THREE COLLETOTRICHUM SPECIES RESPONSIBLE FOR ANTHRACNOSE ON SYNSEPALUM DULCIFICUM (MIRACLE FRUIT)

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**ABSTRACT**

In 2016 and 2017, fruit rot and two different leaf diseases (leaf spot and leaf blight) were found on Synsepalum dulcificum (miracle fruit) in Tokyo, Kanagawa and Kagoshima prefectures of Japan. From the lesions, abundant conidial masses and acervuli of three Colletotrichum species, two of which produced sexual state, were observed. We conducted a pathogenicity assay using these Colletotrichum species on healthy fruits and leaves of S. dulcificum. Our artificial inoculation tests showed symptoms of disease on tested fruit and leaf and indicated all three Colletotrichum species as causal agents of anthracnose on S. dulcificum. Based on morphological characters and molecular phylogenetic analyses using ITS, GAPDH, ACT, CAL and TUB2 loci, these species were identified as Colletotrichum aenigma (MAFF 246750), C. siamense (MAFF 246751) and C. karstii (MAFF 245966). They have been previously reported as plant pathogenic fungi elsewhere in the world. This is the first report of fruit rot, leaf blight and leaf spot on S. dulcificum caused by these three Colletotrichum species.

**Keywords:** Colletotrichum aenigma, C. karstii, C. siamense, fruit rot, leaf anthracnose, miracle fruit, molecular phylogeny.

**INTRODUCTION**

The genus Colletotrichum is one of the most important plant pathogenic fungal groups in the world. The genus causes diseases on a wide variety of woody and herbaceous plants and is the principal cause of serious plant diseases especially in tropical and the sub-tropical regions (Da Silva and Michereff, 2013; De Silva et al., 2016; Lima et al., 2013). Colletotrichum has recently been voted as the world’s eighth most economically important fungal pathogens, based on perceived scientific and economic criteria (Dean et al., 2012). Interestingly, previous studies showed that one species of Colletotrichum can cause disease on multiple host plants, and multiple species can jointly infect a single host (Nguyen et al., 2009; Sharma and Shenoy, 2016). According to Rojas et al. (2010), Colletotrichum spp. are the principal cause of damping-off, leaf spot, seedling blight as well as pre- and post-harvest fruit rot. These disease symptoms appear in developing and mature plant tissues of diverse hosts such as fruit, vegetables and ornamental plants (Da Silva and Michereff, 2013; Zivkovic et al., 2010). For the purposes of plant quarantine, Colletotrichum-infected commodities are not suitable for import or export due to the potential revenue loss (Sharma and Shenoy, 2016). Precise identification plays an important role for understanding the epidemiology of Colletotrichum species and developing effective disease control methods. Yokosawa et al. (2017), for instance, mentioned that the different levels of resistance to several fungicides was observed among members of the Colletotrichum gloeosporioides species complex. Traditional identification systems in Colletotrichum were mainly based on morphological and cultural characters as well as host association (Alizadeh et al., 2015; Lima et al., 2013). However, morphology alone is inadequate to provide sufficient and informative characters for an accurate identification (Alizadeh et al., 2015). Therefore, molecular analyses with multiple loci coupled with morphological characters is now the preferred method for precise identification of the Colletotrichum species (Cai et al., 2009).

Synsepalum dulcificum (Sapotaceae) is commonly known as miracle fruit, miraculous berry or sweet berry (Akinmoladun, 2016; Shi et al., 2016). This plant originates...
from tropical West Africa (Akinmoladun, 2016; Rodrigues et al., 2016; Shi et al., 2016). It has subsequently been treated as an important plant because of an active compound in the fruit called miraculin. Miraculin is a single polypeptide chain, which is used to modify taste in food and to control obesity (Akinmoladun, 2016).

