Colletotrichum anthracnose of *Amaryllidaceae*

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Yang, Y.L., Liu, Z.Y., Cai, L., Hyde, K.D., Yu, Z.N. and Mckenzie, E.H.C. (2009). *Colletotrichum* anthracnose of *Amaryllidaceae.* Fungal Diversity 39: 123-146.

Twenty strains representing eight species of *Colletotrichum* were isolated from lesions of various species of amaryllids (monocotyledons, *Amaryllidaceae*) in Yunnan, Guangxi and Guizhou Provinces in China and Chiang Rai Province, Thailand. They are characterized through morphological studies and from phylogenetic analyses based on actin, β-tubulin (tub 2), calmodulin (CAL), chitin synthase A (CHS I), glyceraldehyde-3-phosphate dehydrogenase (GPDH) and the rDNA internal transcribed spacer (ITS) gene sequence data. *Colletotrichum cliviae*, *C. hippeastri* and *C. hymenocallidis* are new species described and illustrated in this paper based on morphological characters and multi-gene sequence data. Pathogenicity testing indicated that the three new taxa from *Amaryllidaceae* are not host-specific.

Key words: anthracnose, multigene phylogeny, new species, *Amaryllidaceae*, pathogenicity

Article Information
Received 30 November 2009
Accepted 3 December 2009
Published online 9 December 2009
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Introduction

Colletotrichum is among the most important genera of plant pathogenic fungi worldwide and species cause disease symptoms commonly known as anthracnose on a wide range of important crops, fruits and ornamental plants (Bailey *et al*., 1992). Some of the better known species include *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara, *C. gloeosporioides* (Penz.) Penz. & Sacc., *C. acutatum* J.H. Simmonds ex J.H. Simmonds, *C. falcatum* Went, *C. nupharicola* D.A. Johnson, Carris & J.D. Rogers and *C. nymphaeae* (Pass.) Aa. Some plant pathogenic species have high significance to quarantine because they could be introduced into countries where the disease does not occur (Farr *et al*., 2006).

The taxonomy and phylogeny of the genus *Colletotrichum* is confused (Hyde *et al*., 2009; Cai *et al*., 2009). Historically, if anthracnose was found on a host substratum for which no records of that fungus/host relationship were known, then the species was interpreted as new, and formally described (Cannon *et al*., 2000). This practice was later rejected (von Arx 1957; Sutton 1980; Baxter *et al.*, 1983), but new species are still occasionally described in *Colletotrichum* (Sivanesan *et al.,* 1993; Nakanura *et al*., 2006; Liu *et al*., 2007). There are now more than 688 epithets listed in *Index fungorum* (www. index fungorum.org/names /names.asp). Based on a morphological study, von Arx (1957) included more than 600 synonyms under *C. gloeosporioides* and 84 synonyms under *C. dematium*. Sutton (1992) listed 39 accepted species in *Colletotrichum* each with a short description.

In recent years, molecular tools have been employed to infer the evolutionary relationships of *Colletotrichum* species. Based on nu-rDNA ITS sequence data and morphological characteristics, some species have been segregated from the *Colletotrichum gloeosporioides* complex, such as *C. boninense* Moriwaki, Toy. Sato & Tsukib. which has been

shown to cause disease of eight host plants in Japan (Moriwaki *et al*., 2003). Although ITS sequence data may help in *Colletotrichum* species identification, it cannot alone be used to adequately address species delimitation for closely related species (Crouch *et al*., 2009a). Researchers have recently tried to examine multiple genes sequence data to distinguish species in *Colletotrichum* (Du *et al*., 2005; Johnston *et al*., 1997; Crouch *et al*., 2006, 2009b,c; Farr *et al*., 2006; Shenoy *et al*., 2007a,b; Than *et al*., 2008a,b; Moriwaki and Tsukiboshi, 2009). Using multilocus phylogenetics, Crouch *et al*. (2006; 2009b) separated *C. cereale* and *C. eleusines* from *C. graminicola* and introduced six new *Colletotrichum* species from warm-season grass hosts. Multigene sequence analyses are helpful in clarifying the confusion in the *Colletotrichum* systematics.

Amaryllids (members of the *Amaryllidaceae*) are bulbous herbs with distichous or rarely rosulate leaves and a lateral, solid, naked scape which is terminated by an umbel-like cluster of showy flowers (Meerow *et al*., 1998). Several amaryllids have economic value as medicine, while species and/or hybrids of *Nerine*, *Amaryllis* and *Crinum* are cultivated in many countries for their elegant flowers (Dahlgren *et al*., 1985).

Colletotrichum species causing anthracnose on *Amaryllidaceae* have been reported from Japan (Horie *et al*., 1990; Moriwaki *et al*., 2003) and China (Liu *et al*., 2000). The disease symptoms include depressed reddish brown lesions on leaves or scapes, limiting the commercial production of ornamental and medicinal plants (Liu *et al*., 2000). *Colletotrichum boninense* Moriwaki, Toy. Sato & Tsukib., *C. crassipes* (Speg.) Arx, *C. dematium* (Pers.) Grove and *C. capsici* (Syd.) E.J. Butler & Bisby have been reported to infect *Amaryllis* sp., *Crinum asiaticum* var. *sinicum* and *Clivia miniata* (Sutton, 1980; Moriwaki *et al*., 2003; http://194.203.77.76/herbIMI/Name.asp). In this study we use combined morphological and molecular characteristics to identify and characterize *Colletotrichum* species associated with anthracnose of *Amaryllidaceae* in China and Thailand.

Materials and methods

Isolation of Colletotrichum

Colletotrichum samples were collected from anthracnose lesions on *Clivia*, *Crinum*, *Hippeastrum*, *Hymenocallis* (*Amaryllidaceae*) in Guangxi, Guizhou and Yunnan provinces, China and Chiang Rai Province, Thailand from June 2008 to September 2009 (Table 3). Isolation was carried out through two methods depending on the status of fungal sporulation. Isolates were obtained from lesions without visible sporulation using the procedure described by Photita *et al*. (2005). Single-spore isolations from infected leaves or scapes with sporulation were also carried out using the procedure described by Choi *et al*. (1999). Spore masses were picked up with a sterilized wire loop and streaked on to the surface of water agar (WA) plates which were then incubated overnight. A single germinated spore was picked up with a sterilized needle and transferred onto potato dextrose agar (PDA). Pure cultures were stored at 4°C on PDA slants. Isolates were deposited in Guizhou Academy of Agricultural Sciences, China and Mae Fah Luang University (MFLU) Culture Collection and National Centre for Genetic Engineering and Biotechnology (BIOTEC), Thailand**.**

Morphological and cultural characterization

Starter cultures were prepared by plating each isolate onto PDA at 25°C. Five 4 mm plugs were aseptically cut from actively sporulating areas near the growing edge of a 5-day-old culture of each isolate using a sterile cork borer. Each plug was placed onto PDA plates (Petri dishes diameter: 90 × 15 mm) and grown in alternating 12 hours near UV/12 hours dark at 25ºC (Sutton, 1980). Colony diameter was measured at day six (for the fastest growing cultures, at day four). Growth rate was calculated as the 6-day or 4-day average of mean daily growth (mm per day). After 7-10 days, size and shape of 50 conidia harvested from culture were assessed. The colour of the conidial masses and zonation were recorded at day seven. Mycelial appressoria were produced using a slide culture technique (Sutton, 1980),

where 10 mm squares of potato carrot agar (PCA) were placed in an empty Petri dish, the edge of the PCA was inoculated with small sections of mycelia, and a cover slip was placed over the inoculated agar. After 5-14 days, appressoria formed across the underside of the cover slip and their shape and size were then recorded. Conidial appressoria of some strains with cylindrical spores were also recorded. Conidial appressoria were induced by inoculating with conidia in two drops of distilled water on a microscope slide, then placing it inside Petri dishes containing moistened cotton with distilled sterile water, and incubated at 25ºC in darkness. After incubating 24 hours, conidial appressoria were formed from germ tube and then measured.

