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Identification of the *Colletotrichum* species associated with mango diseases and a universal LAMP detection method for *C. gloeosporioides* species complex

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Abstract: The *Colletotrichum gloeosporioides* species complex contains plant pathogens linked to Anthracnose diseases afflicting various crops. In this study, we designed a loop-mediated isothermal amplification assay (LAMP) primer set based on calmodulin gene coding region sequences from taxonomically authorized isolates of species from this complex to rapidly detect the presence of fungi associated with Anthracnose diseases. This test can be employed at any point between cultivation and sale. Moreover, we examined the specificity and detectable range of this primer set using isolates selected from species of the genus *Colletotrichum*. This test was able to specifically detect members of the *C. gloeosporioides* species complex, including *C. gloeosporioides*, *C. aotearo*a, *C. fructicola*, *C. horii*, *C. kahawae*, *C. musae*, *C. siamense*, *C. theobromicola*, and *C. tropicale.*

Keywords: *Anthracnose, diagnosis, phylogeny, plant disease*

INTRODUCTION

Species of fungi from the genus *Colletotrichum* are known to cause Anthracnose diseases in various plants [Cannon et al., 2012; Dean et al., 2012; Jayawardena et al., 2016]. *Colletotrichum gloeosporioides* sensu lato (s. lat.) is a polyxenic plant pathogen from a species complex consisting of 51 closely related species [Weir et al., 2012; Jayawardena et al., 2021]. According to Far and Rossman [2021], *C. gloeosporioides* has been identified in more than 1000 plant species. *C. gloeosporioides* was proposed by Penzig [1882] based on a fungus from citrus known then as *Vermicularia gloeosporioides*, but was transferred to the genus *Colletotrichum* by Penzig and Saccardo in 1884. However, for many years, the authentic isolate – which is important for modern species classification, as well as for accurate identification of the causal fungus responsible for plant disease – has not been available. Moreover, until recently, the phylogenetic relationships among numerous *Colletotrichum* species had been unclear. In 2008, a lectotype and living culture derived from the epitype were designated by Cannon et al. [2008]. Thereafter, isolates that were genetically congruent with this ex-epitype isolate have been classified as *C. gloeosporioide*s sensu stricto (s. str.). Furthermore, *C. gloeosporioides* has been recognized as a species complex (i.e., the *C. gloeosporioides* species complex, or CGSC), which is a group of species clustered within a single clade of a phylogenetic tree [Weir et al., 2012]. Although the CGSC is supported by ITS1-5.8S-ITS2 sequence data [Cannon et al., 2012], to date, morphological and cultural characteristics cannot unequivocally place an isolate in this complex [Weir et al., 2012].

As mentioned above, CGSC species are plant pathogenic fungi that have numerous host plants and can cause devastating crop diseases to citrus, coffee, mango, persimmon, and strawberry plants in temperate and tropic areas. Diagnosis of these diseases often uses ITS1-5.8S-ITS2 sequences in combination with conventional methods, such as morphological and cultural characteristics on various media, and requires significant time and effort. In recent years, loop-mediated isothermal amplification (LAMP) assays [Notomi et al., 2000; 2015] have been introduced to improve the diagnosis efficiency of crop Anthracnose diseases. LAMP amplification uses repetition of two types of elongation reactions via multiple primer sets and is rapid, simple, and highly specific [Notomi et al.,

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2015]. Recent examples of LAMP-based detection of Anthracnose fungal disease include the identification of Anthracnose on strawberry using ITS1-5.8S-ITS2 sequences [Katoh et al., 2016] and the identification of Anthracnose on soybean, grape, pear, rubber, navel orange, and fishtail palm using glutamine synthetase sequences [Wang et al., 2017]. The LAMP primer sets used in these studies were designed to detect several specific crop diseases. However, these sets were designed on the basis of data from relatively few taxonomically unauthorized isolates. Accordingly, untargeted species may be detected from the samples, and the detectable species range using these primer sets would therefore be ambiguous.

In this study, we designated a LAMP primer set based on calmodulin gene coding region (cal) sequences obtained from taxonomically authorized isolates from the CGSC. Furthermore, we evaluated the specificity and detectable range of our newly designed primers by testing the LAMP analyses using against isolates selected from various species complexes of the genus *Colletotrichum*. We aimed to facilitate the rapid detection of the causal fungi responsible for Anthracnose diseases in crops.

MATERIAL AND METHODS

Diseased samples and isolates. We selected 35 isolates identified as *Colletotrichum* species kept at the Culture Collection at the Genetic Resources Center of the National Agriculture and Food Research Organization (NARO, MAFF), Tsukuba, Japan, for use in our LAMP assay trials. These isolates, selected by the phylogenetic relationship indicated by previous studies [Cannon et al., 2012; Damm et al., 2012; Suwannarat et al., 2017] as well as current taxonomic position [Jayawardena et al., 2016], included those from various host plants. They consisted of the *Colletotrichum acutatum* species complex (CASC; eight isolates of seven species), the *C. boninense* species complex (CBSC; two isolates of two species), the *C. caudatum* species complex (CCSC; one isolate of one species), the *C. dematium* species complex (CDSC; two isolates of two species), the *C. gloeosporioides* species complex (CGSC; 13 isolates of nine species), the *C. gigasporum* species complex (CISC; one isolate of one species), the *C. graminicola* species complex (CMSC; two isolates of two species), the *C. orbiculare* species complex (COSC; one isolate of one species), the *C. spaethanum* species complex (CSSC: one isolate of one species), the *C. truncatum* species complex (CTSC; one isolate of one species), the *C. destructivum* species complex (CVSC; one isolate of one species), and singleton species present on the phylogenetic tree (three isolates of three species) (Tab. 1).