In 2016 and 2017, we found fruit rot and two different leaf symptoms of *S. dulcificum* in Tokyo, Kanagawa and Kagoshima prefectures, Japan (Figure 1). The fruit rot was first observed in a greenhouse of the botanical garden in Kanagawa prefecture. During our research, the disease was constantly observed to cause damage to the host plant. From microscopic examination of plant symptoms, conidial masses and acervuli of the genus *Colletotrichum* were prominent. Two leaf symptoms, leaf blight and leaf spot, were observed in Tokyo and Kagoshima prefectures respectively. An initial symptom of leaf blight was small lesion at the tip of the leaf, and the lesion then developed and increased in size towards the petiole. Morphological features of the genus *Colletotrichum* such as conidial masses and setae on acervuli, were observed from the symptoms. The leaf spot was first started as tiny black dots at leaf margin. The black dots then developed and produced big spots and chlorosis areas encompassed by a dark brown line. Both diseased leaves were eventually defoliated.

Figure 1. Original symptoms caused by *Colletotrichum* spp. on *S. dulcificum*. a: Fruit rot (white arrow). b: Leaf spot. c: Leaf blight.

Although *S. dulcificum* is a notable tropical plant, there have not been many studies focusing on its pathology until now. To the best of our knowledge, the only leaf disease reported on *S. dulcificum* was caused by *Pestalotiopsis synsepali* (Chen et al., 2002). Damm et al. (2012) found *C. karstii* on leaf of *S. dulcificum*, but its pathogenicity on *S. dulcificum* has been unknown. The aims of this study were: (1) to identify these three *Colletotrichum* species causing of anthracnose on *S. dulcificum* based on morphology and molecular analyses; (2) to determine their pathogenicity to *S. dulcificum*.

**MATERIALS AND METHODS**

**Sampling and fungal isolation:** Fruit rot of *S. dulcificum* was observed in a greenhouse of the botanical garden located Kamakura, Kanagawa, in 2016. From its symptom, conidial masses were collected and suspended in sterile water. The prepared conidial suspension was then spread over the surface of water agar (WA). After 24 hours, a single germinating spore was transferred onto Difco™ potato dextrose agar (PDA; Detroit, MI, USA).

Two different leaf symptoms of *S. dulcificum* were determined in different regions. Leaf spot was observed in a fruit garden at Tanegashima island, Kagoshima in 2016 while leaf blight was found in a greenhouse, in Tokyo in 2017. The aforementioned isolation method was employed both for leaf spot and blight diseases. The isolates from fruit rot (MAFF 246750), leaf blight (MAFF 246751) and leaf spot (MAFF 245966) were obtained and preserved at the Genebank, National Agriculture and Food Research Organization (NARO), Tsukuba, Ibaraki, Japan.

**Pathogenicity assay:** *Colletotrichum* isolates were grown on PDA for seven days at 25 °C. Spores were harvested by using 10 ml of sterilized distilled water to pour into the cultures, and the water was gently swirled to dislodge the conidia. Conidial density was adjusted to get 10^6 conidia/ml by using a haemocytometer (Prihastuti et al., 2009). The wound/non-wound treatments for the pathogenicity assay were performed on healthy fruits...
and leaves of potted *S. dulcificum* seedlings. The wounds were made by pricking the surface of the miracle fruits or leaves with a sterilized needle. The conidial suspension was sprayed on the wounded/non-wounded fruits and leaves, while sterilized distilled water was used as control. The inoculated and non-inoculated fruits and leaves were covered by plastic bags and then placed in a greenhouse under 25-30 °C. Plastic bags were removed after 48 hours. Disease symptoms such as fruit rot, leaf blight and leaf spot were observed after seven days. These experiments were performed with three replicates for each isolate.

**Morphological identification:** These *Colletotrichum* isolates growing on PDA were used for morphological examination. Morphological and cultural characters such as shape and size of conidia and appressoria, and presence or absence of setae were observed on PDA plate growing at 28 °C after one week. Shape and size of 30 conidia from each isolate were evaluated. Images under a stereo microscope (Olympus, Tokyo, Japan) and a compound microscope (Olympus, Tokyo, Japan) were captured with a digital camera (Olympus DP21, Tokyo, Japan). Conidial size was calculated by using ‘image’ software (free download available at http://rsbweb.nih.gov/ij/).

Appressoria were produced by using a slide culture technique. A 10 mm² square block of Synthetic Low-nutrient Agar (SNA) was placed on a sterile slide glass that was kept in an empty petri dish, and the edge of the agar blocks was inoculated on one side with mycelium. The inoculated agar block was covered by a sterile coverslip (Lima et al., 2013). Seven days after inoculation, shape and size of 30 appressoria from each isolate were measured.

