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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 prefecture 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.* d*ulcificum* 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 'imageJ' software (free download available at http://rsbweb.nih.gov/ij/).

Appressoria were produced by using a slide culture technique. A 10 mm^2 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 (MOBIO, 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	JX009683	JX009443	JX010389
C. aenigma	**MAFF 246750**	**LC412412**	**LC412415**	**LC412414**	**LC412413**	**LC412416**
C. aeschynomenes	ICMP 17673*	JX010176	JX009930	JX009721	JX009483	JX010392
C. alatae	CBS 304.67*	JX010190	JX009990	JX009738	JX009471	JX010383
C. alienum	ICMP 12071*	JX010251	JX010028	JX009654	JX009572	JX010411
C. aotearoa	ICMP 18537*	JX010205	JX010005	JX009611	JX009564	JX010420
C. asianum	ICMP 18580*	FJ972612	JX010053	FJ917506	JX009584	JX010406
C. boninense	CBS 123755*	JQ005153	JQ005240	JQ005674	JQ005501	JQ005588
C. changpingense	MFLUCC 15-0022	KP683152	KP852469	-	KP683093	KP852490
C. clidemiae	ICMP 18658*	JX010265	JX009989	JX009645	JX009537	JX010438
C. conoides	CGMCC 3.17615*	KP890168	KP890162	KP890150	KP890144	KP890174
C. cordylinicola	MFLUCC 090551*	JX010226	JX009975	HM470238	HM470235	JX010440
C. endophytica	MFLUCC 13-0418*	KC633854	KC832854	KC810018	KF306258	-
C. fructicola	ICMP 18581*	JX010165	JX010033	FJ917508	FJ907426	JX010405
C. fructicola (syn. *C. ignotum*)	CBS 125397(*)	JX010173	JX010032	JX009674	JX009581	JX010409
C. fructicola (syn. *Glomerrella cingulata* var. *minor*)	CBS 238.49 (*)	JX010181	JX009923	JX009671	JX009495	JX010400
C. fructivorum	CBS 133125*	JX145145	-	-	-	JX145196
C. gloeosporioides	IMI 356878*	JX010152	JX010056	JX009731	JX009531	JX010445
C. grevilleae	CBS 132879*	KC297078	KC297010	KC296963	KC297056	KC296941
C. grossum	CGMCC 3.17614*	KP890165	KP890159	KP890147	KP890141	KP890171
C. hebeiense	MFLUCC13-0726*	KF156863	KF377495	-	KF377523	KF288975
C. henanense	CGMCC 3.17354*	KJ955109	KJ954810	KJ954662	KM023257	KJ955257
C. hippeastri	CBS 241.78	JX010293	JX009932	JX009740	JX009485	JX009838
C. horii	NBRC 7478*	GQ329690	GQ329681	JX009604	JX009438	JX010450
C. jiangxiense	CGMCC 3.17363*	KJ955201	KJ954902	KJ954752	KJ954471	KJ955348
C. kahawae subsp*. ciggaro*	ICMP 18539*	JX010230	JX009966	JX009635	JX009523	JX010434
C. kahawae subsp. *ciggaro* (syn. *Glomerella cingulata* var. *migrans*)	CBS 237.49 (*)	JX010238	JX010042	JX009636	JX009450	JX010432
C. kahawae subsp. *ciggar*o (syn. *Glomerella rufomaculans* var. *vaccinii*)	CBS 124.22 (*)	JX010228	JX009950	JX009744	JX009536	JX010433