DNA extraction and sequencing

DNA was extracted from all isolates growing on PDA at room temperature for 8-10 days using a modified protocol of Chen *et al*. (2007). The gene regions were amplified using the primers listed in Table 1. The PCR amplifications were performed in a 25 µl mixture containing 9.5µl ddH_2O, 12.5 µl 2×PCR Master Mix (TIANGEN Co. China), 1 µl of DNA templates, 1 µl of each primers (10 µM). The reactions were performed with a thermal cycler (Mycler™, Bio-Rad, Hercules, CA, USA) using the following thermal program: 94°C for 5 min, followed by 35 cycles of denaturation (94°C for 30 s), annealing (30 s at 59°C for Actin, CAL and β-tubulin, 56°C for GPDH and CHS I, and 53°C for ITS), elongation (72°C for 90 s), and a final 7 min extension at 72°C. PCR products were examined by electrophoresis stained with ethidium bromide, and purified according to the manufacturer's instructions of a TIANGEL Mini Purification kit (TIANGEN Co. China). Purified samples were sequenced using the above-mentioned PCR primers in an Applied Biosystems 3730xl DNA Analyzers at Sinomax Co., China. Two of gene amplicon, CAL and β-tubulin, were cloned into DH5α (TIANGEN Co., China) with pMD18-T vector (Takara), and then sequenced at the same company.

Molecular phylogenetic analysis

Phylogenetic analysis was performed using six gene regions. The accession numbers of all sequences are listed in Table 3. Multiple sequence alignments were generated using ClustalX 2.0.10 (Larkin *et al*., 2007). Gaps were treated as missing data.

Each of the single and combined sequence alignments were analyzed using maximum parsimony (MP) in PAUP* 4b10. Ambiguously aligned regions were excluded from all analyses. Trees were inferred using the heuristic search option with tree bisection-reconnection (TBR) branch swapping and 1000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all multiple parsimonious trees were saved. 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 of the trees resulting from the parsimony analyses were assessed by bootstrap analysis with 1000 replicates. Trees were figured in Treeview. When analyzing ITS and β-tubulin sequence, some reference sequences were obtained from GeneBank (Table 2).

The model of evolution was estimated by using Mrmodeltest 2.2 (Nylander, 2004). Posterior probabilities (PP) (Rannala and Yang, 1996; Zhaxybayeva and Gogarten, 2002) were determined by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). Six simultaneous Markov chains were run for 3,000,000 generations and trees were sampled every 100^{th} generations (resulting 30,000 total trees). The first 2,000 trees, which represented the burn-in phase of the analyses, were discarded and the remaining 28,000 trees were used for calculating posterior probabilities (PP) in the majority rule consensus tree.

Pathogenicity testing and host range

A representative isolate of each species from *Amaryllidaceae* was selected for pathogenicity testing (Table 6). For testing the host range, 12 plant species were selected belonging to 6 families (Table 6), most being hosts of *Colletotrichum* species that are morphologically similar to our isolates from *Amaryllidaceae*. Single spore cultures of each isolate were

Table 1. Primers used for PCR amplification and DNA sequencing.

Gene	primer	Primer sequences	Reference
ITS	ITS1	TCCGTAGGTGAACCTGCGG	White *et al*., 1990
	ITS4	TCCTCCGCTTATTGATATGC	
Actin	ACT-512F	ATGTGCAAGGCCGGTTTCGC	Carbone and Kohn., 1999
	ACT-783R	TACGAGTCCTTCTGGCCCAT	
CAL	CL1	GARTWCAAGGAGGCCTTCTC	Johnston, *pers. comm.*
	CL2	TTTTTGCATCATGAGTTGGAC	
CHS I	CHS I-79F	TGGGGCAAGGATGCTTGGAAGAAG	Carbone *et al*., 1999
	CHS I-354R	TGGAAGAACCATCTGTGAGAGTTG	
GPDH	GDF1	GCCGTCAACGACCCCTTCATTGA	Guerber *et al.*, 2003
	GDR1	GGGTGGAGTCGTACTTGAGCATGT	Templeton *et al.*, 1992
β-tubulin	T1	AACATGCGTGAGATTGTAAGT	O'Donnell and Cigelnik, 1997
	βt2b	ACCCTCAGTGTAGTGACCCTTGGC	Glass and Donaldson, 1995

grown on PDA for 7 days at 25°C. Spores were then harvested by adding 10 ml of sterilized the concentration adjusted to 10^6conidia/ml using a haemocytometer, and used as the standard inoculum for pathogenicity and host range testing. The conidial suspension was filtered through two layers of muslin cloth (Than *et al*., 2008a). Healthy leaves or fruits were picked and washed with tap water; and then disinfected in 1% sodium hypochlorite for 5-7 minutes (disinfection time was adjusted according to whether the surface was smooth or coarse). Disinfected leaves and fruits were washed three times with distilled, sterilized water, and then dried with sterilized filter paper. Leaves or fruits were kept individually in a 12 cm diam Petri dish or tissue culture bottle (for tomato and grape fruit) with a swab of cotton wall (about 1 cm in diam.) containing distilled water to maintain humidity.

The inoculation method has been described by Than *et al*. (2008a). The wound /drop inoculation method involved pin pricking the leaf or fruit wall to a 1 mm depth and then placing 6 µl of conidia suspension (10^6conidia/ml) onto the wound /non wound. Control fruits and leaves were inoculated with 6 µl of sterilized, distilled water onto the wound. Each plant was inoculated with four replicates per selected strain. The inoculated fruits and leaves were then incubated at 25°C for 24 hours and then at room temperature (22-27°C). After 6-7 days and 14-15 days of incubation leaves and fruits were checked for lesions with a stereomicroscope on a superclean bench. Spore masses or acervuli on leaves or fruits were checked with a compound microscope and spores were transferred to PDA medium, and distilled water onto the culture, which was then gently swirled to dislodge the conidia with then incubated at 25°C for checking the colony and spore characters. All infected leaves or fruits were sterilized and then disposed.