**DNA extraction, sequencing, and analysis:** Our obtained cultures were grown on PDA for seven days, and mycelia were scraped from the colony surfaces. Genomic DNA was extracted from the harvested mycelia using UltraClean® Microbial DNA Isolation Kit (MOBI0, Laboratories, Inc., California, USA) based on the instruction of the manufacturer. Sequences were obtained from five loci, namely internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), actin (*ACT*), calmodulin (*CAL*), and β-tubulin 2 (*TUB2*). The loci were amplified and sequenced using the primer pairs: ITS-1/ITS-4 for ITS (Gardes and Bruns, 1993), GDF/GDR for *GAPDH* (Guerber et al., 2003), ACT-521F/ACT-783R for *ACT* (Carbone and Kohn, 1999), CL1C/CL2C for *CAL* (O'Donnell et al., 2000) and T1/T2 for *TUB2* (O'Donnell and Cigelnik, 1997).

The PCR conditions for ITS amplification were 4 minutes at 95 °C; then 35 cycles of 95 °C for 30 seconds, 52 °C for 30 seconds, 72 °C for 45 seconds and final extension at 72 °C for 7 minutes. Different annealing temperatures were used for other loci: *GAPDH* at 60 °C; *ACT* at 58 °C; *CAL* at 59 °C and *TUB2* at 55 °C (Weir et al., 2012). All PCR amplification products were separated by using electrophoresis in 0.7 % agarose gel in 1.0x Tris-acetate acid EDTA (TAE) buffer, and pictures were taken under UV light after staining the gel with ethidium bromide for 10 to 15 minutes. PCR products were purified using ExoSap-IT PCR Clean-up kit (GE Healthcare Life Science, Buckinghamshire, UK), following the manufacturer’s instructions. DNA sequencing was performed by 3130xl Genetic Analyzers (Applied Biosystems, California, USA) using BigDye v.3.1 chemistry (Life Technologies, California, USA).

Sequence queries were submitted to the BLAST search engine of NCBI GeneBank (https://www.ncbi.nlm.nih.gov/). Phylogenetic trees were constructed using data from this study with other sequences extracted from GeneBank (Table 1 and 2). The consensus sequences of each region were aligned using Mesquite version 3.2 (Maddison, 2017). All ambiguously aligned regions were excluded from the analyses by eyes. The analyses were first performed on ITS region. Phylogenetic analyses were performed on the combined dataset of five mentioned loci by maximum likelihood (ML) method using RAxML (Version 0.6.0). Branch and branch node support was determined using 100 bootstrap replicates (Stamatakis et al., 2008).

**RESULTS**

**Pathogenicity assay:** The pathogenicity assay showed that MAFF 246750 isolated from fruit rot produced dark brown lesions around wounded area (Figure 2). Seven days after inoculation, all tested fruits developed the symptoms of fruit rot. From the symptoms, the inoculated fungus was re-isolated. Non-wounded fruits did not show any symptoms.

The assay conducted on leaves indicated that both isolates, MAFF 245966 and MAFF 246751, were able to cause leaf diseases on miracle fruit.
Table 1. Isolates in the phylogenetic analysis of the *Colletotrichum gloeosporioides* species complex.