C. kahawae subsp. *kahawae*	IMI 319418*	JX010231	JX010012	JX009642	JX009452	JX010444
C. musae	CBS 116870*	JX010146	JX010050	JX009742	JX009433	HQ596280
C. nupharicola	CBS 470.96*	JX010187	JX009972	JX009663	JX009437	JX010398
C. proteae	CBS 132882*	KC297079	KC297009	KC296960	KC296940	KC297101
C. psidii	CBS 145.29*	JX010219	JX009967	JX009743	JX009515	JX010443
C. queenslandicum	ICMP 1778*	JX010276	JX009934	JX009691	JX009447	JX010414
C. rhexiae	CBS 133134*	JX145128	-	-	-	JX145179
C. salsolae	ICMP 19051*	JX010242	JX009916	JX009696	JX009562	JX010403
C. siamense	ICMP 18578*	JX010171	JX009924	FJ917505	FJ907423	JX010404
C. siamense	**MAFF 246751**	**LC412417**	**LC412420**	**LC412419**	**LC412418**	**LC412421**
C. siamense (syn. *C. hymenocallidis*)	CBS 125378 (*)	JX010278	JX010019	JX009709	GQ856775	JX010410
C. siamense (syn. *jasmini- sambac*)	CBS 130420 (*)	HM131511	HM131497	JX009713	HM131507	JX010415
C. syzygicola	MFLUCC 10-0624*	KF242094	KF242156	KF254859	KF157801	KF254880
C. temperatum	CBS 133122*	JX145159	-	-	-	JX145211
C. theobromicola	CBS 124945 *	JX010294	JX010006	JX009591	JX009444	JX010447
C. theobromicola (syn. *C. fragariae*)	CBS 142.31 (*)	JX010286	JX010024	JX009592	JX009516	JX010373
C. theobromicola (syn. *C. gloeosporioides* f. *stylosanthis*)	MUCL 42294 (*)	JX010289	JX009962	JX009597	JX009575	JX010380
C. ti	ICMP 4832*	JX010269	JX009952	JX009649	JX009520	JX010442
C. tropicale	CBS 124949*	JX010264	JX010007	JX009719	JX009489	JX010407
C. viniferum	GZAAS 5.08601*	JN412804	JN412798	JQ309639	JN412795	JN412813
C. wuxiense	CGMCC 3.17894*	KU251591	KU252045	KU251833	KU251672	KU252200
C. xanthorrhoeae	BRIP 45094*	JX010261	JX009927	JX009653	JX009478	JX010448
Glomerella cingulata "f.sp. *camelliae*"	ICMP 10646	JX010225	JX009993	JX009629	JX009563	JX010437

*= 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 glycealdahyde-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				
		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 glycealdahyde-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.

Figure 2. Fruits of *S. dulcificum* inoculated with strain MAFF 246750 after seven days (**a)** and control fruits (**b)**.

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.

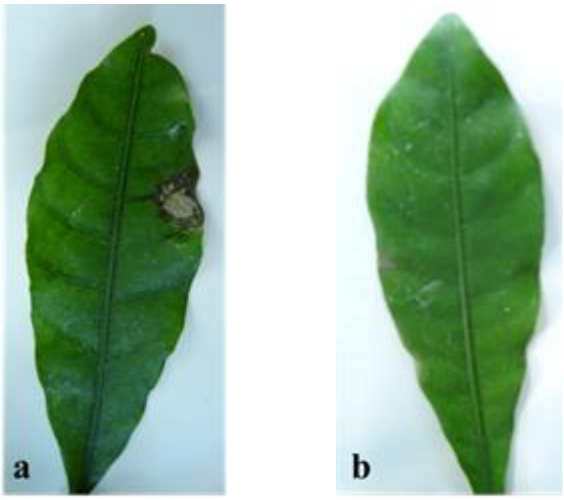

Figure 3. Pathogenicity assay of MAFF 245966 on leaves of *S. dulcificum* after seven days. **a**: Inoculated leaf. **b**: Control leaf.

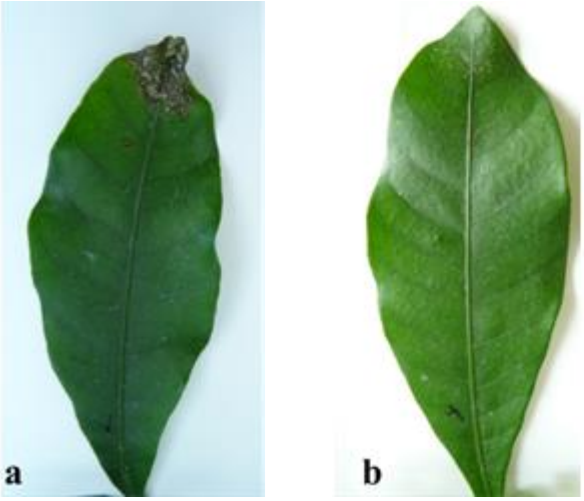

Figure 4. Pathogenicity assay of MAFF 246751 on leaves of *S. dulcificum* after seven days. **a**: Inoculated leaf. **b**: Control leaf.

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).

