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Novel species of *Colletotrichum* revealed by morphology and molecular analysis

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Abstract – *Colletotrichum* species are widely known as key anthracnose pathogens of several economic plants. In this study, *Colletotrichum* species associated with leaf anthracnose isolated from various plants in Thailand were subjected to morphological and molecular analyses. The ITS rDNA regions of these strains were sequenced and aligned with those of type strains in the genus in order to establish if they can be assigned to any known species. Strains that could not be identified were further sequenced for partial actin (ACT), β–tubulin (TUB2) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) genes and employed in a phylogenetic analysis to reveal their relationships with other closely related taxa. The multilocus sequence analysis, together with a critical examination of the phenotypic characters, revealed three new species. These are introduced as *C. brevisporum*, *C. tropicicola* and *C. thailandicum* and formally described, illustrated and compared with similar taxa.

Anthracnose / multilocus phylogeny / plant disease / systematics / pathogenicity

INTRODUCTION

Colletotrichum is an important, cosmopolitan, phytopathogenic genus causing anthracnose disease of a wide range of economically important crops, ornamentals, perennials, herbaceous plants and grasses (Sutton, 1992; Freeman

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et al., 1998; Than *et al*., 2008; Crouch *et al*., 2009; Damm *et al*., 2009; Hyde *et al*., 2009b; Prihastuti *et al*., 2009; Wikee *et al*., 2011). It is well known that a single host species can be infected by more than one *Colletotrichum* species and an individual *Colletotrichum* species may infect several different host species (Cai *et al*., 2009; Crouch & Beirn, 2009; Hyde *et al.,* 2009a; Phoulivong 2011; Yang *et al*., 2011). *Colletotrichum* species affect all above ground plant parts and cause yield and quality reduction. For example, *Colletotrichum* spp. cause extensive pre- and postharvest damage to chilli fruits, with yield losses up to 50% (Manandhar *et al*., 1995; Pakdeevaraporn *et al*., 2005). Roots may also be affected. For example, *C. acutatum* J.H. Simmonds has been isolated from necrotic roots of stunted, chlorotic strawberry plants and also from the rhizosphere of diseased plants (Freeman & Katan, 1997). *Colletotrichum graminicola* (Ces.) G.W. Wilson may also infect the roots of maize as a soil-borne pathogen but is symptomless on above-ground plant parts (Sukno *et al*., 2008).

Identification of *Colletotrichum* based on morphology is problematic due to the few morphological traits that can be used to separate species (Phoulivong *et al*., 2010; Cai *et al*., 2011; Ko Ko *et al*., 2011). Size and shape of conidia and appressoria, production of sclerotia, setae, acervuli, as well as cultural characters such as colony colour, growth rate and texture are the principal morphological characters used to separate species (Hyde *et al*., 2009a; Phoulivong *et al*., 2010). The presence of a teleomorph stage may be also important in identification but it is rarely formed in culture (Hyde *et al*., 2009b).

Colletotrichum classification is presently undergoing substantial revision and several species have been introduced following typification of species in some of the important species complexes, such as *C. gloeosporioides* (Penz.) Penz. & Sacc. (Cannon *et al.,* 2008), *C. falcatum* Went (Prihastuti *et al*., 2010), *C. musae* (Berk. & M.A. Curtis) Arx (Su *et al.,* 2011) and *C. coccodes* (Wallr.) S. Hughes (Liu *et al*., 2011). Molecular characteristics have become increasingly important in the identification of *Colletotrichum* species (Cai *et al*., 2009; Hyde *et al*., 2010; Phoulivong *et al*., 2010). The internal transcribed spacer (ITS) is the most widely sequenced region, but ITS sequences alone cannot be used for confident species delineation, especially for the *C. gloeosporioides* complex (Cai *et al*., 2009). Gazis *et al*. (2011) also demonstrated that ITS used for species delimitation in environmental surveys would underestimate diversity. Cai *et al*. (2009) estimated that >86% of so-called *C. gloeosporioides* in GenBank had considerable phylogenetic divergence from the type specimen (Cannon *et al*., 2008) based on ITS sequence analysis, and most likely represented other *Colletotrichum* species. Within the *C. graminicola* species complex, ITS similarity comparison also results in a high identification error (Crouch *et al*., 2009). Multi-locus phylogeny has been widely applied to decrease subjectivity in species identification and based on this several new species have been described, e.g., *C. asianum* Prihast., L. Cai & K.D. Hyde, *C. fructicola* Prihast., L. Cai & K.D. Hyde, *C. siamense* Prihast., L. Cai & K.D. Hyde (Prihastuti *et al*., 2009), *C. cliviae* Y.L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai, *C. hippeastri* Yan L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai *C. hymenocallidis* Yan L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai (Yang *et al*., 2009), *C. simmondsii* R.G. Shivas & Y.P. Tan (Shivas & Tan, 2009), and *C. jasminigenum* Wikee, K.D. Hyde, L. Cai & McKenzie (Wikee *et al*., 2011). In the present study, we introduce three new species from Thailand based on multi-locus phylogenetic analysis and morphology.

MATERIALS AND METHODS

Collection and fungal isolation. Infected leaf samples were collected from Chiang Mai, Chiang Rai and Nakhon Si Thammarat provinces in Thailand. The samples were incubated in moist chambers to promote sporulation. The fungi were isolated by picking conidia directly from conidial masses on lesions, suspending in sterilized water and streaking onto water agar (WA). After overnight incubation at room temperature, single germinated conidia were transferred to potato dextrose agar (PDA, Criterion®, Santa Maria, USA) plates (adapted from Choi *et al*., 1999 and Chomnunti *et al*., 2011). Cultures are deposited in Mae Fah Luang University Culture Collection, BCC (BIOTEC, Bangkok), LC (Dr Cai Lei's personal culture collection under MTA no MTA0001[Dr. Cai]) and CBS (Centraalbureau de Schimmelcultures - under MTA no MTA0004[CBS]).