| Species                          | Accession number*  | GenBank number | ITS          | GAPDH          | CAL          | ACT          | TUB2          |
|----------------------------------|--------------------|----------------|--------------|----------------|--------------|--------------|---------------|
| *C. aenigma*                     | ICMP 18608*        |                | JX010244     | JX010044       | X009683     | X009443     | X010389       |
| *C. aenigma*                     | MAFF 246750        | LC412412       | LC412415     | LC412414       | LC412413    | LC412416     |
| *C. aescynomenes*                | ICMP 17673*        | JX010176       | JX009930     | JX009721       | X009483     | X010392     |
| *C. alatae*                      | CBS 304.67*        | JX010190       | JX009990     | JX009738       | X009471     | X010383     |
| *C. alienum*                     | ICMP 12071*        | JX010251       | JX10028      | JX009654       | X009572     | X010411     |
| *C. aotearoa*                    | ICMP 18537*        | JX010205       | JX010005     | JX009611       | X009564     | X010420     |
| *C. asianum*                     | ICMP 18580*        | FJ972612       | JX010053     | FJ917506       | X009584     | X010406     |
| *C. boninense*                   | CBS 123755*        | JQ005153       | JQ005240     | JQ005674       | J005501     | J005588     |
| *C. changpingense*               | MFLUCC 15-0002     |                | KP683152     | KP852469       | -           | KP683093    | KP852490     |
| *C. cidemiae*                    | ICMP 18658*        | JX010265       | JX009989     | JX009645       | X009537     | X010438     |
| *C. conoides*                    | CGMCC 3.17615*     | KP890168       | KP890162     | KP890144       | KP890174    |
| *C. cordylinicola*               | MFLUCC 090551*     | JX010226       | JX009975     | HM470238       | HM470235    | X010440     |
| *C. endophytica*                 | MFLUCC 13-0418*    | KC633854       | KC832854     | KC810018       | F306258     |
| *C. fructicola*                  | ICMP 18581*        | JX010165       | JX010033     | FJ917508       | FJ907426    | X010405     |
| *C. fructicola* (syn. *C. ignotum*) | CBS 125397(*)  | JX010173       | JX010032     | JX009674       | X009581     | X010409     |
| *C. fructicola* (syn. *Glomerella cingulata* var. minor) | CBS 238.49(*) | JX010181       | JX009923     | JX009671       | X009495     | X010400     |
| *C. fructivorum*                 | CBS 133125*        | JX145145       | -           | -              | -           | X145196     |
| *C. gloeosporioides*             | IMI 356878*        | JX010152       | JX010056     | JX009731       | X009531     | X010445     |
| *C. grevilleae*                  | CBS 132879*        | KC297078       | KC297010     | KC296963       | KC297056    | KC296941    |
| *C. grossum*                     | CGMCC 3.17614*     | KP890165       | KP890159     | KP890147       | KP890141    | KP890171    |
| *C. hebeiense*                   | MFLUCC 13-0726*    | KP156863       | KP377495     | -              | KP377523    | KP288975    |
| *C. henanense*                   | CGMCC 3.17354*     | KJ955109       | KJ954810     | KJ954662       | KM023257    | KJ55257     |
| *C. hippeastri*                  | CBS 241.78         | JX010293       | JX009932     | JX009740       | X009485     | X009838     |
| *C. horii*                       | NBRC 7478*         | GQ329690       | GQ329681     | JX009604       | JX009438    | X010450     |
| *C. jiangxiense*                 | CGMCC 3.17363*     | KJ955201       | KJ954902     | KJ954752       | KJ954741    | KJ955348    |
| *C. kahawae* subsp. ciggaro      | ICMP 18539*        | JX010230       | JX009966     | JX009635       | X009523     | X010434     |
| *C. kahawae* subsp. ciggaro (syn. *Glomerella cingulata* var. migrans) | CBS 237.49(*) | JX010238       | JX010042     | JX009636       | JX009450    | X010432     |
| *C. kahawae* subsp. ciggaro (syn. *Glomerella rufomaculans* var. vaccinii) | CBS 124.22(*) | JX010228       | JX009950     | JX009744       | X009536     | X010433     |
| Species                                      | Culture Collection | JX010231 | JX010146 | JX010012 | JX009642 | JX009452 | JX010444 |
|---------------------------------------------|--------------------|----------|----------|----------|----------|----------|----------|
| *C. kahawae* subsp. *kahawae*              | IMI 319418*        |          |          |          |          |          |          |
| *C. musae*                                 | CBS 116870*        |          |          |          |          |          |          |
| *C. nupharicola*                           | CBS 470.96*        |          |          |          |          |          |          |
| *C. proteae*                               | CBS 132882*        |          |          |          |          |          |          |
| *C. psidii*                                | CBS 145.29*        |          |          |          |          |          |          |
| *C. queenslandicum*                        | ICMP 1778*         |          |          |          |          |          |          |
| *C. rhexiae*                               | CBS 133134*        |          |          |          |          |          |          |
| *C. salsolae*                              | ICMP 19051*        |          |          |          |          |          |          |
| *C. siamense*                              | ICMP 18578*        |          |          |          |          |          |          |
| *C. siamense* (syn. *C. hymenocallidis*)   | CBS 125378 (*)     |          |          |          |          |          |          |
| *C. siamense* (syn. *jasmini-sambac*)      | CBS 130420 (*)     |          |          |          |          |          |          |
| *C. syzygicola*                            | MFLUCC 10-0624*    |          |          |          |          |          |          |
| *C. temperatum*                            | CBS 133122*        |          |          |          |          |          |          |
| *C. theobromicola*                         | CBS 124945 *       |          |          |          |          |          |          |
| *C. theobromicola* (syn. *C. fragariae*)   | CBS 142.31 (*)     |          |          |          |          |          |          |
| *C. theobromicola* (syn. *C. gloeosporioides f. stylosanthis*) | MUCL 42294 (*) |          |          |          |          |          |          |
| *C. ti*                                    | ICMP 4832*         |          |          |          |          |          |          |
| *C. tropicale*                             | CBS 124949*        |          |          |          |          |          |          |
| *C. viniferum*                             | GZAAS 5.08601*     |          |          |          |          |          |          |
| *C. wuxiens*                               | CGMCC 3.17894*     |          |          |          |          |          |          |
| *C. xanthorrhoeae*                         | BRIP 45094*        |          |          |          |          |          |          |
| Glomerella cinngulata “f.sp. camelliae”    | ICMP 10646         |          |          |          |          |          |          |