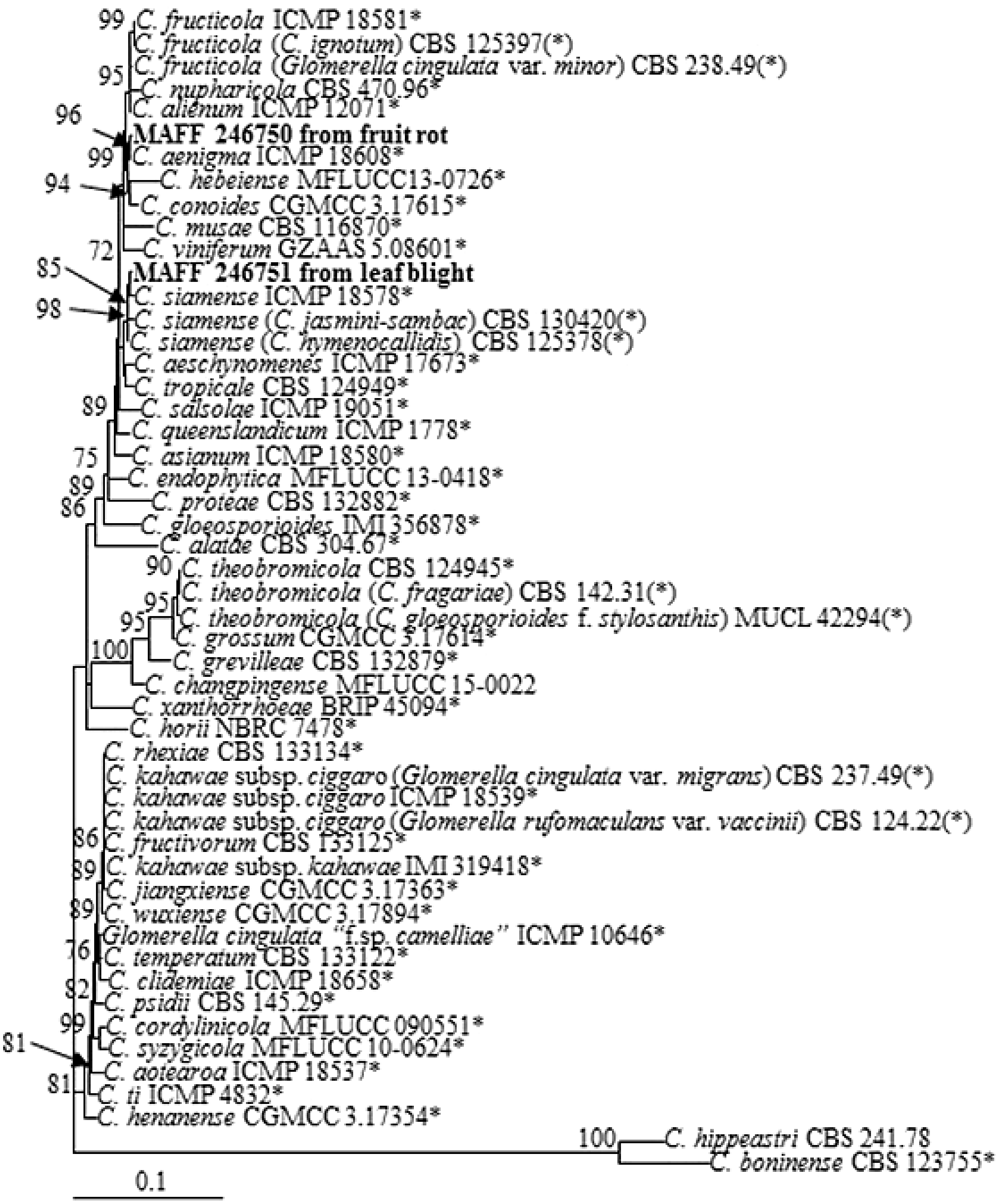

Figure 5. ML phylogenetic analysis of ITS, *GAPDH*, *CAL, ACT* and *TUB2* sequences for the two isolates of *Colletotrichum*, MAFF 246750 from fruit rot and MAFF 246751 from leaf blight on *S. dulcificum*. * = ex-type culture, (*) = ex-type culture of synonymized taxon.

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. 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).