In addition, we also collected diseased fruit of mango (*Mangifera indica* L.) from orchards in Okinawa prefecture, Japan, and from a market for fruit imported from the Philippines, located in Tokyo, Japan. To establish cultures originating from a single conidium, conidia from lesions were suspended in sterilized distilled water and spread on 2 % water agar medium using a flame-sterilized microspatula. After incubation at 20 °C for 24 h, germinating conidia were individually transferred to potato-dextrose agar (PDA; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) using a flame-sterilized microtube under a dissecting microscope at ×100 magnification [Nakashima et al., 2016]. Deposits of the newly obtained isolates were made at Mie University (MUCC), Tsu, Mie, Japan (Tab. 2).

Fungal DNA extraction and phylogenetic analysis. The genomic DNA of all examined isolates was extracted using an Ultra Clean Microbial DNA isolation kit (MoBio Laboratories, CA, USA), according to the manufacturer's instructions. Five partial nuclear genes were subjected to PCR amplification and sequencing: the internal transcribed spacer regions and the intervening 5.8S rRNA gene (ITS) of the nrDNA operon, actin (*act*A), calmodulin (*cal*), chitin synthase (*chs-1*), and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*). PCR was performed using the primers listed in table 3 on a BioRad T100 Thermal Cycler (Biorad Laboratories, Inc., CA, USA). The PCR mixture consisted of 1–10 ng genomic DNA, 0.25 unit Bioline Taq DNA Polymerase (Bioline, London, UK), 1.25 μL 10× NH_4 reaction buffer (Bioline), 2.5 mM $MgCl_2$ (Bioline), 10 μM of each dNTP (Bioline) and 0.16 μL of each primer in a total volume of 12.5 μL. The PCR cycling conditions were as follows: initial denaturation (94 °C, 3 min); 40 cycles of amplification (denaturation at 94 °C for 30 s); annealing (Tab. 4); extension at 72 °C for 45 s), and final extension (72 °C, 5 min). The resulting fragments were sequenced in both directions using their respective PCR primers and the BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems Life Technologies, Carlsbad, CA, USA). DNA sequencing amplicons were purified using Sephadex G-50 Superfine columns (Sigma-Aldrich, St. Louis, MO) in MultiScreen HV plates (Millipore, Billerica, MA). Purified sequence reactions were analyzed on an Applied Biosystems 3730xl DNA Analyzer (Life Technologies, Carlsbad, CA, USA) installed at the Advanced Science Research Promotion

Table 1. A list of strains used for detection of LAMP in this study.

Species Complex	Species		Isolate No. in MAFF	Isolate No. in MUCC	Host	Turbidity	Fluorescence
CASC	*Colletotorichum*	*acutatum* s. lat.	744062	2394	*Fragaria x ananassa*	−	−
		carthami	239359	2441	*Calendula officinalis*	−	−
		fioriniae	306283	2443	*Fragaria x ananassa*	−	−
			240052	23	*Hovenia dulcis*	−	−
		godetiae	241297	2444	*Malus pumila*	−	−
		nymphaeae	306523	2445	*Amygdalus persica*	−	−
		scovillei	242692	2446	*Capsicum annuum*	−	−
CBSC	*Colletotorichum*	*boninense*	305972	2393	*Crinum asiaticum var. asiaticum*	−	−
		karstii	237232	2449	*Hydrangea macrophylla*	−	−
CCSC	*Colletotorichum*	*zoysiae*	238574	2395	*Zoysia pacifica*	−	−
CDSC	*Colletotorichum*	*circinans*	238028	2397	*Allium fistulosum*	−	−
		trichellum	237991	2406	*Hedera rhombea*	−	−
CGSC	*Colletotorichum*	*aotearoa*	243690	2452	*Strelitzia reginae*	+	+
		fructicola	235088	2448	*Fragaria x ananassa*	+	+
		gloeosporioides	237219	2401	*Actinidia chinensis var. deliciosa*	+	+
			306534	2438	*Citrus unshiu*	+	+
		horii	243424	2439	*Diospyros kaki*	+	+
		kahawae	237014	2450	*Hibiscus cannabinus*	+	+
		musae	239086	2440	*Musa x paradisiaca*	+	+
		siamense	240628	797	*Hibiscus hamabo*	+	+
			240597	606	*Vanilla mexicana*	+	+
		theobromicola	305994	2451	*Coffea arabica*	+	+
		tropicale	240593	598	*Dimocarpus longan*	+	+
			240632	802	*Sphagneticola trilobata*	+	+
			240629	798	*Xanthosoma sagittifolium*	+	+
CISC	*Colletotorichum*	*gigasporum*	238783	2400	*Chrysanthemum morifolium*	−	−
CMSC	*Colletotorichum*	*echinochloae*	511473	2399	*Echinochloa esculenta*	−	−
		graminicola	511343	2402	*Zea mays*	−	−
COSC	*Colletotorichum*	*orbiculare*	306518	2404	*Cucumis melo*	−	−
CSSC	*Colletotorichum*	*spaethianum*	239500	2408	*Polygonatum falcatum*	−	−
CTSC	*Colletotorichum*	*truncatum*	306552	2407	*Fagopyrum esculentum*	−	−
CVSC	*Colletotorichum*	*destructivum*	239947	2398	*Antirrhinum majus*	−	−
singleton	*Colletotorichum*	*chlorophyti*	305748	2396	*Vigna radiata*	−	−
		hsienjenchang	243051	2403	*Phyllostachys reticulata*	−	−
		sansevieriae	239721T	2405	*Sansevieria trifasciata*	−	−