Morphological studies. All isolates were cultured on PDA at 27°C under fluorescent light (12 hours light/12 hours dark). Colony diameter of three replicate cultures was measured daily for 7 days. Growth rate was calculated as the 7-day average of mean daily growth. After 7 days, colony size and colour of the conidial masses were recorded. Size and shape of 30 conidia were determined after 7 days incubation. A slide culture technique (Johnston & Jones, 1997) was used for the production of appressoria, the shape and size of which were studied.

DNA extraction, PCR and sequencing. Mycelium was obtained by scraping the surface of 5-day-old cultures on PDA. Genomic DNA was extracted from fresh mycelium using a modified protocol of Lacap *et al*. (2003). The primers used for PCR amplifications were: complete rDNA-ITS region (ITS): ITS5 / ITS4 (White *et al.,* 1990); partial glycerol-3-phosphate-dehydrogenase (GPADH): GDF1 / GDR1 (Templeton *et al.,* 1992); partial actin (ACT): ACT512F / ACT783R (Carbone & Kohn, 1999); partial bêta-tubulin (TUB2), T1 / Bt2b (O'Donnell & Cigelnik, 1997; Glass & Donaldson, 1995).

The cycling parameters were initiated at 94°C for 5 minutes followed by 35 cycles of denaturation (at 94°C for 30 seconds), annealing (30 seconds at 52°C for ITS and TUB2, and 56°C for ACT, GAPDH), elongation (72°C for 90 seconds), and a final extension (72°C for 10 minutes). PCR products were purified and sequenced by the SinoGenoMax, Beijing, China.

Sequence alignment and phylogenetic analyses. Sequences from forward and reverse primers were aligned to obtain a consensus sequence. Combined ITS, GAPDH, ACT and TUB2 sequence dataset of the three new species, along with reference sequences obtained from GenBank (Table 1), were aligned by Clustal X (Thompson *et al.,* 1997). Alignments were optimized manually in BioEdit for maximum alignment and to minimize gaps (Hall, 1999).

Phylogenetic analyses were performed by using PAUP* 4.0b10 (Swofford, 2002). Ambiguously aligned regions were excluded from all analyses. Unweighted parsimony (UP) analysis was performed. Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all multiple parsimonious trees were saved. Shimodaira-Hasegawa test (SH test) (Shimodaira & Hasegawa, 1999) was performed in order to determine whether trees differed significantly. Descriptive tree statistics such as tree length [TL], consistency index [CI], retention index [RI], rescaled consistency index [RC] and homoplasy index [HI] were calculated for trees generated under different optimality criteria. Clade stability was assessed in a bootstrap analysis with 1,000

replicates, each with 10 replicates of random stepwise addition of taxa. Trees were figured in Treeview (Page, 1996).

Model of evolution was estimated by using Mrmodeltest 2.2 for the combined dataset of ACT, GAPDH, ITS, TUB2 (Nylander *et al*., 2004). Posterior probabilities (PP) (Rannala & Yang, 1996; Zhaxybayeva & Gogarten, 2002) were determined by Markov Chain Monte Carlo sampling (MCMC) in MrBayes 3.0b4 (Huelsenbeck & Ronquist, 2001), using the estimated model of evolution. Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100th generation (resulting 10,000 total trees). The first 2,000 trees which represented the burn-in phase of the analyses, were discarded and the remaining 8,000 trees used for calculating posterior probabilities (PP) in the majority rule consensus tree.

RESULTS

Phylogenetic analysis: Blast searches were made using ITS sequences of the six strains, and no identical sequences could be identified in GenBank. The combined dataset of ACT, TUB2, GAPDH and ITS comprised 1723 characters including alignment gaps. Ambiguously aligned regions were excluded from all analyses. The SH test showed that the four trees generated from parsimonious analysis were not significantly different, one of the most parsimonious trees (TL = 685, CI = 0.604, RI = 0.841, RC = 0.508, HI = 0.396) is shown in Fig. 1. The phylogram from the combined dataset shows that all the three new taxa appear as distinct lineages and cluster with *C. cliviae*, *C. dracaenophilum* D.F. Farr & M.E. Palm and *C. yunnanense* Xiao Ying Liu & W.P. Wu, respectively. The tree generated from Baysian analysis shows similar topology as four trees from parsimonious analysis and is, therefore, not shown. The two *C. brevisporum* sp. nov. strains form a sister clade to *C. cliviae* supported by high bootstrap support (100%) and Bayesian posterior probabilities (100%). *C. tropicicola* sp. nov. forms a sister clade to *C. brevisporum* and *C. cliviae* with 57% boostrap support. *C. thailandicum* sp. nov. also appeared as a distinct lineage basal to *C. yunnanense*, *C. dracaenophilum*, *C. cliviae*, *C. brevisporum* and *C. tropicicola*. The three new species group in a monophyletic clade that also includes *C. yunnanense*, *C. dracaenophilum* and *C. cliviae,* with moderate support.

Taxonomy: Six new strains were isolated from different hosts. Differences in colony colour, conidia size, and appressoria shape and size among these *Colletotrichum* isolates allowed them to be separated into three morphological groups, corresponding to three new species, which are described below. Sequences generated from this study were deposited in GenBank (Table 1).

Colletotrichum brevisporum S. Phoulivong, P. Noireung, L. Cai & K.D. Hyde, **sp. nov.** **Fig. 2**

MycoBank: MB564156

Etymology: *brevisporum* refers to the short conidia.