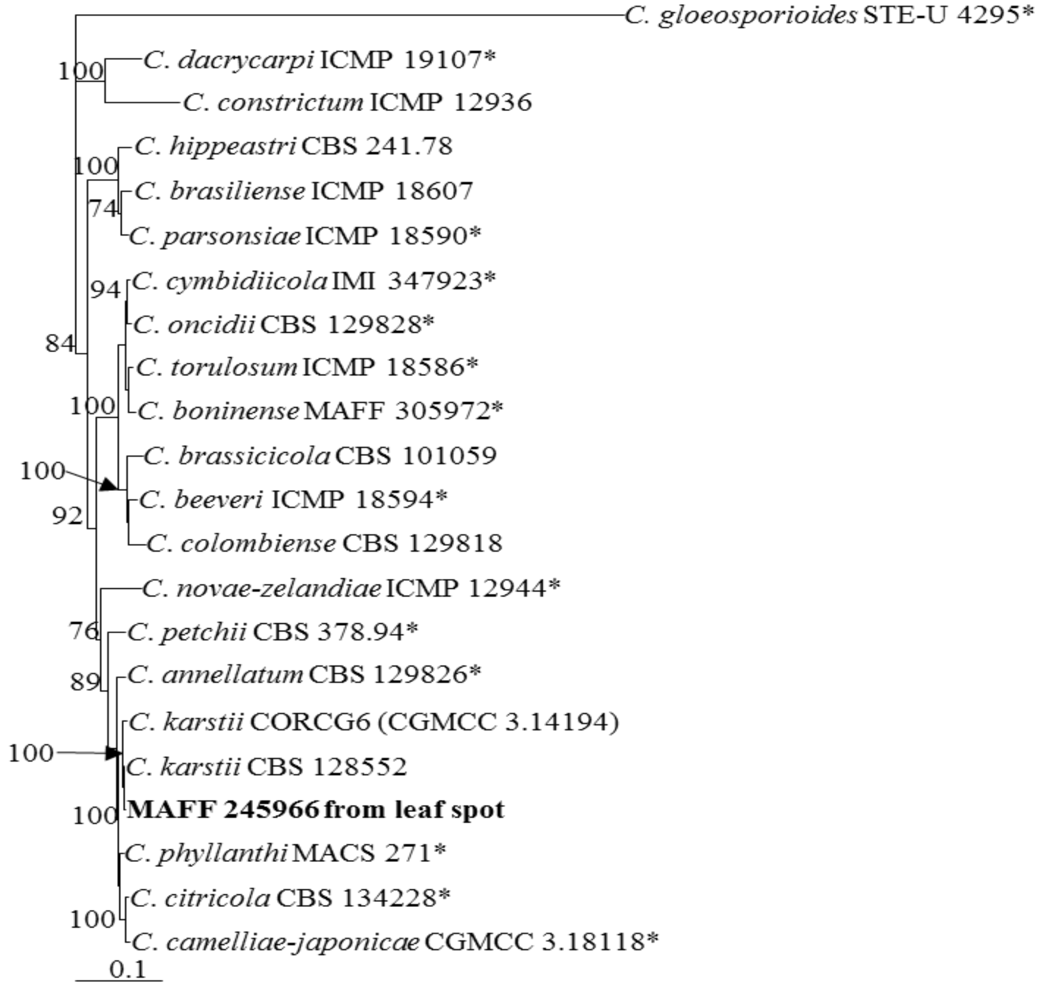

Figure 6. ML phylogenetic analysis of ITS, *GAPDH, CAL, ACT* and *TUB2* sequences for the isolate of *Colletotrichum* MAFF 245966 from leaf spot on *S. dulcificum*. * = ex-type culture.

***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 unitunicate, 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.