Note: Species Complex: CASC: *Colletotrichum acutatum*, CBSC: *C. boninense*, CCSC: *C. caudatum*, CDSC: *C. dematium*, CGSC: *C. gloeosporioides*, CISC: *C. gigasporum*, CMSC: *C. graminicola*, COSC: *C. orbiculare*, CSSC: *C. spaethanum*, CTSC: *C. truncatum*, CVSC: *C. destructivum*, singleton: singleton species
MAFF: Culture Collection at the Genetic Resources Center of the National Agriculture and Food Research Organization (NARO, MAFF), Tsukuba, Japan.
MUCC: Culture collection, herbarium of Mie University (TSU), Mie, Japan.

Table 2. A List of *Colletotrichum* species used for phylogeny analysis.

Species complex	Names	Host	Isolate no.	Taxonomic states
CBSC	*Colletotrichum boninense*	*Cymbidium* sp.	ICMP 17904	ex-type
	Colletotrichum hippeastri	*Hippeastrum vittatum*	CBS 125376	ex-type
CGSC	*Colletotrichum aenigma*	*Persea americana*	ICMP 18608	ex-type
	Colletotrichum aeschynomenes	*Aeschynomene virginica*	ICMP 17673	ex-type
	Colletotrichum alienum	*Malus domestica*	ICMP 12071	ex-type
	Colletotrichum asianum	*Coffea arabica*	ICMP 18580	ex-type
	Colletotrichum fructicola	*Coffea arabica*	ICMP 18581	ex-type
	syn. *C. ignotum*	*Tetragastris panamensis*	ICMP 18646	ex-type
	syn. *Glomerella cingulata* var. *minor*	*Ficus edulis*	ICMP 17921	ex-type
	Colletotrichum musae	*Musa* sp.	ICMP 19119	ex-type
	Colletotrichum nupharicola	*Nuphar lutea subsp. polysepala*	ICMP 18187	
	Colletotrichum tropicale	*Theobroma cacao*	ICMP 18653	ex-type
	Colletotrichum siamense	*Coffea arabica*	ICMP 18578	ex-type
	syn. *C. hymenocallidis*	*Hymenocallis americana*	ICMP 18642	ex-type
	Colletotrichum gloeosporioides	*Citrus sinensis*	ICMP 17821	ex-type
	Colletotrichum queenslandicum	*Carica papaya*	ICMP 1778	ex-type
	Colletotrichum alatae	*Dioscorea alata*	ICMP 17919	ex-type
	Colletotrichum aotearoa	*Coprosma* sp.	ICMP 18537	ex-type
	Colletotrichum horii	*Diospyros kaki*	ICMP 17970	ex-type
	Colletotrichum theobromicola	*Theobroma cacao*	ICMP 18649	ex-type
	syn. *C. fragariae*	*Fragaria* × *ananassa*	ICMP 17927	ex-type
	syn. *C. gloeosporioides* f. *stylosanthis*	*Stylosanthes viscosa*	ICMP 17957	ex-type
	Colletotrichum psidii	*Psidium* sp.	ICMP 19120	ex-type
	Colletotrichum ti	*Cordyline* sp.	ICMP 4832	ex-type
	Colletotrichum clidemiae	*Clidemia hirta*	ICMP 18658	ex-type
	Colletotrichum cordylinicola	*Cordyline fruticosa*	ICMP 18579	ex-type
	C. kahawae subsp. *ciggaro* syn. *Glomerella cingulata* var. *migrans*	*Hypericum perforatum*	ICMP 17922	ex-type
	Colletotrichum xanthorrhoeae	*Xanthorrhoea preissii*	ICMP 17903	ex-type
	Glomerella cingulata "f.sp. *camelliae*"	*Camellia sasanqua*	ICMP 18542	

Center, Mie University, Mie, Japan. The DNA sequences generated were analyzed, and consensus sequences were computed using MEGA version 7 [Kumar et al., 2016]. All novel sequences generated in this study were deposited in the DNA Data Bank of Japan (Tab. 5).

Preliminary identification of field samples based on morphology and ITS phylogeny (data not shown) identified isolates that likely belonged to the CGSC. The phylogenetic relationships of the newly obtained isolates from field samples was determined by using the combined multi-loci sequences and sequences retrieved from GenBank and TreeBASE (www.treebase.org). In particular, we used data from study number S12535, which was analyzed in the comprehensive study of CGSC fungi [Weir et al., 2012]. *Colletotrichum hippeastri* ICMP17920 and *Colletotrichum boninense* ICMP17904 were selected as outgroups for sequence alignment. Sequences were aligned using MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/index.html). The resulting alignments were manually checked and improved where necessary using MEGA version 7. Parsimony analyses were used to estimate phylogenetic relationships in the dataset. Parsimony analyses were conducted using PAUP* v. 4.0b10 [Swofford, 2003]. Alignment gaps were treated as a fifth character, and all characters were unordered and of equal weight. The robustness of the obtained trees was evaluated by 1000 bootstrap replicates using PAUP*. Tree scores including tree length (TL), consistency index (CI), retention index (RI), and the rescaled consistency index (RC) were also calculated. The generated trees were visualized using FigTree version 1.4.2 (Institute of Evolutionary Biology, University of Edinburgh, http://tree.bio.ed.ac.uk/software/figtree).