*= ex-type culture,  (*)= ex-type culture of synonymized taxon
BRIP = Queensland Plant Pathology Herbarium (Australia); CBS: Culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CGMCC: Chinese General Microbiological Culture Collection Center, Beijing, China; GZAAS: Guizhou Academy of Agriculture Science, Guizhou Province, China; ICMP: International Collection of Microorganisms from Plants, Auckland, New Zealand; IMI = CABI Genetic Resource Collection (UK); MAFF: Genebank Project, the Genetic Resources Center, NARO (National Agriculture and Food Research Organization), Tsukuba, Japan; MFLUCC = Mae Fah Luang University Culture Collection (Thailand); MUCL = Belgian Coordinated Collections of Microorganisms, (agro) industrial fungi & yeasts (Belgium); NBRC = Biological Resource Center, National Institute of Technology and Evaluation (Japan); ITS: internal transcribed spacers and intervening 5.8S nrDNA; GAPDH: partial glyceraldehyde-3-phosphate dehydrogenase gen; CAL: partial calmodulin; ACT: partial actin gene; TUB2: partial beta-tubulin gene. Sequences generated in this study are emphasized in bold.
Table 2. Isolates in the phylogenetic analysis of the *Colletotrichum boniense* species complex.

| Species                      | Accession number* | GenBank number | GenBank number | GenBank number | GenBank number |
|------------------------------|-------------------|----------------|----------------|----------------|----------------|
|                              |                   | ITS            | GAPDH          | CAL            | ACT            | TUB2           |
| *C. annellatum*              | CBS 129826*       | JQ005222       | JQ005309       | JQ005743       | JQ005570       | JQ005656       |
| *C. beeveri*                 | ICMP 18594*       | JQ005171       | JQ005258       | JQ005692       | JQ005519       | JQ005605       |
| *C. boninense*               | MAFF 305972*      | JQ005153       | JQ005240       | JQ005674       | JQ005501       | JQ005588       |
| *C. brasiliense*             | ICMP 18607        | JQ005235       | JQ005322       | JQ005756       | JQ005583       | JQ005669       |
| *C. brassicicola*            | CBS 101059        | JQ005172       | JQ005259       | JQ005693       | JQ005520       | JQ005606       |
| *C. camelliae-japonicae*    | CGMCC 3.18118*    | KX853165       | KX893584       | -              | KX893576       | KX893580       |
| *C. citricola*               | CBS 134228*       | KC293576       | KC293736       | KC293696       | KC293616       | KC293656       |
| *C. colombiense*             | CBS 129818        | JQ005174       | JQ005261       | JQ005695       | JQ005522       | JQ005608       |
| *C. constrictum*             | ICMP 12936        | JQ005237       | JQ005324       | JQ005758       | JQ005585       | JQ005671       |
| *C. cymbidiicola*            | IMI 347923*       | JQ005166       | JQ005253       | JQ005687       | JQ005514       | JQ005600       |
| *C. dacrycarpi*              | ICMP 19107*       | JQ005236       | JQ005323       | JQ005757       | JQ005584       | JQ005670       |
| *C. gloeosporioides*         | STE-U 4295*       | JQ005152       | JQ005239       | JQ005673       | JQ005500       | JQ005587       |
| *C. hippeastri*              | CBS 241.78        | JQ005232       | JQ005319       | JQ005753       | JQ005580       | JQ005666       |
| *C. karstii*                 | CBS 128552        | JQ005188       | JQ005275       | JQ005709       | JQ005536       | JQ005622       |