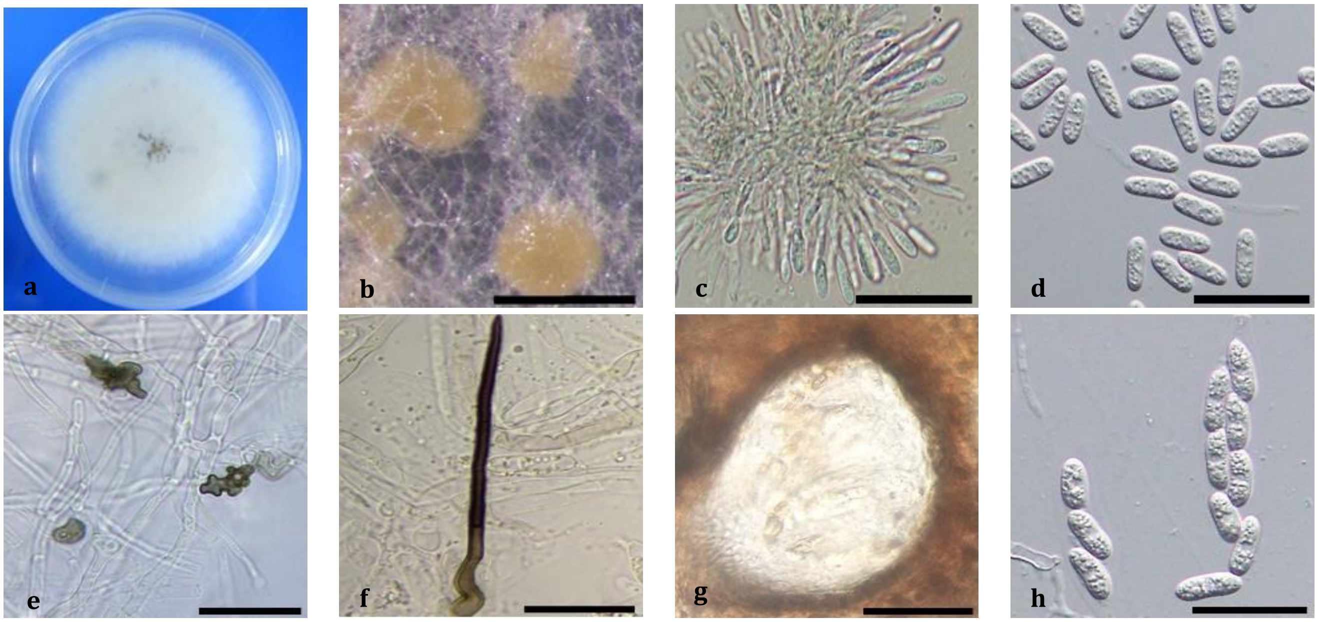

Figure 7. *Colletotrichum aenigma*. **a**: Colony on PDA after seven days (reverse). **b**: Conidial masses. **c**: Conidiophores. **d**: Conidia. **e**: Appressoria. **f**: Seta. **g**: Perithecium. **h**: Ascospores. Scale bars **b**= 200 µm. **c**, **d**, **g**, **h** = 50 µm. **e**, **f** = 20 µm.