LAMP Primer design. On the basis of the alignment of the combined data matrix retrieved from GenBank as well as from previous studies [Weir et al., 2012], the cal gene coding region was selected for development of specific LAMP primers to detect the CGSC. The primer set was designed using PrimerExplorer software

Table 3. Details of PCR primers used in this study for amplification and sequencing.

Locus	Primer	Sequence (5′–3′)	Annealing (°C)	Reference
ITS	V9G	TTA CGT CCC TGC CCT TTG TA	48	de Hoog & Gerrits van den Ende 1998
	ITS4	TCC TCC GCT TAT TGA TAT GC		White et al. 1990
ACT	ACT-512F	ATG TGC AAG GCC GGT TTC GC	58	Carbone & Kohn 1999
	ACT-783R	TAC GAG TCC TTC TGG CCC AT		Carbone & Kohn 1999
CAL	CL1C	GAA TTC AAG GAG GCC TTC TC	59	Weir et al. 2012
	CL2C	CTT CTG CAT CAT GAG CTG GAC		Weir et al. 2012
CHS-1	CHS-79F	TGG GGC AAG GAT GCT TGG AAG AAG	59	Carbone & Kohn 1999
	CHS-345R	TGG AAG AAC CAT CTG TGA GAG TTG		Carbone & Kohn 1999
GAPDH	GDF	GCC GTC AAC GAC CCC TTC ATT GA	59	Templeton et al. 1992
	GDR	GGG TGG AGT CGT ACT TGA GCA TGT		Templeton et al. 1992

Table 4. Details of LAMP primers used in this study.

Location	Primer	Sequence (5′–3′)
FIP	cg_cal_FIP	GTGCCCAGCTCCTTTGTAGTGAGTCGAGGCATGCAGAATCG
BIP	cg_cal_BIP	ATGCGCTCTCTCGGCCAGAACGACCTCGTTGATCATGTCC
F3	cg_cal_F3	ATACACCAGCGGCATTCG
B3	cg_cal_B3	GATGGTGCCGTTGTTGTC

version 5 [https://primerexplorer.jp] provided by Eiken Chemical Co., Ltd. (Tokyo, Japan). Primers were designed to have the minimum degree of mutation in the target sequence, especially at the upper stream of each primer (Fig. 1).

The genomic DNA samples of all isolates were diluted with Sterile ultra-pure water (ddH_2O) to 0.1–10 ng/mL and were added to a reaction mixture. The LAMP

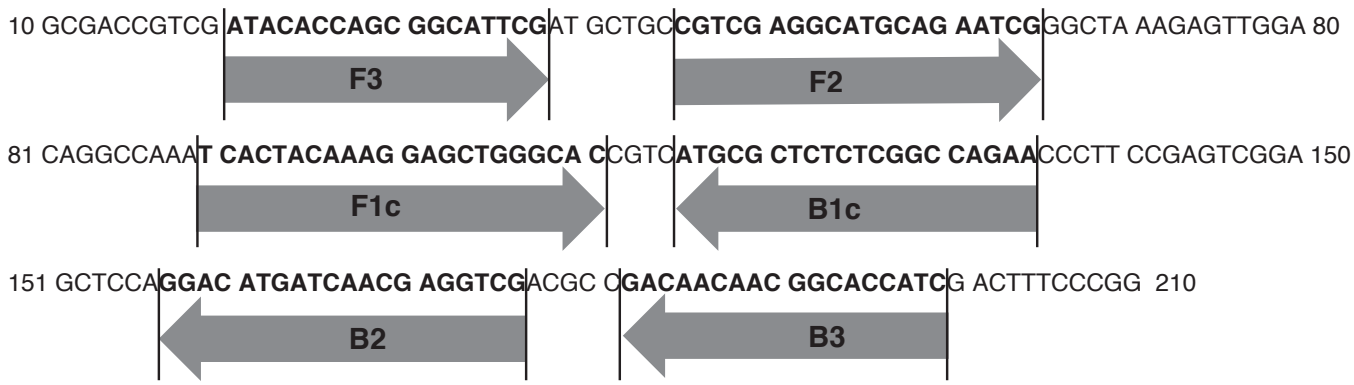

Figure 1. Location and sequence of loop-mediated isothermal amplification (LAMP) primers targeting the *Colletotrichum gloeosporioides* species complex at the cal coding region. The locations of two outer (F3 and B3) and two inner (FIP: F1c-F2, and BIP: B1c-B2) primers are indicated. Arrows indicate the extension direction.

Table 5. A List of newly obtained isolates of *Colletotrichum* species.