Results

Collection of Colletotrichum species

Colletotrichum isolates used in this study are listed in Table 3. Twenty strains of *Colletotrichum* were isolated from anthracnose associated with *Clivia*, *Crinum*, *Hippeastrum* and *Hymenocallis* (*Amaryllidaceae*). Morphological examination classified twenty isolates into 6 morphogroups; while analysis of ITS sequence reveal 8 phylogenetic lineages (see below). For comparison, reference strains of several important *Colletotrichum* species were included in this study including *Colletotrichum simmondsii* R.G. Shivas & Y.P. Tan (holotype, BRIP 28519), *C. truncatum* (Schwein.) Andrus & W.D. Moore (CBS 120709), *C. coccodes* (Wallr.) S. Hughes (CPOS1), *C. gloeosporioides* (epitype, CBS 953.97) and *C. trichellum* (Fr.) Duke (HKUCC 10378).

Phylogenetic analysis

The ITS dataset comprised 570 characters after alignment, of which 138 characters were parsimony informative (24.2%). The Kishino-Hasegawa (KH) test showed that 556 trees generated from parsimonious analysis were not significantly different. One of the most parsimonious trees (TL =549, CI = 0.692, RI = 0.867, RC = 0.600, HI = 0.308) (Table 4.) is shown in Fig. 1. The dataset of combined six genes comprised 2973 characters after align-

Table 2. ITS and β-tubulin sequences obtained from GenBank for analysis.

Anamorph/ teleomorph	Strain	GenBank no. ITS	GeneBank no. β-tub2	Host	Country of origin	Reference
Colletotrichum agaves	AR3920	DQ286221		*Agave striata*	Mexico	Farr *et al.*, 2006
	CBS318	DQ286219		*Agave americana*	Netherlands	Farr *et al.*, 2006
C. asianum	[2]BPD-I4	FJ972612		*Coffea arabica*	Thailand	Prihastuti *et al*., 2009
	BML-I14	FJ972615		*Coffea arabica*	Thailand	Prihastuti *et al*., 2009
C. boninense	[2]MAFF 305972	AB051400		*Crinum asiaticum* var. *sinicum*	Japan	Moriwaki *et al*., 2003
	[3]MAFF 306094	AB051403		*Crinum asiaticum* var. *sinicum*	Japan	Moriwaki *et al*., 2003
C. caudatum	STE-U 5300		AY376575	*Cymbopogon martinii*	India	Lubbe *et al.*, 2004
C. circinans	BBA67864	AJ301955		*Allium schoenoprasum*	Germany	Nirenberg *et al*., 2002
C. coccodes	BBA70879	AJ301957		*Solanum tuberosum*	Germany	Nirenberg *et al*., 2002
	ATCC 58682	FJ545227		*Capsicum annuum*	New York, USA	Unpublished
C. dematium	[1]CBS125.25	*		*	*	Damm *et al.*, 2009
	CBS125340	*		*	*	Damm *et al.*, 2009
C. crassipes	STU-4445	AY376530	AY376578	*Dryandra* sp.	Madeira	Lubbe *et al.*, 2004
	STE-U 5302	AY376529	AY376577	*Dryas octopetala*	Switzerland	Lubbe *et al.*, 2004
C. destructivum	CD-hz 01	EU070911		*Phaseolus limensis*	China	Unpublished
	ATCC11995	AF320563		*Nicotiana tabacum*	Canada	Shen *et al*., 2001
C. dracaenophilum	MEP1537	DQ286207		*Dracaena* sp.	China	Farr *et al.* 2006
	CBS 121453	EU003533		*Dracaena sanderiana*	Bulgaria	Unpublished
C. fructicola	[2]BPD-I16	FJ972603		*Coffea arabica*	Thailand	Prihastuti *et al*., 2009
	BPD-I12	FJ972611		*Coffea arabica*	Thailand	Prihastuti *et al*., 2009
C. gloeosporioides	[1]IMI 356878	EU371022		*Citrus sinensis*	Italy	Cannon *et al*., 2008
	BBA71473	AJ301988		*Citrus aurantiacus*	Germany	Nirenberg *et al*., 2002
	STE-U 5297		AY376582	*Citrus* sp.	Belize	Lubbe *et al*., 2004
	STE-U 4295		AY376580	*Citrus* sp.	Italy	Lubbe *et al*., 2004

Table 2 (continued). ITS and β-tubulin sequences obtained from GenBank for analysis.

Anamorph/ teleomorph	Strain	GenBank no. ITS	GeneBank no. β-tub2	Host	Country of origin	Reference
Colletotrichum. graminicola	MAFF 306612	AB233343		unkown	Japan	Unpublished
	DR 1	AF059676		*Poa annua*	USA	Unpublished
	STE-U 5298		AY376587	*Zea mays*	Zimbabwe	Lubbe *et al.*, 2004
C. lupini	BBA 70385	AJ301935		*Lupinus angustifolius*	Germany	Nirenberg *et al*., 2002
	BBA 63879	AJ301930		*Lupinus mutabilis*	Germany	Nirenberg *et al*., 2002
C. orbiculare	MAFF306684	AB275876		*Cucumis melo*	Japan	Unpublished
C. siamense	[2]BML-I2	FJ972613		*Coffea arabica*	Thailand	Prihastuti *et al*., 2009
	BML-I15	FJ972614		*Coffea arabica*	Thailand	Prihastuti *et al*., 2009
C. spaethianum	[2]CBS167.49	*		*	*	Damm *et al.*, 2009
	CBS_100063	*		*	*	Damm *et al.*, 2009
C. spinaciae	BBA71333	AJ301973		*Spinacia oleracea*	Germany	Nirenberg *et al*. 2002
C. sublineolum	BBA71362	AJ301978		*Sorghum bicolor*	Germany	Nirenberg *et al*., 2002
C. trichellum	BBA71091	AJ301989		*Hedera helix*	Germany	Nirenberg *et al*., 2002
C. truncatum	[1]CBS151.35	*		*	*	Damm *et al.*, 2009
	CBS120709	*		*	*	Damm *et al.*, 2009
C. yunnanense	[2]AS3.9617	EF369490		*Buxus* sp.	China	Liu *et al*., 2007
	[3]AS3.9616	EF369491		*Buxus* sp.	China	Liu *et al*., 2007
Glomerella acutata	STE-U 4466		AY376567	*Hakea sericea*	South Africa	Lubbe *et al.*, 2004
G. lagenaria	BBA71046	AJ301965		*Cucumis sativus*	Germany	Nirenberg *et al*., 2002
Fusarium oxysporum	ATCC MYA-3970	FJ614650		unknown	USA	Unpublished

[1], epitype; [2], holotype; [3], paratype; *, See the same issue.

Table 3. Isolates of *Colletotrichum* used in this study, with GeneBank no. of six genes.