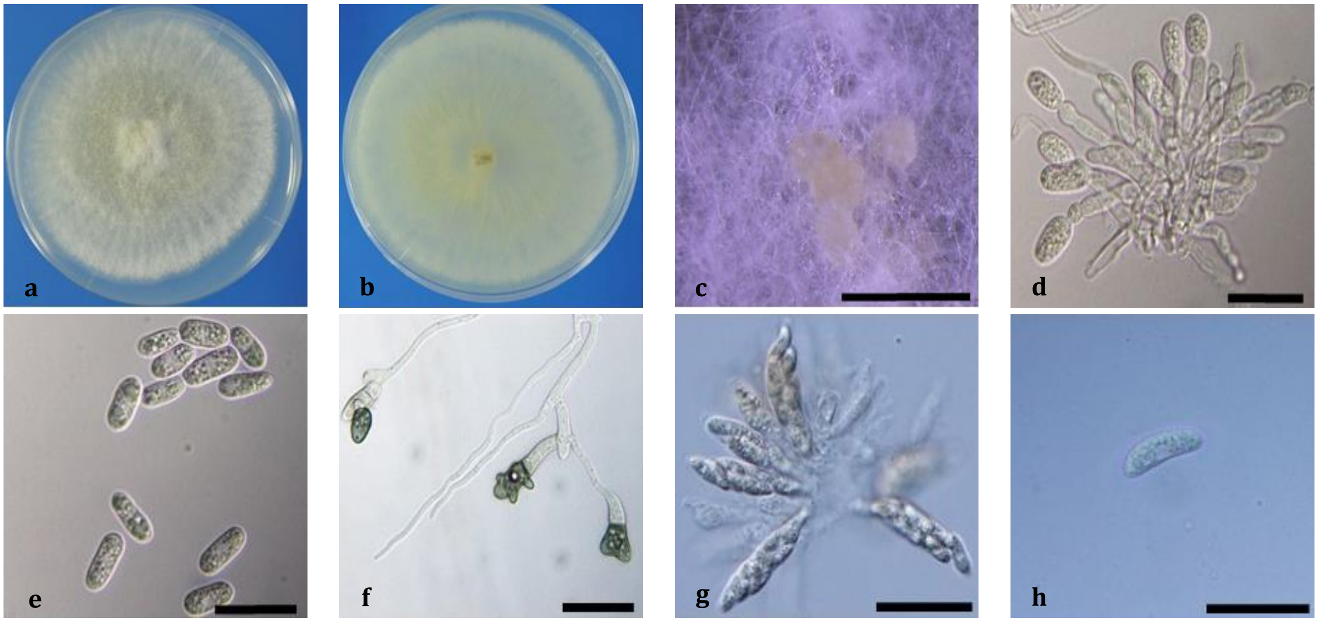

Figure 8. *Colletotrichum karstii*. **a**: Colony on PDA after seven days (surface). **b**: Colony on PDA for seven days (reverse). **c**: Conidial mass. **d**: Conidiophores. **e**: Conidia. **f**: Appressoria. **g**: Asci. **h**: Ascospore. Scale bars: **c** = 200 µm. **d**, **e**, **f**, **h** = 20 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 septates, 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.

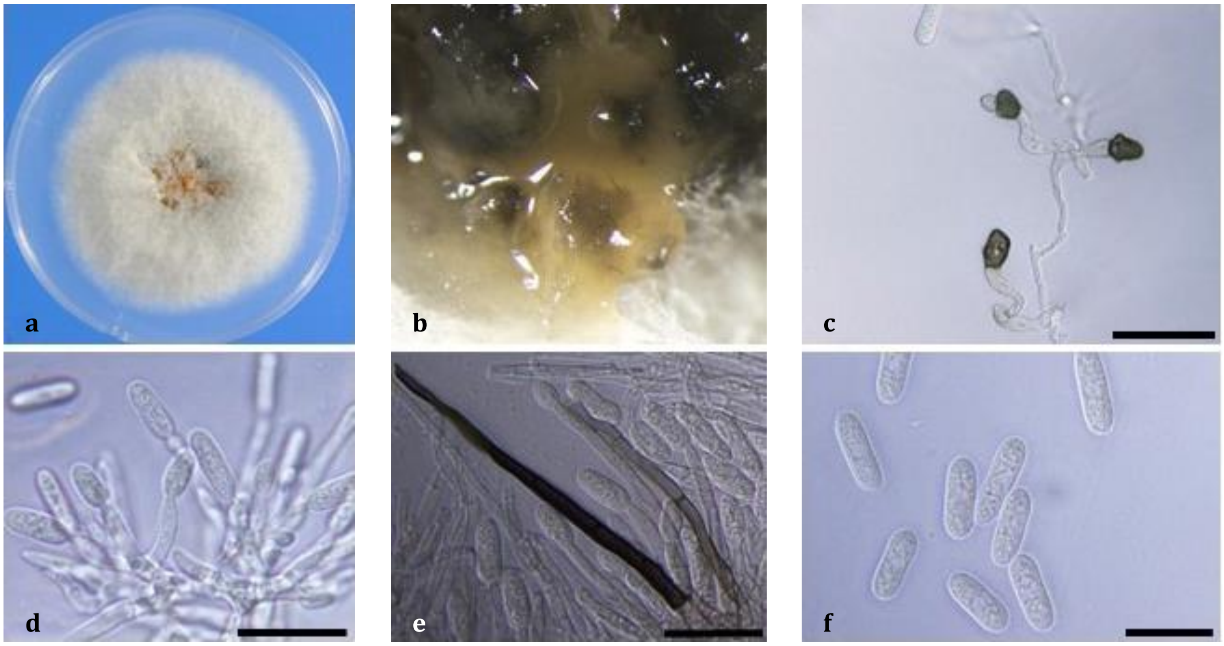

Figure 9. *Colletotrichum siamense*: **a**. Colony on PDA after seven days (surface). **b**: Conidial mass. **c**: Appressoria. **d**: Conidiophores. **e**: Setae. **f**: Conidia. Scale bars: **c**, **d**, **e**, **f** = 20 µm.

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 (Prihastuti *et al.*, 2009), and this species has now been recorded on many hosts (Honger *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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REFERENCES

Akinmoladun, A. C. 2016. Effect of *Synsepalum dulcificum* berry extract on oxidative stress and hepatotoxicity indices, following subacute administration in normal rats. FUTA Journal of Research in Sciences, 12: 167-177.

Alizadeh, A., M. Javan-Nikkhah, R. Zare, K. B. Fotouhifar, U. Damm and E. H. Stukenbrock. 2015. New records of *Colletotrichum* species for the mycobiota of Iran. Mycologia Iranica, 2: 95-109.

