU199259 |
| *C. nymphaeae*  | CBS 126528       | *Capsicum sp.*       | Indonesia   | JQ48219     | JQ948880 | JQ949870 |
| *C. queenslandicum* | BRIP 63699       | *Capsicum annuum*    | Australia   | KU923681    |           | KU923697 |
| *C. queenslandicum* | BRIP 63700       | *Capsicum annuum*    | Australia   | KU923682    |           | KU923698 |
| Boninense complex |                  |                       |             |             |         |         |
| *C. karsti*     | CAUOS1           | *Capsicum sp.*       | China       | KP890103    | KP890134 | KP890110 |
| *C. karsti*     | CBS 128545       | *Capsicum annuum*    | New Zealand | JQ005207    | JQ005294 | JQ005641 |
| Gloeosporioides complex |     |                       |             |             |         |         |
| *C. aeschynomenes* | OBrC1            | *Solanum melongena*  | India       | KU239115    | KU239576 | KU239241 |
| *C. aeschynomenes* | ICMP 17673       | *Aeschynomene virginica* | USA       | NR120133    | JX009930 | JX010392 |
| *C. alienum*    | CBS 112991       | *Leucadendron sp.*   | Portugal    | KC297070    | KC297001 | KC297098 |
| *C. endophyticum* | UOM 1137        | *Protea sp.*         | Portugal    | KC297076    | KC297000 | KC297096 |
| *C. fruticola*  | CPC 28644        | *Capsicum annuum*    | Thailand    | MH728809    | MH707467 | MH846566 |
| *C. fruticola*  | CPC 30253        | *Capsicum annuum*    | Taiwan      | MH728817    | MH707463 | MH846559 |
| *C. grossum*    | CAUG7            | *Capsicum annuum*    | China       | KP890165    | KP890159 | KP890171 |
| *C. makassarii* | CPC 28556        | *Capsicum annuum*    | Indonesia   | MH728815    | MH728821 | MH846561 |
| *C. makassarii* | CPC 28612        | *Capsicum annuum*    | Indonesia   | MH728812    | MH728820 | MH846563 |
| *C. psidii*     | ICMP 19120       | *Psidium sp.*        | Italy       | JX010463    |         |         |
| *C. siamense*   | CPC 30210        | *Capsicum annuum*    | Indonesia   | MH707472    | MH707453 | MH846548 |
| *C. tainanense* | CPC 30221        | *Capsicum annuum*    | Thailand    | MH707475    | MH707456 | MH846551 |
| *C. tainanense* | CPC 28607        | *Capsicum annuum*    | Taiwan      | MH728818    | MH728823 | MH846558 |
| *C. tainanense* | UOM 1290         | *Capsicum annuum*    | Taiwan      | MH728805    | MH728819 | MH846570 |
| *C. tropicalis* | CPC 28607        | *Capsicum annuum*    | Indonesia   | MH728814    | MH707464 | MH846562 |
| *C. viniferum*  | UOM 1002         | *Capsicum annuum*    | Indonesia   | MH728807    | MH707469 | MH846568 |
| *C. viniferum*  | CAUG27           | *Capsicum sp.*       | China       | KP145440    | KP145412 | KP145468 |
| Orchideaerum complex |         |                       |             |             |         |         |
| *C. plurivorum* | CPC 28638        | *Capsicum annuum (leaf)* | Thailand   | MH805810    | MH805816 | MH805824 |
| *C. plurivorum* | UOMM2            | *Capsicum annuum*    | Malaysia    | MH805815    | MH805821 | MH805827 |
| Truncatum complex |                  |                       |             |             |         |         |
| *C. truncatum*  | CBS 151.35       | *Phaseolus lunatus*  | USA         | GU227862    | GU228254 | GU228156 |
| *C. truncatum*  | CBS 120709       | *Capsicum frutescens* | India       | GU227877    | GU228269 | GU228171 |
| Monilochaetes infuscans (Outgroup) | CBS 869.96 | Unknown | Unknown | GU180626 | JX546612 | JQ005864 |
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). *Colletotrichum* 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

### 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, Mertoypudan            | 343                 | *C. annum*     | Fruit |

Note: *)Magelang as comparative sample
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                                  | Absent       | 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 | Range | Present | Absent |
| 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 |
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).
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, straight 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, \textit{tub2}, and \textit{gapdh} sequence alignment showing separation of \textit{Colletotrichum} isolates into \textit{C. siamense}, \textit{C. scovillei} and \textit{C. truncatum}. \textit{Colletotrichum} J2 and Mg could not be separated using these three genes; the scale bar shows the number of substitutions per nucleotide position; \textit{Monilochaetes infuscans} CBS 869.96 is used as outgroup.
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).

### 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 |

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.
Similarly in gloeosporioides complex reported in Thailand and India, the species complex comprises *C. siamense*, *C. fruticola*, and *C. asiaticum* (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. alieum* 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; Wikke 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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