| *C. karstii*                 | CORCG6 (CGMCC 3.14194) | HM585409 | HM585391 | HM582013 | HM581995 | HM585428 |
| *C. karstii*                 | MAFF 245966       | LC412407       | LC412410       | LC412409       | LC412408       | LC412411       |
| *C. novae-zelandiae*         | ICMP 12944*       | JQ005228       | JQ005315       | JQ005749       | JQ005576       | JQ005662       |
| *C. oncidii*                 | CBS 129828*       | JQ005169       | JQ005256       | JQ005690       | JQ005517       | JQ005603       |
| *C. parsonsiae*              | ICMP 18590*       | JQ005233       | JQ005320       | JQ005754       | JQ005581       | JQ005667       |
| *C. petchii*                 | CBS 378.94*       | JQ005223       | JQ005310       | JQ005744       | JQ005571       | JQ005657       |
| *C. phyllanthi*              | MACS 271*         | JQ005221       | JQ005308       | JQ005742       | JQ005569       | JQ005655       |
| *C. torulosum*               | ICMP 18586*       | JQ005164       | JQ005251       | JQ005685       | JQ005512       | JQ005598       |

* = ex-type culture,

CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CGMCC: China General Microbiological Culture Collection Center; ICMP: International Collection of Microorganisms from Plants, Landcare Research, Private Bag 92170, Auckland, New Zealand; MACS: Collection of Microorganisms, Pune, India; MAFF: Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, Japan; IMI = International Mycological Institute, Kew, UK; STE-U: Culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa; ITS: internal transcribed spacers and intervening 5.8S nrDNA; GAPDH: partial glyceraldehyde-3-phosphate dehydrogenase gen; CAL: partial calmodulin; ACT: partial actin gene; TUB2: partial beta-tubulin gene. Sequences generated in this study are emphasized in bold.
The symptoms were first medium brown to dark brown on wounded area and then enlarged on the rest of the leaves (Figure 3 and 4). Both inoculated fungi were re-isolated from the symptoms. On the control and non-wounded leaf, both MAFF 246751 and MAFF 245966 did not provide any symptom.

**Phylogenetic analyses of the combined datasets:**

Sequence similarity searches of ITS region using BLAST were performed to identify *Colletotrichum* isolates. Comparisons of ITS sequences of isolates from *S. dulcificum* with sequences in GeneBank showed that MAFF 246750 and MAFF 246751 belong to the *Colletotrichum gloeosporioides* species complex while MAFF 245966 belongs to the *Colletotrichum boninense* species complex (Data not shown). Because the two species complexes are phylogenetically diverse groups, we carried out separate phylogenetic analyses of the two species complexes as follows. *C. hippeastri* and *C. boninense* were selected as outgroup for the *Colletotrichum gloeosporioides* species complex tree, *C. gloeosporioides* for the *Colletotrichum boninense* species complex tree.

DNA sequences we used for the *C. gloeosporioides* species complex tree were concatenated to form a matrix of 2616 bp. The locus boundaries in the alignment were ITS:1-551, *GAPDH*: 552-836, *CAL*: 837-1605, *ACT*: 1606-1907, and *TUB2*: 1908-2616. A phylogenetic analysis of the *C. gloeosporioides* species complex showed that MAFF 246750 from fruit rot and MAFF 246751 from leaf blight were clearly separated from each other (Figure 5).
Figure 5. ML phylogenetic analysis of ITS, GAPDH, CAL, ACT and TUB2 sequences for the two isolates of Colletotrichum, MAFF 246750 from fruit rot and MAFF 246751 from leaf blight on S. dulcificum. * = ex-type culture, (*) = ex-type culture of synonymized taxon.