Taxon	Specimen no.	Host	Site	GeneBank no. ITS	GeneBank no. β-tubulin	GeneBank no. CAL	GeneBank no. CHS I	GeneBank no. GPDH	GeneBank no. Actin
Colletotrichum cliviae	CSSK4	*Clivia miniata*	Kunming, Yunan, China	GQ485607	GQ849440	GQ849464	GQ856722	GQ856756	GQ856777
C. cliviae	CSSS1	*Clivia miniata*	Shuicheng, Guizhou, China	GU109479	GU085869	GU085863	GU085865	GU085867	GU085861
C. cliviae	CSSS2	*Clivia miniata*	Shuicheng, Guizhou, China	GU109480	GU085870	GU085864	GU085866	GU085868	GU085862
C. trunctatum	CSSX2	*Crinum asiaticum*	Jinghong, Yunan, China	GQ485595	GQ849424	GQ849457	GQ856736	GQ856750	GQ856766
C. boninense	CSSN 1	*Crinum asiaticum*	Nanning, Guangxi, China	GQ485597	GQ849437	GQ849462	GQ856728	GQ856743	GQ856774
C. boninense	CSSX8	*Crinum asiaticum*	XTFPG, Jinghong, Yunan, China	GQ485596	GQ849433	GQ849460	GQ856727	GQ856742	GQ856771
C. fructicola	CSSX7	*Crinum asiaticum*	XTFPG, Jinghong, Yunan, China	GQ485604	GQ849435	GQ849459	GQ856734	GQ856760	GQ856770
C. hippeastri	CSSG1	Hippeastrum vittatum	Guiyang, Guizhou, China	GQ485599	GQ849446	GQ849469	GQ856725	GQ856764	GQ856788
C. hippeastri	CSSG2	Hippeastrum vittatum	Guiyang, Guizhou, China	GQ485598	GQ849445	GQ849470	GQ856726	GQ856765	GQ856789
C. hymenocallidis	CSSN 2	*Hymenocallis americana*	Nanning, Guangxi, China	GQ485600	GQ849438	GQ849463	GQ856730	GQ856757	GQ856775
C. hymenocallidis	CSSN 3	*Hymenocallis americana*	Nanning, Guangxi, China	GQ485601	GQ849439	GQ849451	GQ856729	GQ856759	GQ856776
C. trunctatum	CSSX4	*Hymenocallis americana*	Jinghong, Yunan, China	GQ485590	GQ849425	GQ849450	GQ856738	GQ856755	GQ856768
C. trunctatum	CSSX9	*Hymenocallis americana*	XTFPG, Jinghong, Yunan, China	GQ485594	GQ849436	GQ849461	GQ856737	GQ856752	GQ856772
C. spaethianum	CSSX3	*Hymenocallis americana*	Jinghong, Yunan, China	GQ485584	GQ849432	GQ849456	GQ856719	GQ856745	GQ856767
C. spaethianum	CSSX5	*Hymenocallis americana*	Jinghong, Yunan, China	GQ485586	GQ849426	GQ849448	GQ856718	GQ856747	GQ856769
C. spaethianum	CSSX10	*Hymenocallis americana*	XTFPG, Jinghong, Yunan, China	GQ485585	GQ849427	GQ849449	GQ856720	GQ856746	GQ856773
C. trunctatum	CSST3	*Hymenocallis* sp.	Chiang Rai, Thailand	GQ485592	GQ849442	GQ849471	GQ856740	GQ856754	GQ856779
C. trunctatum	CSST5	*Hymenocallis* sp.	Chiang Rai, Thailand	GQ485591	GQ849428	GQ849458	GQ856741	GQ856751	GQ856781
C. siamense	CSST1	*Hymenocallis* sp.	Chiang Rai, Thailand	GQ485602	GQ849441	GQ849467	GQ856731	GQ856758	GQ856778
C. siamense	CSST4	*Hymenocallis* sp.	Chiang Rai, Thailand	GQ485603	GQ849443	GQ849465	GQ856732	GQ856761	GQ856780
*C. trunctatum**	CBS 120709	*Capsicum frutescens*	Coimbatore, India	GQ485593	GQ849429	GQ849453	GQ856739	GQ856753	GQ856783
*C. simmondsii**	[1]BRIP 28519	*Carica papaya*	Yandina, Queensland, Australia	GQ485606	GQ849430	GQ849454	GQ856735	GQ856763	GQ856784
C. gloeosporioides	[1]CBS 953.97	*Citrus sinensis*	Italy	GQ485605	GQ849434	GQ849452	GQ856733	GQ856762	GQ856782
C. coccodes	CPOS1	Solanum tuberosum	Shuicheng, Guizhou, Guizhou	GQ485588	GQ849444	GQ849468	GQ856723	GQ856744	GQ856787
C. trichellum	HKUCC 10378	Unknown	Unknown	GQ485589	GQ849447	GQ849466	GQ856724	GQ856749	GQ856786

Abbreviations: BRIP, Queensland Department of Primary Industries Plant Pathology Herbarium; CBS, Centraalbureau voor Schimmelcultures, Netherlands, HKUCC, The University of Hong Kong Culture Collection; XTFPG, Xishuangbanna Tropical Flowers and Plants Garden; *, See Shivas and Tan 2009 for recent name changes.[1], epitype.

Table 4. Descriptive tree statistics of Actin, CAL, GDPH, CHS I, tub2 and ITS determined by *Paup*.

	Actin	β-tubulin	CAL	CHS I	GPDH	ITS	Combined six genes
Characters	271	744	791	250	245	570	2973
NPIC	96	333	312	59	162	138	1081
NPIC/Character	35.4%	44.8%	39.4%	23.6%	66.1%	24.2%	36.3%
Length	216	954	780	141	448	548	2547
CI	0.745	0.644	0.701	0.638	0.719	0.692	0.707
RI	0.898	0.876	0.892	0.848	0.889	0.867	0.877
RC	0.670	0.564	0.625	0.541	0.639	0.600	0.620
HI	0.255	0.356	0.299	0.362	0.281	0.308	0.293

Abbreviation: NPIC, Number of (included) parsimony informative characters.

ment, of which, 1081 characters were parsimony informative (36.3%). KH tested showed that 2 trees inferred from parsimonious analysis were not significantly different. The phylograms deduced from dataset of combined six gene regions is shown in Fig. 2, and descriptive tree statistics are listed in Table 4. The phylograms inferred from single gene Actin, CHS I, β-tubulin, CAL and GPDH show essentially similar topology as that from ITS and combined datasets and are, therefore, not shown.

The isolates of *Colletotrichum* associated with anthracnose on *Amaryllidaceae* clustered into 8 clades based on phylograms inferred from ITS region (Fig. 1). Clade 1 was monophyletic with high bootstrap support (97%) and comprised isolates CSSX3, CSSX5 and CSSX10 representing *Colletotrichum spaethianum* (Allescher) Damm, P.F. Cannon & Crous. Isolates CSSK4, CSSS1 and CSSS2 constituted another monophyletic lineage (clade 2) with 100% bootstrap support and appeared as a sister clade to *Colletotrichum orbiculare* (Berk. & Mont.) Arx. Isolates CSSG1 and CSSG2 clustered in the monophyletic clade 3 with 96% bootstrap support and appeared basal to *Colletotrichum boninens*e. Isolates CSSN1 and CSSX8 clustered with two strains of *Colletotrichum boninense* (holotype and paratype) from *Crinum asiaticum* var. *sinicum* in clade 4 with 92% bootstrap support. Clade 5 (100%) included isolates CSSX2, CSSX4, CSSX9, CSST3 and CSST5. These five strains formed a distinct lineage together with two strains of *Colletotrichum truncatum* (CBS151.35 (epitype) and CBS120709). Isolates CSST1 and CSST4 clustered with the type specimens of *Colletotrichum siamense* Prihastuti, L. Cai & K.D. Hyde (Clade 6). In addition, the phylogenetic position of isolates CSSX7, CSSN2 and CSSN3 remain uncertain in the ITS tree, in which, CSSX7 were close to *C. fructicola* Prihastuti, L. Cai & K.D. Hyde.