Cai, L., K. D. Hyde, P. W. J. Taylor, B. Weir, J. Waller, M. M. Abang, J. Z. Zhang, Y. L. Yang, S. Phoulivong and Z. Y. Liu. 2009. A polyphasic approach for studying *Colletotrichum*. Fungal Diversity, 39: 183-204.

Carbone, I. and L. M. Kohn. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia, 91: 553-556.

Chen, Y., G. Wei and W. Chen. 2002. New species of *Pestalotiopsis*. Mycosystema, 21: 316-323.

Da Silva, C. d. F. B. and S. J. Michereff. 2013. Biology of *Colletotrichum* spp. and epidemiology of the anthracnose in tropical fruit trees. Revista Caatinga, 26: 130-138.

Damm, U., P. F. Cannon, J. H. C. Woudenberg, P. R. Johnston, B. S. Weir, Y. P. Tan, R. G. Shivas and P. W. Crous. 2012. The *Colletotrichum boninense* species complex. Studies in Mycology, 73: 1-36.

De Silva, D. D., P. K. Ades, P. W. Crous and P. W. J. Taylor. 2016. *Colletotrichum* species associated with chili anthracnose in Australia. Plant Pathology, 66: 254-267.

Dean, R., J. A. L. Van Kan, Z. A. Pretorius, K. E. Hammond-Kosack, A. Di Pietro, P. D. Spanu, J. J. Rudd, M. Dickman, R. Kahmann and J. Ellis. 2012. The top 10 fungal pathogens in molecular plant pathology. Molecular Plant Pathology, 13: 414-430.

Diao, Y. Z., C. Zhang, F. Liu, W. Z. Wang, L. Liu, L. Cai and X. L. Liu. 2017. *Colletotrichum* species causing anthracnose disease of chili in China. Persoonia-Molecular Phylogeny and Evolution of Fungi, 38: 20-37.

Gan, P., N. Nakata, T. Suzuki and K. Shirasu. 2016. Markers to differentiate species of anthracnose fungi identify *Colletotrichum fructicola* as the predominant virulent species in strawberry plants in Chiba Prefecture of Japan. Journal of General Plant Pathology, 83: 14-22.

Gardes, M. and T. D. Bruns. 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Molecular Ecology, 2: 113-118.

Guerber, J. C., B. Liu, J. C. Correll and P. R. Johnston. 2003. Characterization of diversity in *Colletotrichum acutatum* sensu lato by sequence analysis of two gene introns, mtDNA and intron RFLPs, and mating compatibility. Mycologia, 95: 872-885.

Honger, J. O., S. K. Offei, K. A. Oduro, G. T. Odamtten and S. T. Nyaku. 2016. Identification and molecular characterisation of *Colletotrichum* species from avocado, citrus and pawpaw in Ghana. South African Journal of Plant and Soil, 33: 177-185.

Ichinose, R., H. Hoshi, T. Ono, Y. Hirooka and H. Horie. 2016. Anthracnose diseases found on *Aesculus indica*, *Cornus kousa* and *Acer platanoides* in Japan. Annual report of the kanto-tosan plant protection society. 63-123.

Lima, N. B., M. V. de A. Batista, M. A. De Morais, M. A. G. Barbosa, S. J. Michereff, K. D. Hyde and M. P. S.

Câmara. 2013. Five *Colletotrichum* species are responsible for mango anthracnose in northeastern Brazil. Fungal Diversity, 61: 75-88.

Liu, F., M. Wang, U. Damm, P. W. Crous and L. Cai. 2016. Species boundaries in plant pathogenic fungi: A *Colletotrichum* case study. BMC Evolutionary Biology, 16: 81-86.

Maddison, W. P. 2017. Mesquite: A modular system for evolutionary analysis; 2007. Version 3.2. http://mesquiteproject.org.

Meetum, P., C. Leksomboon and M. Kanjanamaneesathian. 2015. First report of *Colletotrichum aenigma* and *C. siamense*, the causal agents of anthracnose disease of dragon fruit in Thailand. Journal of Plant Pathology, 97: 402.

Nguyen, P. T. H., O. Vinnere Pettersson, P. Olsson and E. Liljeroth. 2009. Identification of *Colletotrichum* species associated with anthracnose disease of coffee in Vietnam. European Journal of Plant Pathology, 127: 73-87.