The tree also indicated that the most closely related species to MAFF 246750 and MAFF 246751 were C. aenigma and C. siamense with 96% and 98% bootstrap support, respectively. The ITS, GAPDH, CAL, ACT, and TUB2 sequences obtained for the C. boninense species complex tree were concatenated to form an alignment of 2178 bp. The locus boundaries in the alignment were ITS: 1-551, GAPDH: 552-850, CAL: 851-1307, ACT: 1308-1602, TUB2: 1603-2178. A maximum likelihood tree of the concatenated dataset is shown in Figure 6. In this tree, MAFF 245966 fell into the C. karstii clade supported by 100% bootstrap value.
**Morphology**

*Colletotrichum aenigma* isolated from fruit rot on *S. dulcificum*: Colonies on PDA were flat with entire edges, white to grey and cottony with scattered pale orange conidial mass near the center. On the PDA reverse side, colonies were colorless to white and black spots occurred toward center. Asexual and sexual morphology were observed on PDA after seven days. Conidia were 14.5-19.5 x 4-6.5 µm (average 16.6 x 5.3, n = 30) in size, straight and cylindrical with broadly round ends.

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**Figure 6.** ML phylogenetic analysis of ITS, GAPDH, CAL, ACT and TUB2 sequences for the isolate of *Colletotrichum MAFF 245966* from leaf spot on *S. dulcificum*. * = ex-type culture.

Setae were dense, dark brown and smooth with 2-4 septate. Appressoria were lobed and 8.5-15.0 x 5.5-9.0 µm (average 11.6 x 7.1, n = 30) in size (Figure 7). In terms of teleomorph state, perithecia were oval and brown to dark brown color. Asci contained eight ascospores were clavate and 96.0-108.0 x 10-14.0 µm (average 103.6 x 12.8, n = 7) in size. Ascospores were hyaline, aseptate, smooth, ellipsoidal and 14.0-20.0 x 4.5-8.0 µm (average 16.9 x 6.5, n = 30) (Figure 7) in size. These morphological characters agreed with *Colletotrichum aenigma* described by Weir *et al.* (2012).

*Colletotrichum karstii* isolated from leaf spot on *S. dulcificum*: Colonies on PDA after one week at 25 °C were white to slightly grey and produced aerial mycelium at the center and scatter of tufts. On the reverse side, the colony is yellowish color near the center, colourless toward the edge. Conidia were in yellowish mass. Conidiophores were hyaline, smooth and cylindrical. Conidia on PDA plate after one week were hyaline, smooth-walled, aseptate, straight, cylindrical with broadly round ends and 14.0-18.0 x 5.5-8.0 µm in size. Setae were not observed. Appressoria on SNA were pale to medium brown, bud
shape to bullet-shaped, smooth walled and 7.0-12.0 x 3.5-9.0 µm (average 8.7 x 5.6, n = 30) in size. Asci were unisticate, clavate-shaped, tapering, smooth walled, 51.0-70.0 x 9.0-14.0 µm (average 59.8 x 11.8, n = 7) in size and contained eight ascospores. Ascospores were aseptate, hyaline, smooth walled, fusiform to ovoid, slightly curved with rounded ends, and 15.0-17.0 x 5.0-6.0 µm (average 17.0 x 6.2, n = 30) in size (Figure 8). These morphological characters agreed with *Colletotrichum karstii* (Yang *et al.*, 2011), with the exception of color of conidial mass and the shape of ascospores.

Figure 7. *Colletotrichum aenigma*. a: Colony on PDA after seven days (reverse). b: Conidial masses. c: Conidiophores. d: Conidia. e: Appressoria. f: Seta. g: Perithecium. h: Ascospores. Scale bars b = 200 µm. c, d, g, h = 50 µm. e, f = 20 µm.