Names	Host	Site	Isolate no.	Collected place	Accession no.				
					ITS	ACT	CAL	CHS-1	GAPDH
Colletotrichum asianum	*Mangifera indica*	Fruit	MUCC 2555	Tokyo*, Japan	LC474407	LC474424	LC474441	LC474475	LC474458
	M. indica	Fruit	MUCC 2562	Tokyo*, Japan	LC474408	LC474425	LC474442	LC474476	LC474459
	M. indica	Fruit	MUCC 2563	Tokyo*, Japan	LC474409	LC474426	LC474443	LC474477	LC474460
	M. indica	Leaf	MUCC 2574	Okinawa, Japan	LC474410	LC474427	LC474444	LC474478	LC474461
	M. indica	Fruit	MUCC 2576	Okinawa, Japan	LC474411	LC474428	LC474445	LC474479	LC474462
	M. indica	Fruit	MUCC 2593	Tokyo*, Japan	LC474414	LC474431	LC474448	LC474482	LC474465
	M. indica	Fruit	MUCC 2595	Tokyo*, Japan	LC474415	LC474432	LC474449	LC474483	LC474466
	M. indica	Fruit	MUCC 2597	Tokyo*, Japan	LC474416	LC474433	LC474450	LC474484	LC474467
	M. indica	Fruit	MUCC 2598	Tokyo*, Japan	LC474417	LC474434	LC474451	LC474485	LC474468
	M. indica	Fruit	MUCC 2599	Tokyo*, Japan	LC474418	LC474435	LC474452	LC474486	LC474469
	M. indica	Fruit	MUCC 2600	Tokyo*, Japan	LC474419	LC474436	LC474453	LC474487	LC474470
	M. indica	Fruit	MUCC 2601	Tokyo*, Japan	LC474420	LC474437	LC474454	LC474488	LC474471
	M. indica	Fruit	MUCC 2602	Tokyo*, Japan	LC474421	LC474438	LC474455	LC474489	LC474472
	M. indica	Fruit	MUCC 2603	Tokyo*, Japan	LC474422	LC474439	LC474456	LC474490	LC474473
Colletotrichum siamense	*M. indica*	Fruit	MUCC 2592	Tokyo*, Japan	LC474413	LC474430	LC474447	LC474481	LC474464
Colletotrichum tropicale	*M. indica*	Leaf	MUCC 2580	Okinawa, Japan	LC474412	LC474429	LC474446	LC474480	LC474463
	M. indica	Leaf	MUCC 2636	Okinawa, Japan	LC474423	LC474440	LC474457	LC474491	LC474474

Note: MUCC: Culture collection, herbarium of Mie University (TSU), Mie, Japan.
*: the fruits imported from the Philippines developed anthracnose at a market.

reaction was carried out using a DNA amplification kit B for LAMP analyses (Nippon Gene, Tokyo, Japan). The total volume of each LAMP reaction mixture was 25 µl and contained 1.5 µl 10×buffer B (Nippon Gene), 2.4 µl 5× reaction additive (Nippon Gene), 0.84 µl dNTPs mixture (Nippon Gene), 0.96 µM of each forward inner primer (FIP) and backward inner primer (BIP) (Tab. 5), 0.12 µM of each F3 and B3 primers, 1 µl of the amplification enzyme (Nippon Gene), 1 µl of the diluted PCR products as a DNA template, and 5.86 µl of ddH_2O (Tab. 6). ddH_2O was used as a negative control. The LAMP reaction was performed on detection equipment (LF-8, Nippon Gene) at 67.5 °C for 45 min. DNA amplification was confirmed by the turbidity of LAMP products by absorbance at 465 nm on LF-8. The

Table 6. Reaction Mixture for LAMP reaction in this study.

ddH_2O	5.86 µl
10× Reaction Buffer B	1.5 µl
5× Reaction Additive	2.4 µl
dNTP mixture	0.84 µl
LAMP Primer Mix	2.4 µl
1.25 µl of each F3 (100 µM) B3(100 µM), 10 µl of each FIP and BIP (100 µM), 77.5 µl of ddH_2O	
DNA Template	1.0 µl
Mineral Oil	10 µl
	25 µl

experiment was repeated at least three times. In-tube visualization of amplified products was performed by directly staining double-stranded DNA using SYBR Green-1, an intercalating DNA dye (Takara Bio) after the amplification.

RESULTS AND DISCUSSION

Identification of diseased samples. To test the ability of the LAMP assay to identify and detect pathogens efficiently, we examined four isolates from diseased mango fruits and leaves collected at Okinawa and 14 isolates from symptomatic samples collected at Tokyo.

In all diseased samples, the leaf symptoms were irregular at the leaf margin, scattered and circular to irregular at the lamina, brown to greyish brown, enlarged, 5–30 mm, and formed acervuli visible as black dots. The symptoms on the fruit were observed as blackish brown small dots that later turned to black to dark brown spots, enlarged and confluent, circular to subcircular in shape. These spots were slightly concave and covered the whole fruit given enough time. Acervuli formed on the spots and bore slimy orange conidial masses.

Colonies were classified into three groups based on cultural characters assessed 7 days after inoculation on PDA. Representative isolates are shown in Fig. 2. MUCC2555: colonies reaching 55 mm in diameter, white to olivaceous at the center, and white aerial