Conidiogenous cells enteroblastic, hyaline to pale brown, cylindrical to clavate. **Conidia** 12-17 × 5-6 µm ($\bar{x}$ = 14.9 ± 3.3 × 5.9 ± 0.4, n = 30), one-celled, hyaline, cylindrical with round ends, smooth-walled, guttulate. **Spore germination** on PDA mostly near apex of conidia. **Appressoria** in slide culture 10-13 × 8-11 µm

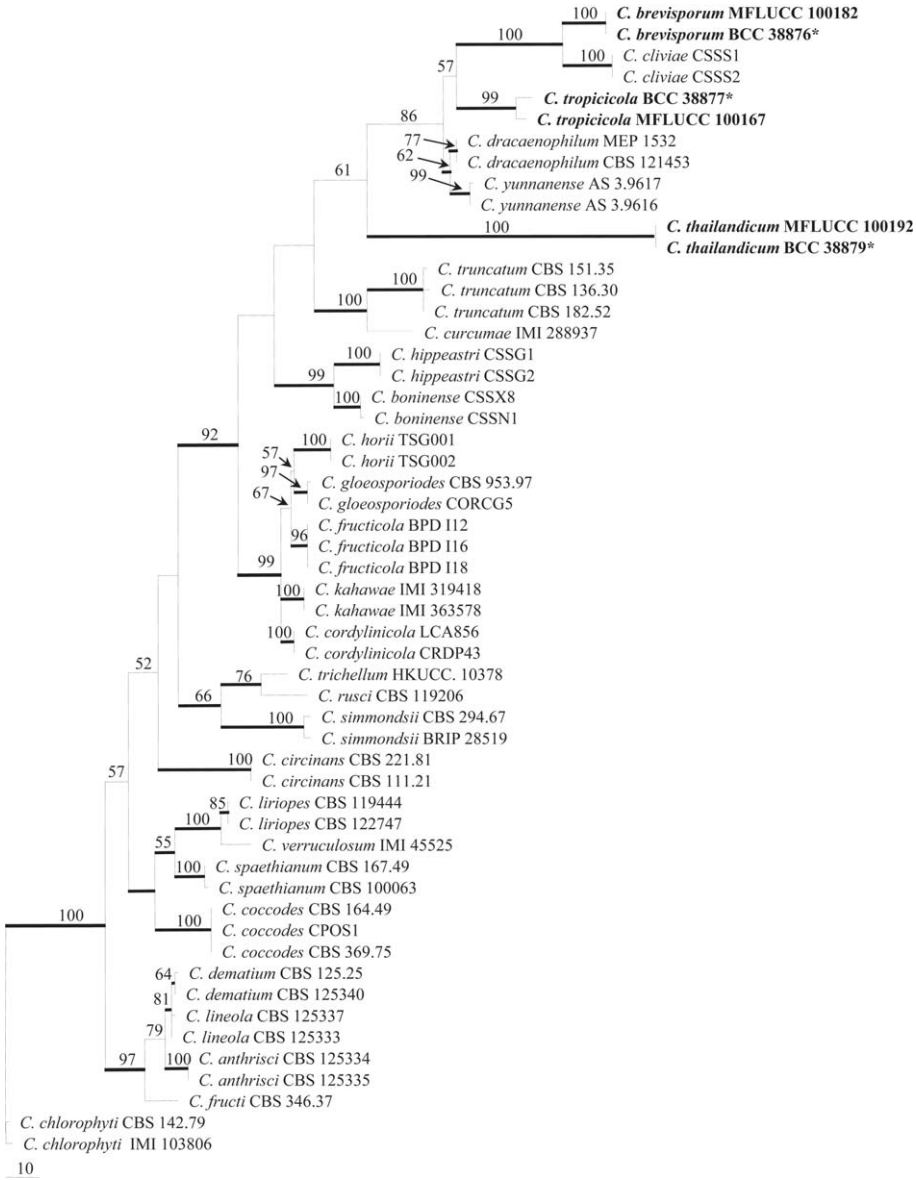

Fig. 1. One of the four most parsimonious trees generated from maximum parsimony analysis based on combined ACT, GAPDH, ITS and TUB2 sequences, showing the phylogenetic relationships of three new species, *C. brevisporum*, *C. tropicicola* and *C. thailandicum*. Values above the branches are parsimony bootstrap (> 50%). Thickened branches represent significant Bayesian posterior probability (≥ 95%). The tree is rooted with *C. chlorophyti*.

($\bar{x}$ = 11.3 ± 1.5 × 9.8 ± 4.4, n = 10), mostly formed from conidia, brown to dark brown, variable in shape including ovoid, clavate or slightly irregular, often becoming complex with age. **Teleomorph** not produced in culture after 2 months.

Table 1. Strains of *Colletotrichum* studied in this paper with details of host/substrate and location, and GenBank accessions of the sequences (New sequences are in bold)