Phylograms inferred from CAL gene sequences delimited isolate CSSX7 together with *C. fructicola* (strain BPD-I16, holotype) in a distinct lineage (clade 7) with 96% bootstrap.

In phylograms inferred from CHS I, as compared to that from ITS, clades 1-6 are very similar. The phylograms indicated that isolates CSSN2 and CSSN3 represented a separate lineage (clade 8) with 67% bootstrap support. In addition, the bootstrap support for clade 6 (CSST1 and CSST4) is higher than that inferred from ITS (89% vs 58%). The position of isolate CSSX7 was not resolved in this single gene tree (trees not shown).

Phylograms inferred from combined datasets of Actin, β-tubulin, CHS I, CAL, GPDH and the ITS regions support eight well-defined clades (Fig. 2), representing eight distinct species. Clades 1 to 6 correspond with phylograms constructed using ITS and CHS I. The bootstrap and Bayesian posterior probabilities of the six clades are also very high (equal or above 98%). Isolate CSSX7 represented a distinct clade (clade 7) with 100% bootstrap support. In addition, isolates CSSN2 and CSSN3 also represents a distinct clade (clade 8) with 95% bootstrap support and 100% Bayesian posterior probabilities (Fig. 2). Clades 1, 4, 5, 6 and 7 are *Colletotrichum spaethianum, C. boninense*, *C. truncatum*, *C. siamense* and

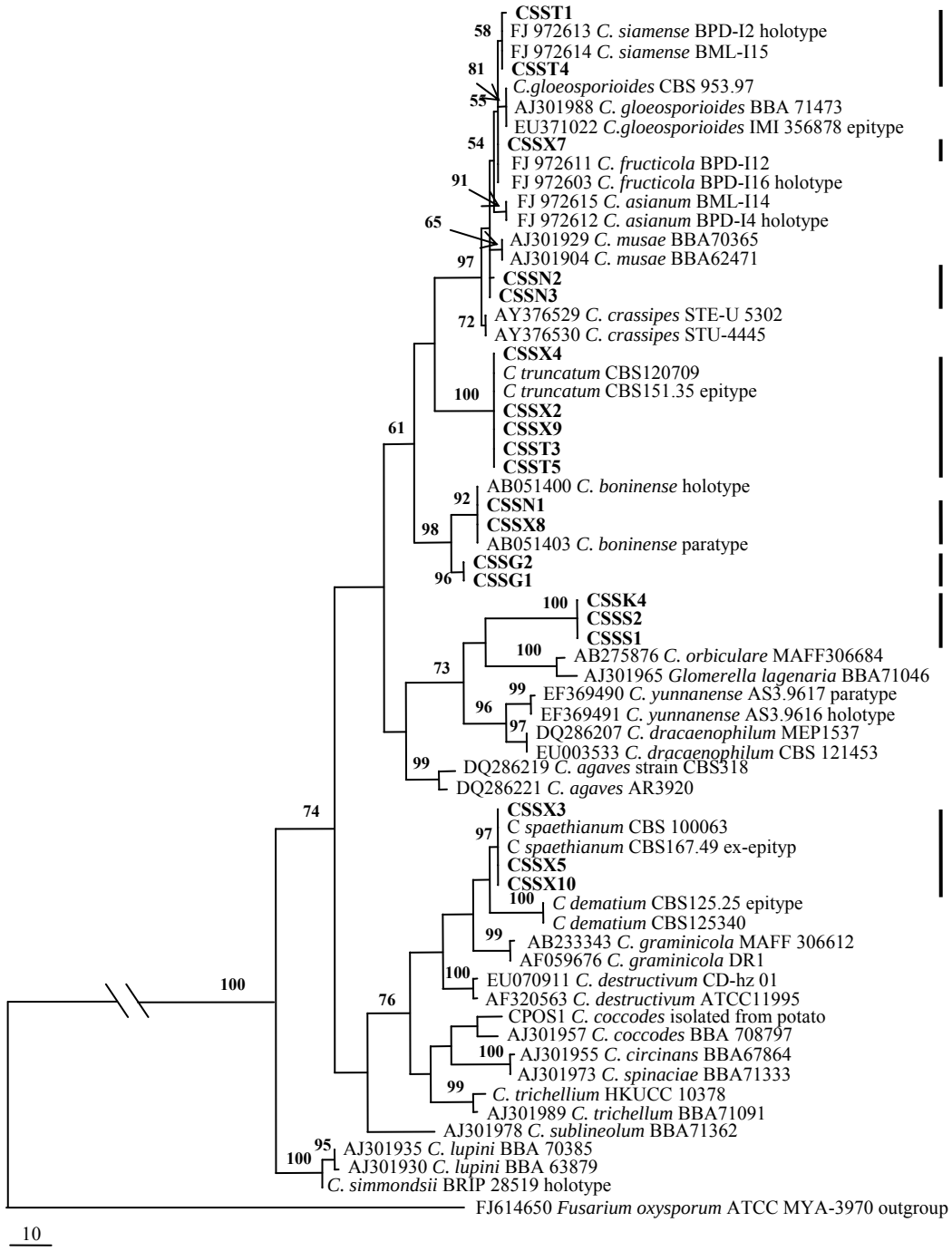

Fig. 1. Maximum parsimony phylograms inferred from ITS sequence data showing phylogenetic relationships among isolates of *Colletotrichum* from *Amaryllidaceae* in southwest China and Chiang Rai, Thailand (bold) and selected sequences of *Colletotrichum* species (of which, some are epitypes). Data analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. Values above the branches are parsimony bootstrap (equal or above 50%). The tree is rooted with *Fusarium oxysporum* (ATCC MYA- 3790).