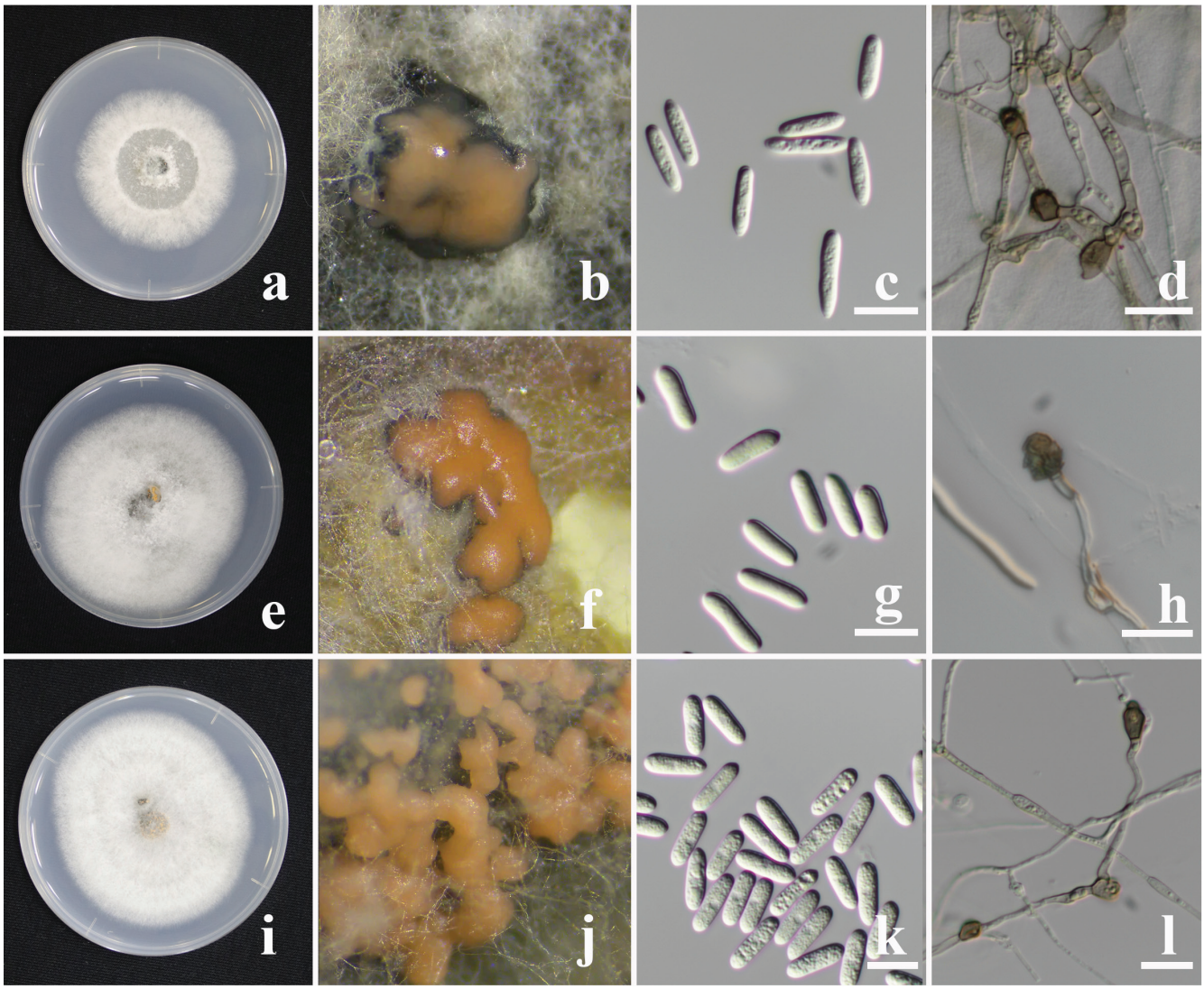

Figure 2. Morphological and phenotypic characters of *Colletotrichum* species isolated from mango.
a–d: *Colletotrichum asianum* (MUCC2555), e–h: *C. siamense* (MUCC2592), i–l: *C. tropicale* (MUCC2636). a, e, i: mycelial colonies on potato-dextrose agar (PDA) 7 days after inoculation; b, f, j: conidial masses on mycelial colony; c, g, j: conidia; d, h, l: appresoria. Bars 10 µm.