Species	*Accession number*	*Host/Substrate*	*Country*	*GenBank accessions*			
				ITS	*ACT*	*TUB2*	*GAPDH*
C. anthrisci	CBS 125334*	*Anthriscus sylvestris*, dead stem	Netherlands	GU227845	GU227943	GU228139	GU228237
	CBS125335	*Anthriscus sylvestris*, dead stem	Netherlands	GU227846	GU227944	GU228140	GU228238
C. boninense	CSSN1	*Crinum asiaticum*, leaf	China	GQ485597	GQ856774	GQ849437	GQ856743
	CSSX8	*Crinum asiaticum*, leaf	China	GQ485596	GQ856771	GQ849433	GQ856742
C. brevisporum	**BCC 38876***	***Neoregalia* sp., leaf**	**Thailand**	**JN050238**	**JN050216**	**JN050244**	**JN050227**
	MFLUCC100182	***Pandanus pygmaeus*, leaf**	**Thailand**	**JN050239**	**JN050217**	**JN050245**	**JN050228**
C. chlorophyti	IMI 103806*	*Chlorophytum* sp.	India	GU227894	GU227992	GU228188	GU228286
	CBS 142.79	Stylosanthes hamata	Australia	GU227895	GU227993	GU228189	GU228287
C. circinans	CBS 221.81*	*Allium cepa*	Serbia	GU227855	GU227953	GU228149	GU228247
	CBS 111.21	*Allium cepa*	USA	GU227854	GU227952	GU228148	GU228246
C. cliviae	CSSS1	*Clivia miniata*, leaf	China	GU109479	GU085861	GU085869	GU085867
	CSSS2	*Clivia miniata*, leaf	China	GU109480	GU085862	GU085870	GU085868
C. coccodes	CBS 164.49	*Solanum tuberosum*	Netherlands	HM171678	HM171666	-	HM171672
	CBS 369.75*	*Solanum tuberosum*	Netherlands	HM171679	HM171667	-	HM171673
	CPOS1	*Solanum tuberosum*	China	GQ485588	GQ856787	GQ849444	GQ856744
C. cordylinicola	BCC 38872*	*Cordyline fructicosa*, leaf	Thailand	HM470246	HM470234	HM470249	HM470240
	MFLU 100132	*Cordyline fructicosa*, leaf	Thailand	HM470247	HM470235	HM470250	HM470241
C. curcumae	IMI 288937*	*Curcuma longa*	India	GU227991	GU227893	GU228187	GU228285
C. dematium	CBS 125.25*	*Eryngium campestre*, dead leaf	France	GU227819	GU227917	GU228113	GU228211
	CBS 125340	*Apiaceae*, dead stem	Czech Rep.	GU227820	GU227918	GU228114	GU228212
C. dracaenophilum	BPI 871498 *	*Dracaena* sp.	China	DQ286209	-	-	-
	CBS 121453	*Dracaena sanderiana*	Bulgaria	EU003533	-	-	-
C. fructi	CBS 346.37*	*Malus sylvestris*	USA	GU227844	GU227942	GU228138	GU228236
C. fruticola	BPDI12	*Coffea arabica*	Thailand	FJ972611	FJ907425	FJ907440	FJ972577
	BPDI16*	*Coffea arabica*	Thailand	FJ972603	FJ907426	FJ907441	FJ972578
	BPDI18	*Coffea arabica*	Thailand	FJ972602	FJ907427	FJ907442	FJ972579
C. gloeosporioides	CBS 953.97*	*Citrus sinensis*	Italy	GQ485605	GQ856782	GQ849434	GQ856762
	CORCG5			HM034809	HM034801	HM034811	HM034807
C. hippeastri	CSSG1*	*Hippeastrum vittatum*, leaf	China	GQ485599	GQ856788	GQ849446	GQ856764
	CSSG2	*Hippeastrum vittatum*, leaf	China	GQ485598	GQ856789	GQ849445	GQ856765
C. horii	TSG001	*Diospyros kaki*	China	AY787483	GU133374	GU133375	GU133378
	TSG002	*Diospyros kaki*	China	AY791890	GU133379	GU133380	GU133383
C. kahawae	IMI 319418*	*Coffea arabica*	Kenya	FJ972608	FJ907432	FJ907446	FJ972583
	IMI 363578	*Coffea arabica*	Kenya	FJ972607	FJ907433	FJ907447	FJ972584
C. lineola	CBS 125333	*Heracleum* sp.	Netherlands	GU227930	GU228126	GU227832	GU228224
	CBS 125337*	*Apiaceae*, dead stem	Czech Rep.	GU227829	GU227927	GU228123	GU228221
C. liriopes	CBS 119444*	*Liriope muscari*	Mexico	GU227804	GU227902	GU228294	GU228196
	CBS 122747	*Liriope muscari*	Mexico	GU227805	GU227903	GU228099	GU228197
C. rusci	CBS 119206*	*Ruscus* sp.	Italy	GU227818	GU227916	GU228112	GU228210

Table 1. Strains of *Colletotrichum* studied in this paper with details of host/substrate and location, and GenBank accessions of the sequences (New sequences are in bold) *(continued)*

Species	*Accession number*	*Host/Substrate*	*Country*	*GenBank accessions*			
				ITS	*ACT*	*TUB2*	*GAPDH*
C. simmondsii	BRIP28519*	*Carica papaya*	Australia	GQ485606	GQ849430	GQ856784	GQ856763
C. spaethianum	CBS 167.49*	*Hosta sieboldiana*, stem	Germany	GU227807	GU228101	GU227905	GU228199
	CBS 100063	*Lilium* sp., infected leaves	South Korea	GU227808	GU228102	GU227906	GU228200
C. thailandicum	**BCC 38879***	***Hibiscus rosa-sinensis*, leaf**	**Thailand**	**JN050242**	**JN050220**	**JN050248**	**JN050231**
	MFLUCC100192	***Alocasia* sp., leaf**	**Thailand**	**JN050243**	**JN050221**	**JN050249**	**JN050232**
C. trichellum	HKUCC 10378	Unknown	Unknown	GQ485589	GQ856786	GQ849447	GQ856749
C. tropicicola	**BCC 38877***	***Citrus maxima*, leaf**	**Thailand**	**JN050240**	**JN050218**	**JN050246**	**JN050229**
	MFLUCC100167	***Paphiopedilum bellatolum*, leaf**	**Thailand**	**JN050241**	**JN050219**	**JN050247**	**JN050230**
C. truncatum	CBS 151.35*	*Phaseolus lunatus*	USA	GU227862	GU227960	GU228156	GU228254
	CBS 182.52	*Glycine max*	USA	GU227866	GU228160	GU227964	GU228258
	CBS 136.30	*Crotalaria juncea*	Trinidad and Tobago	GU227876	GU227974	GU228170	GU228268
C. verruculosum	IMI 45525*	*Crotalaria juncea*	Zimbabwe	GU227806	GU227904	GU228100	GU228198
C. yunnanense	AS3.9616	*Buxus* sp.	China	EF369491	-	-	-
	AS3.9617*	*Buxus* sp.	China	EF369490	-	-	-

Note: ACT: actin; TUB-2: partial ß-tubulin; GAPDH: glyceraldehydes-3-phosphate dehydrogenase; ITS: complete rDNA-ITS region.
*: ex-type cultures

Habitat: leaf disease of *Neoregelia* sp. and *Pandanus pygmaeus* Thouars.
Known distribution: Thailand.
Holotype: THAILAND, Nakhon Si Thammarat Province, Thasala District, Walailak University, on *Neoregelia* sp., 17 January 2008, Sitthisack Phoulivong (MFLU 110011); culture ex-type L57-CgPa1NK = LC0600 = MFLUCC 110115 = BCC 38876.
Additional specimen examined: THAILAND, Chiang Mai Province, San Sai District, San Sai Noi Village, on *Pandanus pygmaeus*, 9 July 2009, Parinn Noireung, (MFLU 110012); living culture BTL23 = LC0870 = MFLUCC 100182.

Colletotrichum tropicicola S. Phoulivong, P. Noireung, L. Cai & K.D. Hyde, **sp. nov.** **Fig. 3**.