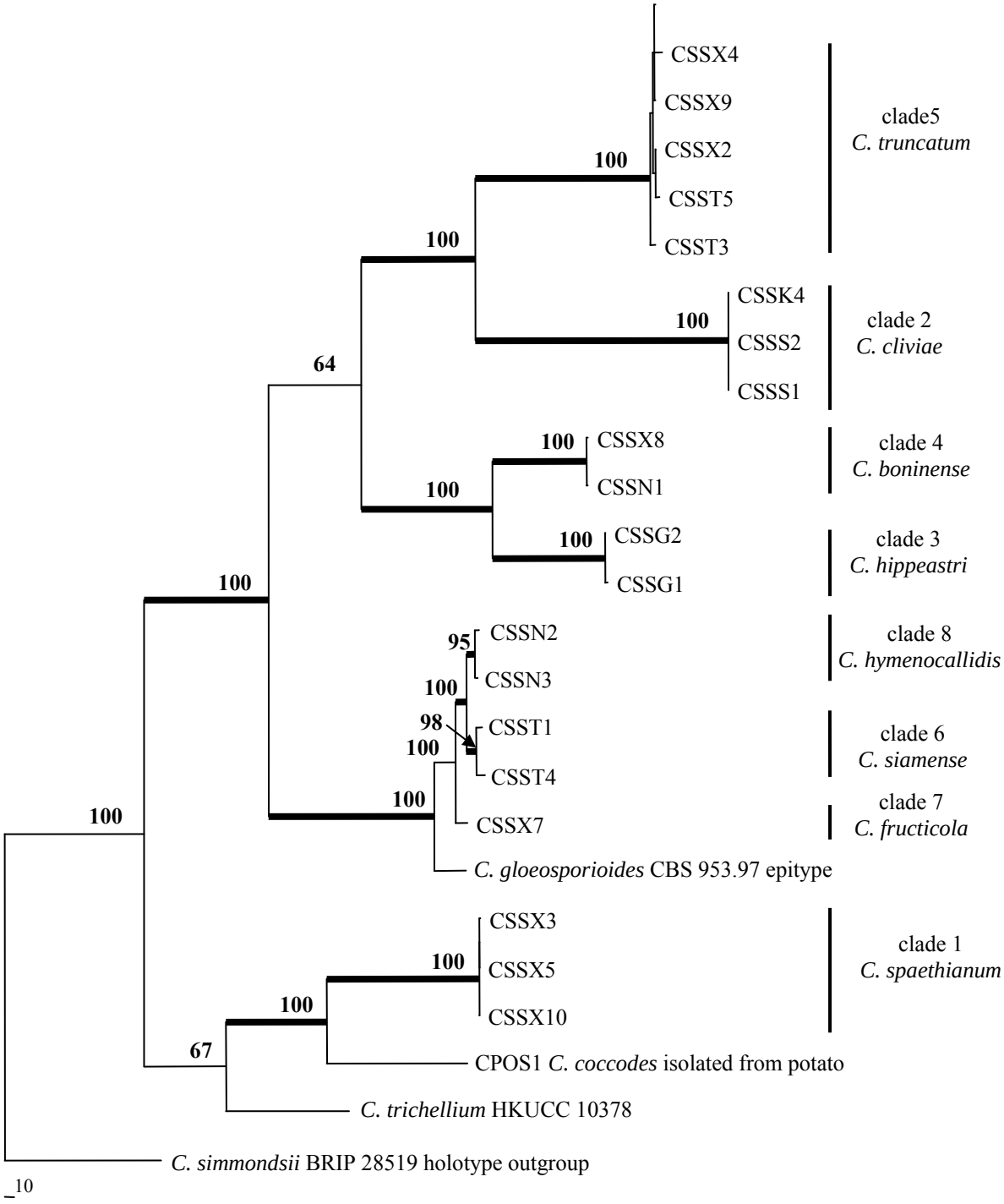

Fig. 2. Maximum parsimony phylograms inferred from combined partial Actin, β-tubulin, CHS I, CAL, GPDH and ITS sequence data, showing phylogenetic relationships among isolates of *Colletotrichum* from *Amaryllidaceae* in southwest China, and Chiang Rai Province, Thailand and selected sequences of *Colletotrichum* species (some are epitypes). Data analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. Values above the branches are parsimony bootstrap (equal or above 50%). Thickened branches represent significant Bayesian posterior probabilities (equal or above 95%). The tree is rooted with *C. simmondsii* (BRIP 28519 epitype). The clade number corresponds with phylograms deduced from ITS.

C. fructicola, respectively. Clades 2, 3 and 8 represent three undescribed *Colletotrichum* species. Full descriptions and illustrations of these three species are provided in the following taxonomy section.

Taxonomy

Eight species of *Colletotrichum* causing anthracnose of plants in *Amaryllidaceae* are listed in (Tables 3, 5). Three of these are clearly different from all currently known

species of *Colletotrichum* (Hyde *et al*., 2009; Sutton, 1992) and are therefore described and illustrated as new species.

Colletotrichum boninense Moriwaki, Toy. Sato & Tsukib**.**

This species occurs on leaves of *Crinum asiaticum* as reddish brown, ellipsoid to irregular spots and forms pink conidial masses with rare setae (Fig. 6A). In this study, although the conidia of *C. boninense* (CSSN1 and CSSX8) are wider than those of the holotype and paratype, their shape and cultural characteristics are similar and they cluster in one clade with high bootstrap support (there is one base difference in the ITS sequence among them and the holotype). In addition, the holotype and paratype of *C. boninense* was collected from *Crinum asiaticum* var. *sinicum* in Japan.

Material examined: CHINA, Yunnan Province, Jinghong, on leaf of *Crinum asiaticum*, 2 August 2008, Y.L. Yang. (GZAAS 080009, ex-living culture CSSX8); China, Guangxi Province, Nanning, on leaf of *Crinum asiaticum*, 19 June 2008, Y.L. Yang. (GZAAS 080006, ex-living culture CSSN1).

Colletotrichum cliviae Y.L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai, **sp. nov.** (Fig. 3)
MycoBank: 515279

Etymology: *cliviae*, in reference to the host *Clivia miniata*.

In agaro decoct tuberosum post 5 dies colonae est 7.4-7.7 cm diam, albae vel griseae. Ad margine griseo-albus, densus, reversum atrobrunnea vel pallens nigri cum pallide bubalinus massa conidiales. Sclerotia presentia, setosa. Setae presentia, basi medie brunneae, 1-3-septatae, 75-105 × 4-6 µm, acuta ad apicem. Conidiophora hyalinae, 22.5-49 × 4-5.5 µm, 1-4-septatae. Conidia in pallide bubalinus massa**,** 19.5-24.5 × 4.5-7µm, aseptata, laevia, hyalina,cylindrica, rectae vel laeviter curvus, ad apicem obtusa, Appressoria medie brunneae, irregularis, crenate vel lobata, 10.5-14.5 × 6-11 µm.

Habitate: in foliis *Clivia miniata*.

Holotypus: Cultura (CSSK4), isolata e foliis morbo affectis *Clivia miniata*, Yunnan, China, 2008. Paratypus: Cultura (CSSS2), isolata e foliis morbo affectis *Clivia miniata*, Guizhou, China, 2009.

Description: On host, acervuli circular to elliptical, arranged irregularly, subepidermal, disrupting outer epidermal cell wall of host, setae present, with pale yellow conidial masses (Fig. 3A). Setae 95-210 × 6-7 µm ($\bar{x}$ = 160 ± 43.2 × 6.5 ± 0.5, n = 5), moderately brown, smooth-walled, 1-3-septate, tapered and paler toward the apex. Conidiophores hyaline, 1-3-celled, branched or unbranched at the base, 18-36.5 × 4-6.5 µm ($\bar{x}$ = 29.4 ± 7.7 × 5.2 ± 0.9, n = 10). Conidia 15-24 × 4.5-6.5 µm ($\bar{x}$ = 20.5 ± 2.2 × 5.8 ± 0.5, n = 20), one-celled, smooth-walled, hyaline, straight, obtuse at the ends.