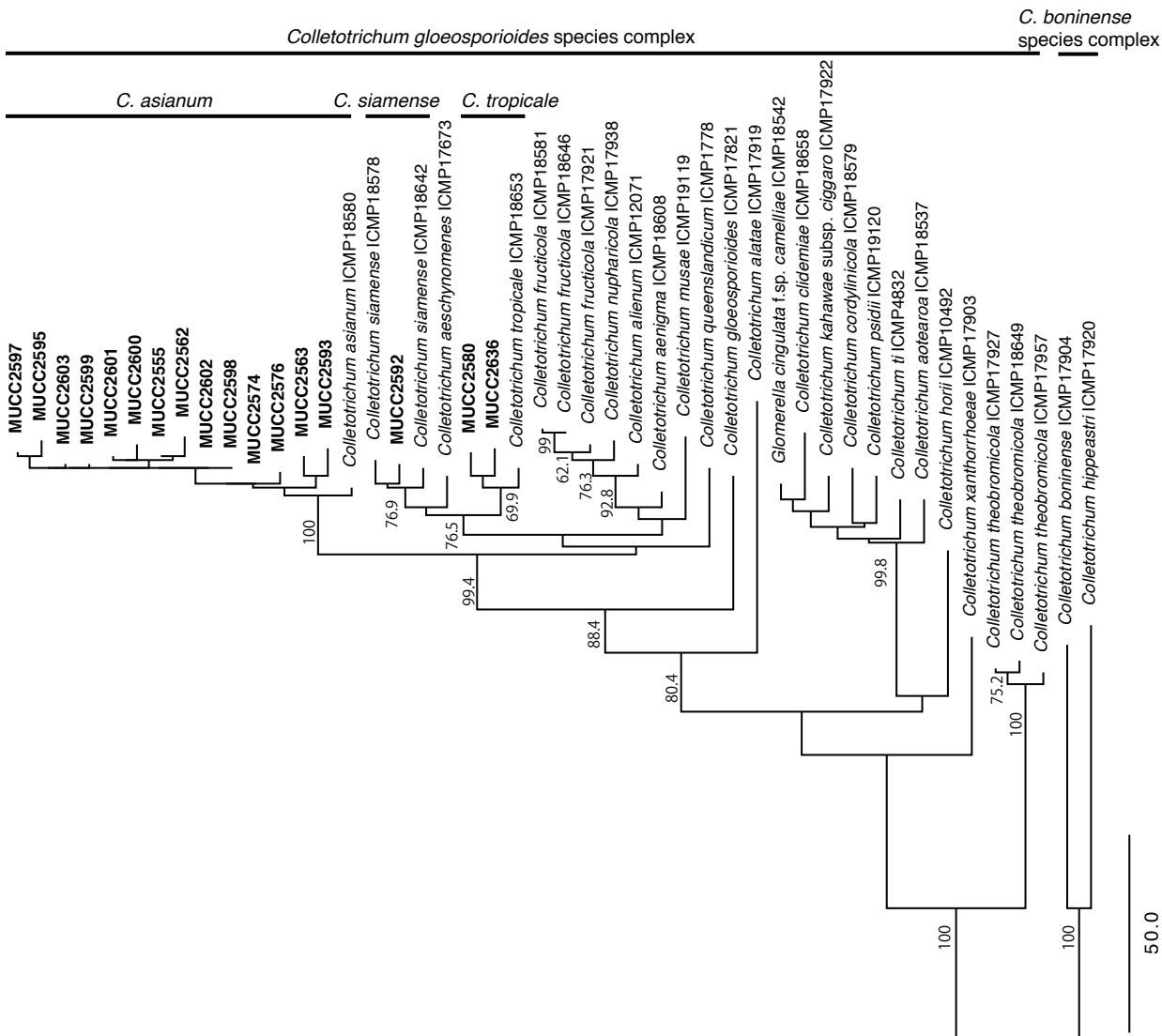

Figure 3. Phylogenetic tree of members of the *Colletotrichum gloeosporioides* species complex generated from analysis of the ITS, act, cal, chs-1, and gapdh sequence dataset. Parsimony (MP) bootstrap values >60% are shown above branches. TL = 1085, CI = 0.689, RI = 0.848, and RC = 0.644. The scale bar indicates the number of nucleotide substitutions. Isolates sequenced in this study are indicated in bold.

mycelia were observed at the margin. Orange conidial masses formed in the center (Fig. 2 a, b). Conidia were hyaline, aseptate, cylindrical to ellipsoid, 16–20 (–25) × 4–5 µm (Fig. 2 c). Appressoria were brown to blackish brown, ovoid, 7–12 × 5–6.5 µm (Fig. 2 d). MUCC2592: colonies reaching 70 mm in diameter, white to olivaceous at the upper surface, dark green to black at the center of the reverse, white to olivaceous at the marginal area of the reverse, and white aerial mycelia were observed. Orange conidial masses formed in the center (Fig. 2 e, f). Conidia were hyaline, aseptate, cylindrical to ellipsoid, 13.5–16 × 4–6 µm (Fig. 2 g). Appressoria were brown to dark brown and ovoid to irregular, 6.5–11 × 5–7.5 µm (Fig. 2 h). MUCC2636: colonies reaching 70 mm in diameter, white to olivaceous at the upper surface, partially dark green to black at the reverse, and white aerial mycelia were observed. Pale orange conidial masses formed in the center (Fig. 2 i, j). Conidia were hyaline, aseptate, cylindrical to ellipsoid, 15–18 × 4.5–6 µm (Fig. 2 k). Appressoria were dark brown, and ovoid to ellipsoid, 8.5–12.5 × 5–6.8 µm (Fig. 2 l).

The phenotypic characters of these isolates were similar to those of *C. asianum* (MUCC2555), *C. siamense* (MUCC2592), and *C. tropicale* (MUCC2636), all of which have been described in previous studies [Prihastuti et al., 2009; Rojas et al., 2010]. The sequence matrix consisted of 46 OTUs composed of five loci (ACT + CAL + CHS + GAPDH + ITS) belonging to the CGSC. The matrix was deposited as S28247 in TreeBASE. The final alignment contained a total of 2318 characters used for the phylogenetic analyses, including alignment gaps. From the analyzed characters, 1434 were constant, 329 were variable and parsimony-uninformative, and 555 were parsimony-informative. The maximum parsimony (MP) analyses generated eight equally most parsimonious trees. An MP tree was selected on the basis of the result of the Kishino-Hasegawa test [Kishino, Hasegawa, 1989] in PAUP* (Fig. 3; TL = 1085, CI = 0.689, RI = 0.848, and RC = 0.644), and the bootstrap support values (MP-BS) were indicated on the branches (Fig. 2; MP-BS > 60% shown). On the tree, 14 isolates from the newly obtained isolates formed a clade of *C. asianum* (MP-BS 100%). MUCC2592 was located within the clade of *C. siamense* (MP-BS 76.9%). MUCC2580 and MUCC2636 formed a clade with *C. tropicale* (MP-BS 69.9%). Taken together, the morphology and phylogeny analyses suggest that MUCC2592 is *C. siamense*, MUCC2580 and MUCC2636 are *C. tropicale*, and the other isolates are *C. asianum*.