MycoBank: MB564159
Etymology: *tropicicola*, refers to the tropical region where the type specimen was collected.
Colonies on PDA attaining 75 mm diam. in 7 days at 27°C, growth rate 6.7-7.2 mm/day ($\bar{x}$ = 6.9 ± 0.2, n = 5), white, reverse white to grey. **Aerial mycelium** sparse, in small tufts, with orange conidial masses. Sclerotia absent. Acervuli absent. Setae absent. **Conidiogenous cells** enteroblastic, hyaline to pale brown, cylindrical to clavate. **Conidia** 15-19 × 6-7 µm ($\bar{x}$ = 16.6 ± 2.6 × 6.5 ± 0.2, n = 30), one-celled, hyaline, cylindrical with round ends, smooth-walled. **Spore germination** on PDA mostly near the apex of the conidia. **Appressoria** in slide culture 13-24 × 7-8 µm ($\bar{x}$ = 18.5 ± 9.2 × 7.1 ± 1.07, n = 10), mostly formed from conidia, brown to dark brown, variable in shape including ovoid, clavate or

slightly irregular, often becoming complex with age. **Teleomorph** not produced in culture after 3 months.

Habitat: on leaf of *Citrus maxima* Merr. and *Paphiopedilum bellatulum* (Reichb. f.) Stein.

Known distribution: Thailand.

Holotype: THAILAND, Chiang Mai Province, Mae Taeng District, Phadeng village, on *Citrus maxima*, 14 March 2009, Sitthisack Phoulivong (MFLU 110013); culture ex-type L58 = CaPe3CM = LC0598 = MFLUCC 110114 = BCC 38877.

Additional specimen examined: THAILAND, on *Paphiopedilum bellatulum*, 16 March 2009, Parinn Noireung (MFLU 110014); living culture BTL07 = LC0957 = MFLUCC 100167.

Colletotrichum thailandicum S. Phoulivong, P. Noireung, L. Cai & K.D. Hyde **sp. nov.** Fig.4.

MycoBank: MB564160

Etymology: *thailandicum*, refers to the country where the type specimen was collected.

Colonies on PDA attaining 75 mm diam. in 7 days at 27°C, growth rate 3.8-8.8 mm/day ($\bar{x}$ = 6.0 ± 1.5, n = 5), white, reverse green to dark green. **Aerial mycelium** sparse, in small tufts, with grey conidial masses. **Sclerotia** absent. **Acervuli** present in culture. **Setae** on PDA 65-185 µm in length ($\bar{x}$ = 95 ± 5.0, n = 10). **Conidiogenous cells** enteroblastic, hyaline to pale brown, cylindrical to clavate. **Conidia** 27-30 × 9-10 µm ($\bar{x}$ = 28.6 ± 0.16 × 9.9 ± 0.46, n = 30), one-celled, hyaline, cylindrical with round ends, smooth-walled, without guttules. **Spore germination** on PDA mostly near apex of conidia. **Appressoria** in slide culture 15-30 × 7-14 µm ($\bar{x}$ = 21.8 ± 5.3 × 10.5 ± 3.0, n = 10), mostly formed from conidia, brown to dark brown, variable in shape including ovoid, clavate or slightly irregular, often becoming complex with age. **Teleomorph** not produced in culture after 3 months.

Habitat: on leaf of *Alocasia* sp. and *Hibiscus rosa-sinensis* L.

Known distribution: Thailand.

Holotype: THAILAND, Chiang Rai Province, Thasud Village, Mae Fah Luang University, on *Hibiscus rosa-sinensis*, 14 May 2009, Sitthisack Phoulivong (MFLU 110015); culture ex-type L62 = HR01MFU = LC0596 = MFUCC 110113 = BCC 38879.

Additional specimens examined: THAILAND, Chiang Mai Province, Sarapee District, on *Alocasia* sp., 20 February 2009, Parinn Noireung (MFLU 110016); living culture CMSP34 = LC0958 = MFLUCC 100192.

DISCUSSION

Species complexes in *Colletotrichum* include *C. acutatum* J.H. Simmonds, *C. boninense* Moriwaki, Toy. Sato & Tsukib., *C. gloeosporioides* and *C. dematium* (Pers.) Grove, and distinct individual species such as *C. coccodes* (Wallr.) S. Hughes, *C. circinans* (Berk.) Voglino, *C. trichellum* (Fr.) Duke, *C. truncatum* and *C. curcumae* (Syd.) E.J. Butler & Bisby (Damm *et al*., 2009; Shivas & Tan 2009; Phoulivong *et al*., 2010). The new species described in this paper can be differentiated from these species complexes and from the distinct individual species, by morphological characters, such as conidial size and shape (Table 2), and by their placement in the phylogenetic tree (Fig. 1).