Figure 8. *Colletotrichum karstii*. a: Colony on PDA after seven days (surface). b: Colony on PDA for seven days (reverse). c: Conidial mass. d: Conidiophores. e: Conidia. f: Appressoria. g: Asci. h: Ascospore. Scale bars: c = 200 µm. d, e, f, h = 20 µm. g = 50 µm.
**Colletotrichum siamense** isolated from leaf blight on *S. dulcificum*: Colonies on PDA after seven days were white, and reverse side was pale pink. Aerial mycelium was greyish white, dense and cottony. Conidial masses were in medium to dark orange at the inoculum point (Figure 9). Setae present, 3-5 septate, pale brown to dark brown and smooth walled. Conidiophores were hyaline, smooth and cylindrical. Conidia were one-celled, smooth-walled, hyaline with obtuse to slight rounded ends and 13.0-19.0 x 3.0-5.5 μm (average 16.3 x 4.4) in size. Appressoria were brown, ovoid, bud-shaped, and 6.0-9.5 x 4.0-6.0 μm (average = 7.9 x 5.0, n = 30) in size. The mycelium produced appressoria on SNA at fifth day. Teleomorph of this fungus did not produce under any condition we used.

Figure 9. *Colletotrichum siamense*: a. Colony on PDA after seven days (surface). b: Conidial mass. c: Appressoria. d: Conidiophores. e: Setae. f: Conidia. Scale bars: c, d, e, f = 20 μm.

**DISCUSSION**

Using ITS is useful in preliminary identification of fungi (Schoch et al., 2012). Our results of BLAST search indicated that our three *Colletotrichum* species belonged to the *C. gloeosporioides* species complex and the *C. boninense* species complex, respectively. The fungus isolated from fruit rot was identified as *Colletotrichum aenigma*. This species has been reported as an anthracnose pathogen on several plants around the world (Diao et al., 2017; Gan et al., 2016; Meetum et al., 2015; Schena et al., 2013; Wang et al., 2016). Database of plant diseases in Japan (http://www.gene.affrc.go.jp/databases-micro_pl_diseases_en.php) showed *C. aenigma* to be associated with anthracnose or other diseases on Buckwheat, Japanese horse chestnut, mango, apple, melon, grape and strawberry. It suggests that this species has a wide geographic distribution and broad host range in Japan. Two isolations obtained from leaf spot and leaf blight were identified as *C. karstii* and *C. siamense*, respectively. *Colletotrichum karstii* has the broadest geographical range in *C. boninense* species complex (Damm et al., 2012). Our study is the second record finding *C. karstii* in Japan after Ichinose et al. (2016). This species has been found on various host plants (Lima et al., 2013). Damm et al. (2012) identified culture strain CBS 128552 found on leaf of *Synsepalum dulcificum* as *C. karstii*. However, the pathogenicity of this species on *S. dulcificum* has not been tested before. Based on the result of our study, we found that this species causes of leaf spot on *S. dulcificum. Colletotrichum siamense* belonging to the *C. gloeosporioides* species complex was first confirmed as pathogen associated with anthracnose of coffee berries in the northern Thailand (Prihartuti et al., 2009), and this species has now been recorded on many hosts (Honder et al., 2016; Sharma and Shenoy, 2013). It is evaluated as a dominant species on
tropical fruits (Sharma and Shenoy, 2013). Recently the taxonomic position of *C. siamense* has been under debate. Prihastuti et al. (2009) and Wikee et al. (2010) found *C. siamense* could be a species complex whereas Liu et al. (2016) indicated *C. siamense* as a single species based on statistical analysis using multi-locus sequence data, cross-mating and genetic recombination test. In this study, our phylogenetic tree showed slight phylogenetic distance between our isolate and *C. siamense* supported by 98% bootstrap value. We therefore tentatively identified it as *C. siamense*.

This study provides the first report of fruit rot, leaf blight and leaf spot caused by three *Colletotrichum* species on *S. dulcificum* based on pathogenicity test, morphological and molecular identification methods. This information of host and pathogen will aid plant pathologists in designing disease control strategies for *S. dulcificum*. Further studies such as host range, disease impact on yield, and control methods for these *Colletotrichum* species above are required to protect *S. dulcificum* from anthracnose.

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