Colonies on PDA, attaining 7.4-7.7 cm ($\bar{x}$ = 7.5 ± 1.1, n = 10) diam. in 5 days at 25°C, growth rate 15.2-16 mm per day ($\bar{x}$ = 15.6 ± 0.2, n = 10); at first white, becoming grey with age, greyish-white at margin, dense, reverse dark brown to greenish-black (Figs 3B, C), with pale buff conidial masses. Sclerotia present, setose. Setae present, brown, 75-105 × 4-6 µm ($\bar{x}$ = 85 ± 10.5 × 5 ± 0.7, n = 5), 1-3-septate, with tapering acute apices. Conidiophores hyaline, 22.5-49 × 4-5.5 µm ($\bar{x}$ = 33.3 ± 8.7 × 4.7 ± 0.6, n = 10), 1-4-celled, branched or unbranched at the base (Figs 3D, G). Conidia in pale buff masses, 19.5-24.5 × 4.5-7 µm ($\bar{x}$ = 21.8 ± 1.4 × 5.7 ± 0.5, n = 100), one-celled, smooth-walled, hyaline, cylindrical, straight or slightly curved, obtuse at the ends (Figs 3E, H-I). Appressoria brown, irregular, crenate or lobed, 10.5-14.5 × 6-11 µm ($\bar{x}$ = 11.7 ± 1.2 × 8.6 ± 1.2, n = 40) (Figs 3F, J-K).

Teleomorph: not produced in culture.

Holotype: CHINA, Yunnan Province, Kunming, on leaf of *Clivia miniata*, 10 August 2008, Y.L. Yang (GZAAS 080005; ex-holotype living culture CSSK4, CBS 125375).

Known host and distribution: *Clivia miniata*, Kunming and Shuicheng, China.

Additional specimens examined: CHINA, Guizhou Province, Shuicheng, on leaf of *Clivia miniata*, 20 September 2009, Y.L. Yang (GZAAS 080018, ex-paratype living culture CSSS2).

Colletotrichum fructicola Prihastuti, L. Cai & K.D. Hyde

On leaves of *Crinum asiaticum* with anthracnose caused by this species occurring as yellowish brown ellipsoid spots, acervuli without setae, with yellowish-white conidial masses (Fig.6D).

Material examined: CHINA, Yunnan Province, Jinghong, on leaf of *Crinum asiaticum*, 1 August 2008, Y.L. Yang (GZAAS 080019, ex-living culture, CSSX7).

Colletotrichum hippeastri Y.L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai, **sp. nov.** (Fig. 4)
MycoBank: 515280.

Table 5. *Colletotrichum* species on *Amaryllidaceae.*

Species and Isolate numbers	Conidial shape and size (µm)	Mycelial appressoria shape and size (µm)	Conidial appressoria shape and size (µm)	Colony characteristics	Mycelial growth rate(mm per day)	Host(s)
C. boninense (CSSN1, CSSX8)	Cylindrical, with a hilum-like base, 13-19.5 × 4.5-8, $\overline{x}$ =16.3 ± 1.2 × 6.6 ± 0.7, n = 100	Irregular, sepia to dark brown, (8.5-)10-12(-15) × 6.5-11, $\overline{x}$ = 11.4 ± 1.5 × 8.5 ± 1.1 n = 20	Irregular, sepia to dark brown, 6.5-9.5 (-11.5) × 5.0-7.5, $\overline{x}$ = 8.8 ± 0.8 × 6.4 ± 0.7, n = 40	White aerial mycelia; reverse cream to reddish orange	9.8-11, $\overline{x}$ = 10.6 ± 0.4, n = 10	*Crinum asiaticum*
C. cliviae (CSSK4, CSSS1, CSSS2)	Cylindrical, straight or slightly curved, obtuse at the ends, 19.5-24.5 × 4.5-7, $\overline{x}$ = 21.8 ± 1.4 × 5.7 ± 0.5, n = 100	Not produced	Dark brown, irregular, crenate or lobed, 10.5-14.5 × 6-11 µm, $\overline{x}$ = 11.7 ± 1.2 × 8.6 ± 1.2, n = 40	White to grey, white at margin, reverse dark brown to greenish black	15.2-16 , $\overline{x}$ = 15.6 ± 0.2, n = 10	*Clivia miniata*
C. fructicola (CSSX7)	Cylindrical, with obtuse to slightly rounded ends, 8.5-16×3.5-5, $\overline{x}$ = 12.7 ± 1.7 × 4.1 ± 0.4, n = 50	Clavate to ovate, medium brown, 7.5-13.5×5-8, $\overline{x}$ = 10 ± 1.5 × 6.3 ± 0.9, n = 20	Clavate to ovate, medium brown, 6-9 × 5.5-7, $\overline{x}$ = 7.2 ± 0.7 × 5.9 ± 0.4, n = 20)	White, grey to dark grey with age, reverse black, circular around at the centre	11.4-13.4 $\overline{x}$ = 12.3 ± 0.7, n = 5	*Crinum asiaticum*
C. hippeastri (CSSG1, CSSG2)	Cylindrical, straight, usually slightly curved, obtuse at the ends, usually constricted near each end or centre, 19.5-40.5 (42.5) × 7-10.5 (-12), $\overline{x}$ = 29.2 ± 5.5 × 8.8 ± 1.0,n = 100	Not produced	Medium to dark brown, irregular, crenate or lobed, occasionally becoming complex, 10.5-17.5 × 8-12.5, $\overline{x}$ = 13 ± 1.7 × 10 ± 1.1,n = 40	Pale white to black, floccose, sparse, reverse pale white-black	13.6-14.2 $\overline{x}$ = 13.9 ± 0.2, n = 10	*Hippeastrum vittatum*