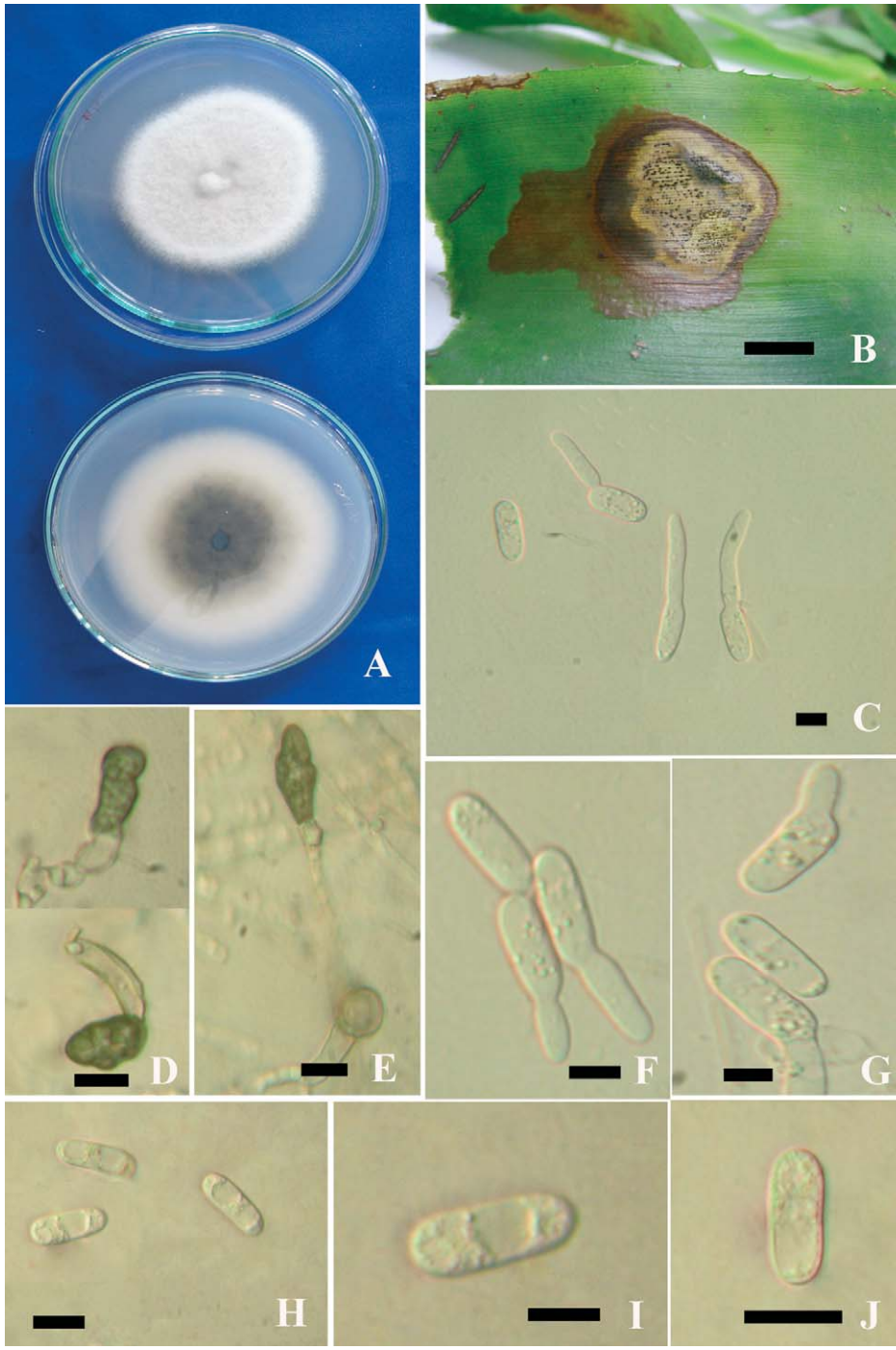

Fig. 2. *Colletotrichum brevisporum* from holotype. **A.** Upper and reverse view of cultures on PDA after 7 days. **B.** Symptom on *Neoregalia* sp. leaf. **D-E.** Appressoria. **C, F-G.** Germinating conidia. **H-I.** Conidia; (Bars: A-B = 1 cm, C-G = 5 µm, H-J = 10).