O'Donnell, K. and E. Cigelnik. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Molecular Phylogenetics and Evolution, 7: 103-116.

O'Donnell, K., H. I. Nirenberg, T. Aoki and E. Cigelnik. 2000. A multigene phylogeny of the *Gibberella fujikuroi* species complex: Detection of additional phylogenetically distinct species. Mycoscience, 41: 61-78.

Prihastuti, H., L. Cai, H. Chen, E. H. C. McKenzie and K. D. Hyde. 2009. Characterization of *Colletotrichum* species associated with coffee berries in northern Thailand. Fungal Diversity, 39: 89-109.

Rodrigues, J. F., R. d. S. Andrade, S. C. Bastos, S. B. Coelho and A. C. M. Pinheiro. 2016. Miracle fruit: An alternative sugar substitute in sour beverages. Appetite, 107: 645-653.

Rojas, E. I., S. A. Rehner, G. J. Samuels, S. A. Van Bael, E. A. Herre, P. Cannon, R. Chen, J. Pang, R. Wang, Y. Zhang, Y.-Q. Peng and T. Sha. 2010. *Colletotrichum gloeosporioides* s.l. associated with *Theobroma cacao* and other plants in Panamá: Multilocus phylogenies distinguish host-associated pathogens from asymptomatic endophytes. Mycologia, 102: 1318-1338.

Schena, L., S. Mosca, S. O. Cacciola, R. Faedda, S. M. Sanzani, G. E. Agosteo, V. Sergeeva and G. Magnano di San Lio. 2013. Species of the *Colletotrichum gloeosporioides* and *C. boninense* complexes associated with olive anthracnose. Plant Pathology, 63: 437-446.

Schoch, C. L., K. A. Seifert, S. Huhndorf, V. Robert, J. L. Spouge, C. A. Levesque, W. Chen, E. Bolchacova, K. Voigt and P. W. Crous. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences, 109: 6241-6246.

Sharma, G. and B. D. Shenoy. 2013. *Colletotrichum fructicola* and *C. siamense* are involved in chilli anthracnose in India. Archives of Phytopathology and Plant Protection, 47: 1179-1194.

Sharma, G. and B. D. Shenoy. 2016. *Colletotrichum* systematics: Past, present and prospects. Mycosphere, 7: 1093-1102.

Shi, Y.-C., K.-S. Lin, Y.-F. Jhai, B.-H. Lee, Y. Han, Z. Cui, W.-H. Hsu and S.-C. Wu. 2016. Miracle Fruit (*Synsepalum dulcificum*) exhibits as a novel anti-hyperuricaemia agent. Molecules, 21: 140-144.

Stamatakis, A., P. Hoover and J. Rougemont. 2008. A rapid bootstrap algorithm for the RAxML web servers. Systematic Biology, 57: 758-771.

Wang, Y.-C., X.-Y. Hao, L. Wang, X. Bin, X.-C. Wang and Y.-J. Yang. 2016. Diverse *Colletotrichum* species cause anthracnose of tea plants (*Camellia sinensis* (L.) O. *Kuntze*) in China. Scientific Reports, 6: 35287.

Weir, B. S., P. R. Johnston and U. Damm. 2012. The *Colletotrichum gloeosporioides* species complex. Studies in Mycology, 73: 115-180.

Wikee, S., L. Cai, N. Pairin, E. H. C. McKenzie, Y.-Y. Su, E. Chukeatirote, H. N. Thi, A. H. Bahkali, M. A. Moslem, K. Abdelsalam and K. D. Hyde. 2010. *Colletotrichum* species from Jasmine (*Jasminum sambac*). Fungal Diversity, 46: 171-182.

Yang, Y., L. Cai, Z. Yu, Z. Liu and K. D. Hyde. 2011. *Colletotrichum* Species on Orchidaceaein Southwest China. Cryptogamie Mycologie, 32: 229-253.

Yokosawa, S., N. Eguchi, K.-i. Kondo and T. Sato. 2017. Phylogenetic relationship and fungicide sensitivity of members of the *Colletotrichum gloeosporioides* species complex from apple. Journal of General Plant Pathology, 83: 291-298.

Zivkovic, S., S. Stojanovic, Z. Ivanovic, N. Trkulja, N. Dolovac, G. Aleksic and J. Balaz. 2010. Morphological and molecular identification of *Colletotrichum acutatum* from tomato fruit. Pesticidi i Fitomedicina, 25: 231-239.