These species are already known as invasive pathogens that can cause anthracnose of mango and can be responsible for plant quarantines in Japan. However, two mango-associated *Colletotrichum* species imported from the Philippines, *C. asianum* and *C. siamense*, were also recognized in this study. Anthracnose of mango caused by *C. asianum* has been reported by Alvarez et al. [2019]. However, to date, *C. siamense* has not been reported from the Philippines. To confirm its distribution in the Philippines, as well as the diversity of the other related species, more detailed field surveys are required.

LAMP assay. The site differences in the F3 and F2 primer regions are 2 of 18 sites and 3 of 20 sites among the species of the CGSC, respectively. By contrast, between species complexes, these differences are 8–9 of 14–19 sites and 9 and above of 20 sites, respectively. As the result of the LAMP assay, all isolates of the CGSC examined in this study were specifically amplified using the newly designed primer set discussed here (Tab. 1). Partial results of the in-tube visualization are shown in figure 4. These newly designed LAMP primers were able to detect *C. gloeosporioides*, *C. aotearoa*, *C. fructicola*, *C. horii*, *C. kahawae*, *C. musae*, *C. siamense*, *C. theobromicola* and *C. tropicale*. In contrast, the primer sets described here were not able to amplify isolates of species belonging to other species complexes. This finding suggests that this primer set can detect the different species of the CGSC. The plant protection station of Japan counts twelve *Colletotrichum* species as quarantine species, including two species of the CGSC, *C. gloeosporioides* and *C. tropicale.*

More broadly, some species from the CGSC are known to cause the Anthracnose of strawberry in the

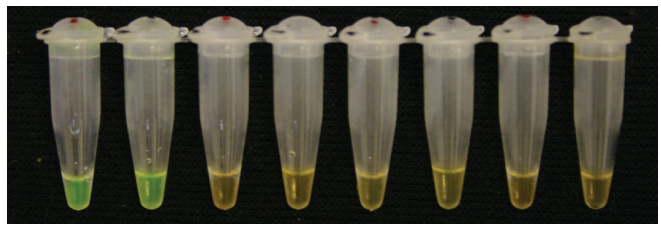

Figure 4. Detection of CGSC species by LAMP analysis. From the left: *C. asianum* (CGSC) on mango fruit, *C. gloeosporioides* (MAFF237219, CGSC), *C. zoysiae* (MAFF238574, CCSC), *C. chlorophyti* (MAFF305748, singleton), *C. circinans* (MAFF238028, CDSC), *C. destructivum* (MAFF239947, CVSC), *C. echinochloae* (MAFF511473, CMSC), and negative control (ddH2O).

world. The development of Anthracnose on crown and runners caused by species of the CGSC is an important disease for strawberry cultivation because strawberry is propagated by the crown. Strawberry growers therefore also require a method for rapid diagnosis of Anthracnose on the crown and runners using symptomatic or asymptomatic samples. We expect that the LAMP primers developed in this study will be helpful for the rapid diagnosis of fungal pathogens, both at the scene of infection and afterward.

DISCLOSURE

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of Japan.

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Mango xəstəlikləri ilə əlaqəli *Colletotrichum* növlərinin identifikasiyası və *C. gloeosporioides* növlər kompleksi üçün universal LAMP aşkarlama metodu

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Colletotrichum gloeosporioides növlər kompleksi müxtəlif bitki mənşəli məhsullara zərər verən antraknoz xəstəliklərini törədən bitki patogenlərini ehtiva edir. Bu tədqiqat işində biz növ kompleksindən növün taksonomik təsdiqlənmiş ştammlarına əsasən antraknoz xəstəliyi ilə əlaqədar olan göbələklərin sürətli təyinatı üçün kalmodulin gen kodlaşdıran regionun sekvensləri əsasında ilmə vasitəsi ilə izotermik gücləndirmə testi (LAMP) dizayn etmişik. Bu test əkin və satış arasında istənilən mərhələdə tətbiq edilə bilər. Həmçinin, biz *Colletotrichum* cinsinə aid növlərin ştammlarından istifadə etməklə praymer dəstinin spesifikliyini və təyin etmə aralığını yoxlamışıq. Bu test xüsusi olaraq, *C. gloeosporioides, C. aotearoa, C. fructicola, C. horii, C. kahawae, C. musae, C. siamense, C. theobromicola* və *C. tropicale* daxil olmaqla *C. gloeosporioides* növlər kompleksini spesifik təyin edə bilir.

Açar sözlər: *Antraknoz, diaqnoz, filogeniya, bitki xəstəliyi*

Идентификация видов *Colletotrichum*, связанных с болезнями манго и универсальный метод обнаружения LAMP для комплекса видов *C. gloeosporioides*

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Colletotrichum gloeosporioides видовой комплекс содержит патогены растений, связанные с антракнозом болезни, поражающие различные культуры. В этом исследовании для быстрого обнаружения наличия грибов связанных с заболевания антракноза, нами разработаны набор праймеров (LAMP) для анализа изотермической амплификации опосредственный петлей на основе последовательностей кодирующей области кальмодулин гена из таксономически авторизованных штаммов видов из этого комплекса. Этот тест можно использовать в любой момент между выращиванием и продажей. Более того, мы изучили специфичность и обнаруживаемый диапазон этого набора праймеров с использованием штаммов, выбранных из видов рода *Colletotrichum*. Этот тест позволяет специально обнаруживать представителей вида *C. gloeosporioides* комплекс, включая *C. gloeosporioides, C. aotearoa, C. fructicola, C. horii, C. kahawae, C. musae, C. siamense, C. theobromicola* и *C. tropicale.*

Ключевые слова: *антракноз, диагностика, филогения, болезнь растений*