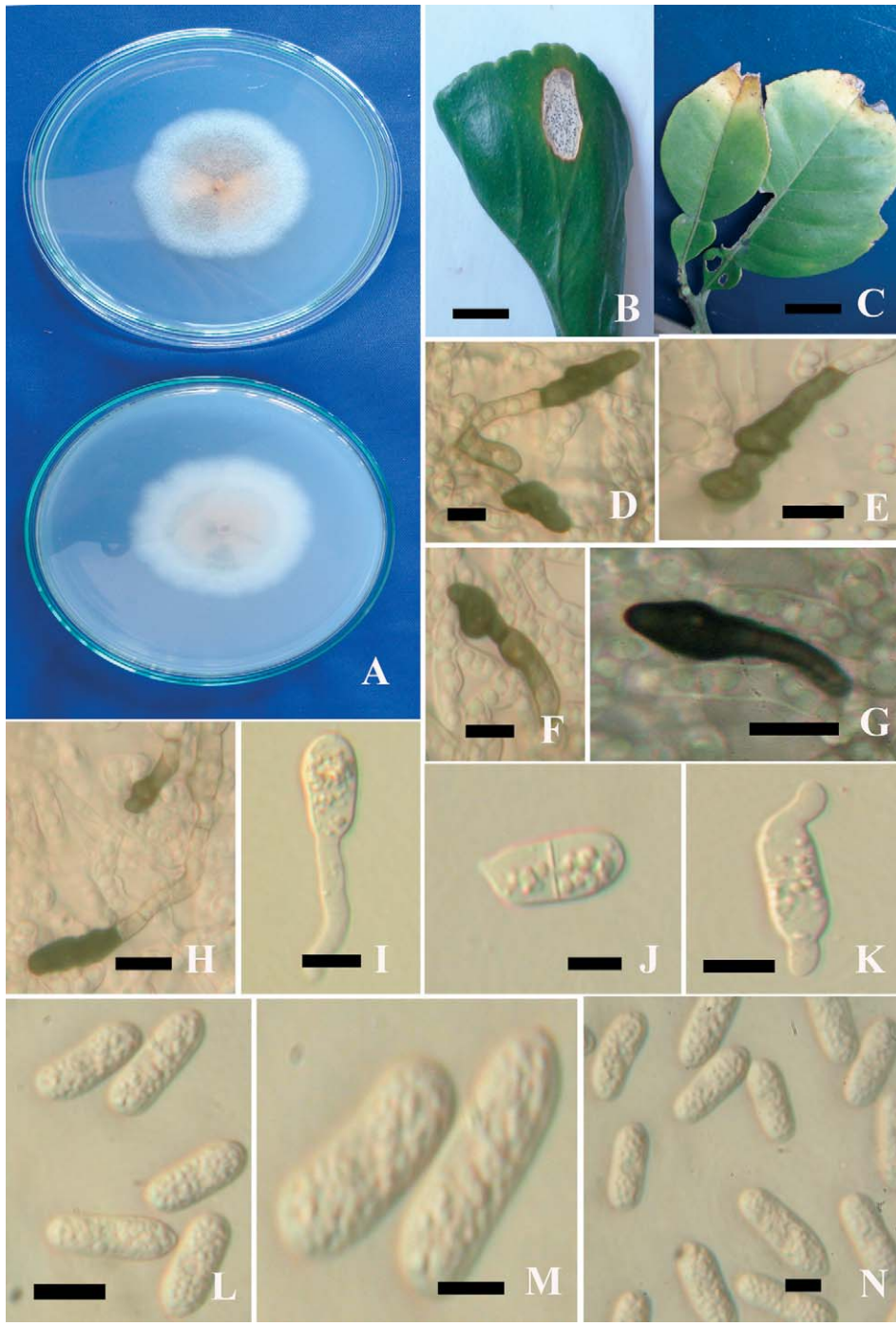

Fig. 3. *Colletotrichum tropicicola* from holotype. **A.** Upper and reverse view of cultures on PDA after 7 days. **B-C.** Symptoms on *Citrus maxima* leaves. **D-H.** Appressoria. **L-N.** Conidia. **I-K.** Germinating conidia; (Bars: B-C = 1 cm, D-H ,K-L = 10 µm, I-J, M-N = 5 µm).

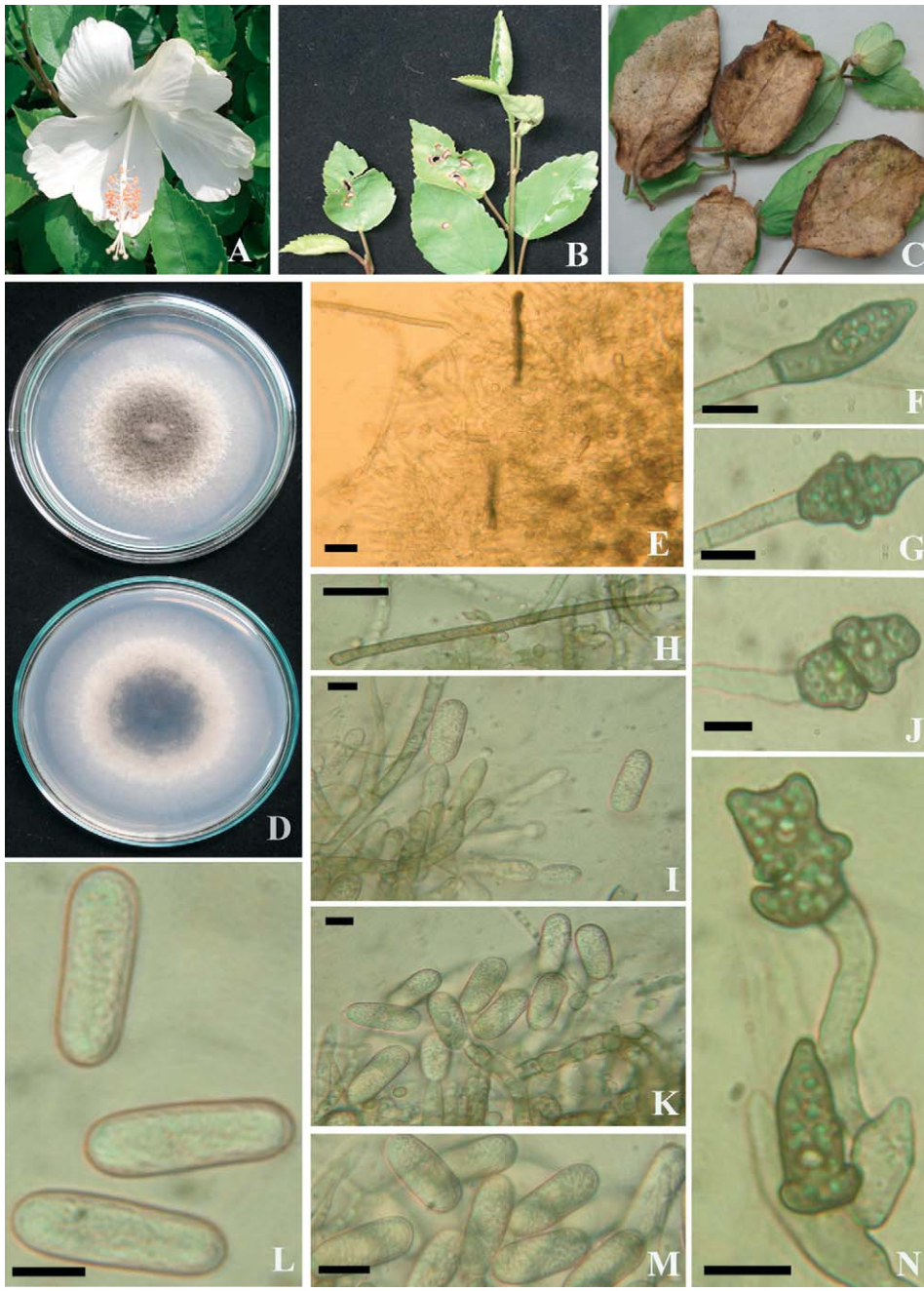

Fig. 4. *Colletotrichum thailandicum* from holotype. **A.** Flower of the host plant. **B-C.** Symptoms on *Hibiscus rosa-sinensis*. **D.** Upper and reverse view of cultures on PDA after 7 days. **E.** Setae. **F-G, J, N.** Appressoria. **H-I,K-M.** Conidia. (Bars: A-C = 1 cm, E-N = 10 µm).

Table 2. Morphological characters of new species compared with phylogenetically related species

Taxa	*Colonies*	*Conidia shape and size (µm)*	*Appressoria shape and size (µm)*	*Growth rate (mm per day)*	*Reference*
C. brevisporum	Aerial mycelium in small tufts, white, sparse, with conidial masses, reverse dark green	Cylindrical with round ends, smooth-walled, hyaline, guttulate, 12-17 × 5-6 µm	Brown to dark brown, variable in shape including ovoid, clavate or slightly irregular, often becoming complex with age, 10-13 × 8-11µm	7.5-9.8, $\overline{x}$ = 8.5	This study
C. cliviae	White to grey, white at margin, reverse dark brown to greenish black	Cylindrical, straight or slightly curved, obtuse at the ends, 19.5-24.5 × 4.5-7µm	Dark brown, irregular, crenate or lobed, 10.5-14.5 × 6-11, $\overline{x}$ = 11.7 × 8.6 µm	15.2-16 , $\overline{x}$ = 15.6	Yang *et al*. 2009
C. dracaenophilum	Pale pink, reverse speckled from profuse sporulation, sparse aerial mycelium, rosy buff to saffron in centre, rosy buff to saffron in reverse	Broadly clavate to cylindrical, frequently slightly curved, hyaline, guttulate, 22-34 × 6.5-9.5 µm	(No information)	(No information)	Farr *et al*. 2006
C. thailandicum	Aerial mycelium in small tufts, white, sparse, with grey conidial masses, reverse green to dark	Cylindrical with round ends, smooth-walled, hyaline, 27-30 × 9-10 µm	Brown to dark brown, variable in shape including ovoid, clavate or slightly irregular, often becoming complex with age, 15-30 × 7-14 µm	3.8-8.8 ($\overline{x}$ = 6.0)	This study
C. tropicicola	Aerial mycelium in small tufts, white, sparse, with white orange conidial masses, reverse slightly white to grey	Cylindrical with round ends, smooth-walled, hyaline, guttulate, 15-19 × 6-7µm	Brown to dark brown, variable in shape including ovoid, clavate or slightly irregular, often becoming complex with age, 13-24 × 7-8 µm	6.7-7.2, $\overline{x}$ = 6.9	This study
C. yunnanense	Edge irregular, white to cream, felted with aerial mycelium, revers straw yellow to hazel, sclerotia present	Cylindrical, slightly clavate or bacilliform, smooth, rounded at each end with abscission scar, hyaline, guttulate, 16-21 × 5-6 µm	Brown to dark, irregularly lobed, 7-12 × 6-8 µm	Slow growing	Liu *et al*. 2007

The new species are most similar in conidial shape to *C. cliviae*, *C. dracaenophilum* and *C. yunnanense* but differ in conidial size. *C. dracaenophilum* has long conidia (mean length > 28 μm) and overlap with those of *C. thailandicum* (Farr *et al.* 2006). Conidia of *C. thailandicum* are, however, wider (9-10 μm vs 6.5-9.5 μm). Conidial shape is cylindrical with round ends in *C. thailandicum*, as compared to broadly clavate to cylindrical, frequently slightly curved in *C. dracaenophilum*. In the phylogenetic tree, *C. thailandicum* forms a separate clade with long branch length, indicating certain distance from *C. dracaenophilum* and *C. yunnanense* (Fig. 1). *Colletotrichum brevisporum* and *C. tropicicola* have short conidia, but their appressoria are significantly different in size and shape (10-13 × 8-11 μm in *C. brevisporum* vs. 13-24 × 7-8 μm in *C. tropicicola*). In the phylogenetic tree *C. brevisporum*, *C. tropicicola* and *C. clivae* cluster in a moderately supported clade and each represented by well supported lineages (Fig. 1). The conidia of *C. brevisporum* (12-17 μm) and *C. tropicicola* (15-19 μm) are shorter than those of *C. cliviae* (19.5-24.5 μm); the latter also grows faster in culture (Yang *et al*., 2009). *Colletotrichum brevisporum* and *C. cliviae* are sister groups with high bootstrap support. The conidia of *C. thailandicum* are larger than *C. yunnanense*, while those of C. yunnanense are similar to *C. brevisporum* and *C. tropicicola*. The appressoria of *C. yunnanense* are regularly lobed which distinguishes it from these species (Liu *et al*., 2007).

A synopsis of the three new species and similar taxa is provided in Table 2. We have not epitypified older names of *Colletotrichum* that are from the same host as our new species because the new species colonize more than one host. The older names were based on host association and thus it would be difficult to decide on an earlier name for these new species. More importantly, living strains do not exist for the older names and characters in the original protologues of species on the hosts colonized differ as detailed below.

Colletotrichum brevisporum is recorded from *Neoregelia* sp. and *Pandanus pygmaeus* which belong to related plant families. We could not find any species of *Colletotrichum* that are described from these hosts, although *C. gloeosporioides* has been reported from *Pandanus utilis* Bory (Farr & Rossman, 2010). *C. pandani* was described from *Pandani veitchii*, but it has narrower conidia than *C. brevisporum* (3.5-4.5 μm vs. 5-6 μm) (Sydow & Sydow, 1913).

C. tropicicola was found on *Citrus maxima* Merr. and *Paphiopedilum bellatolum* Thouars (lady's slipper orchid). It is unlikely to be conspecific with *C. orchidearum* Allesch. since the latter has narrower conidia (4-6 μm vs. 6-7 μm in *C. tropicicola*) (Saccardo & Saccardo, 1906). There are also numerous varieties of *C. orchidearum* but none come from the same host of *C. tropicicola*. Therefore, we prefer to introduce a new species as it is also recorded from *Citrus maxima* and differs from *C. orchidearum* and *C. cliviae* in producing much smaller conidia (Saccardo & Saccardo, 1906; Yang *et al*., 2009).

Several species of *Colletotrichum* have been recorded from *Hibiscus* such as *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore (≡ *C. capsici* (Syd. & P. Syd.) E.J. Butler & Bisby), *C. gloeosporioides*, *C. hibisci* Pollacci, *C hibisci-cannabini* Sawada and *C. hibiscicola* Rangel (Farr & Rossman, 2010). Our phylogenetic analysis has shown that *C. thailandicum* is phylogenetically distinct from *C. truncatum* and *C. gloeosporioides* (Fig. 1). *C. thailandicum* from *Hibiscus rosa-sinensis* produces larger conidia (27-30 × 9-10 μm) than either in *C. hibisci* (11-25 × 4.2 μm) or *C. hibiscicola* (12-20 × 4.6 μm) (Saccardo & Sydow, 1899; Saccardo *et al*., 1931). *Colletotrichum hibisci-cannabini* reported from *Hisbiscus cannabinus* can easily be differentiated from *C. thailandicum* by its smaller conidia (10-24 × 4-7 μm) (Sawada, 1959).

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