Table 5 (continued). *Colletotrichum* species on *Amaryllidaceae.*

Species and Isolate numbers	Conidial shape and size (µm)	Mycelial appressoria shape and size (µm)	Conidial appressoria shape and size (µm)	Colony characteristics	Mycelial growth rate(mm per day)	Host(s)
C. hymenocallidis (CSSN2, CSSN3)	Fusiform, straight, obtuse at the ends, 14-18.5(-20) × 5-6.5, $\overline{x}$ = 15.9 ± 1.1 × 5.1 ± 0.4, n = 100	Ovate to clavate, margin entire, medium brown, 6-13 × 5-7, $\overline{x}$ = 9.4 ± 3 ×5.9 ± 0.7, n = 5	Ovate, sometimes clavate, medium brown, 7-11×5-7.5, $\overline{x}$ = 8.5 ± 0.9 × 6.6 ± 0.6, n = 40	White to becoming pale grey with circles, reverse greenish black	8.8-11, $\overline{x}$ = 9.9 ± 0.8, n = 10	*Hymenocallis americana*
C. siamense (CSST1, CSST4)	Fusiform to cylindrical with obtuse to slightly rounded ends, 13-17.5 × 4-5.5, $\overline{x}$ = 15.3 ± 0.9 × 4.7 ± 0.3, n = 100	Ovate to clavate, medium brown, 6.5-10.5(-12) × 4.5-8, $\overline{x}$ = 8.8 ± 1.3 ×5.9 ± 1, n = 15	Ovoid, medium brown, 6-8 × 4.5-7, $\overline{x}$ = 7 ± 0.7 × 5.9 ± 0.6, n = 20, n = 20	White, becoming pale brown with age, reverse pale yellowish	10.5-12.4 $\overline{x}$ = 11.5 ± 0.5, n = 10	*Hymenocallis* sp.
C. spaethianum (CSSX3, CSSX5, CSSX10)	Falcate, fusiform, gradually tapered towards each end, 13.5-19 × 2.5-4, $\overline{x}$ = 15.8 ± 1.2 × 3.2 ± 0.3, n = 50	Clavate, ovate to irregular, margin entire or irregular lobed, medium, 8.5-15.5 × 5-9.5, $\overline{x}$ = 11.5 ± 1.8 × 7 ± 1.3, n = 60	No data	Pale grey to mouse grey, reverse pale yellow	12.4-13.2, $\overline{x}$ = 13± 0.3, n = 15	*Hymenocallis americana*
C. truncatum (CSSX2,CSSX4, CSSX9, CSST3 CSST5)	Falcate, fusiform, gradually tapered towards each end, (15-) 21.5-29 (-32) ×3-4, $\overline{x}$ = 25.2 ± 2.1 × 3.6 ± 0.2, n = 150	Clavate to ovate, margin entire, becoming complex and forming irregular chains, medium brown to dark brown, 9-15.5(-18) ×5.5(-12), $\overline{x}$ = 13.2 ± 2 ×8.3 ± 1.3 , n = 60	No data	Medium grey to dark grey dense colony, reverse dark brown.	9.8 - 12, $\overline{x}$ = 10.5 ± 0.4, n = 15	*Crinum asiaticum* *Hymenocallis* sp. *Hymenocallis americana*

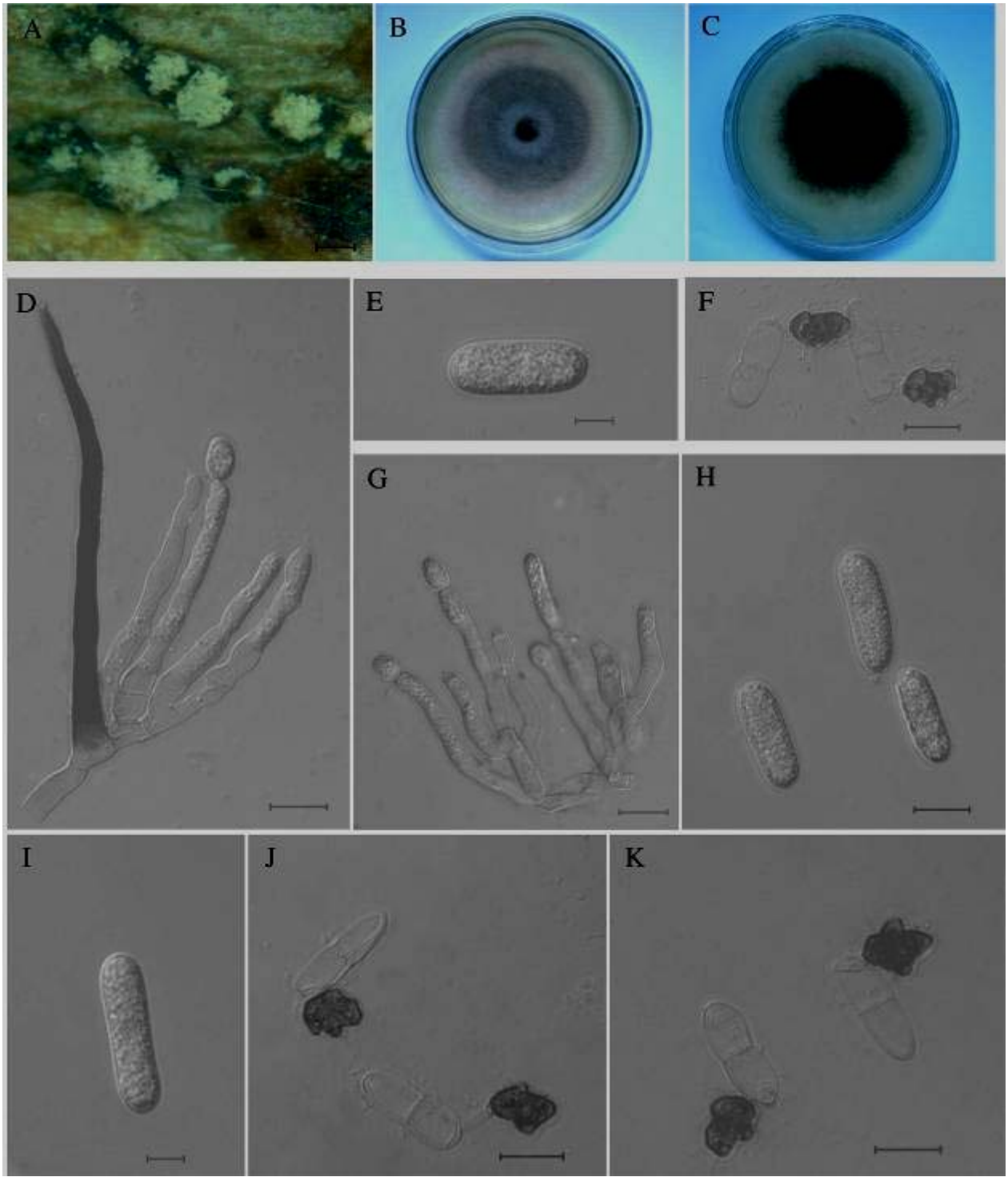

Fig. 3. *Colletotrichum cliviae* (from holotype). **A**, Acervuli on leaf of *Clivia miniata*. **B**, **C**. Colony on PDA after 7 days, upper B and reverse C. **D**, **G**, Seta and conidiophores. **E**, **H, I**, Conidia. **F**, **J**, **K**, Appressoria. Bars: **A** = 100 μm; **E**, **I** = 5 μm; **D**, **F**, **H, J** and **K** = 10 μm.

Etymology: *hippeastri*, in reference to the host *Hippeastrum vittatum.*

In agaro decoct tuberosum post 6 dies colonae est 7.5-7.8 cm diam., griseo-albus vel nigri de margine, sparsae, reversum griseoalbus vel nigri cum pallide bubalinus conidiales massa. Sclerotia presentia, setosae. Setae absentia**.** Conidiophora hyalinae, 20.5-39.5 (-67) × 4.5-6.5 μm, 2-4- cellulae. Conidia in albo massa**,** 19.5-42.5 × 7-12 μm ($\overline{x}$ = 29.2 ± 5.5 ×8.8 ± 1.0, n = 100), aseptata, laevia, hyalinae, cylindrica, rectae, ad apicem obtusa. Ad prope utrinque vel centralis anqustusAppressoria medie brunneae vel fusco-brunneo, irregularis, crenate vel lobata, 10.5-17.5 × 8-12.5 μm ($\overline{x}$ = 13 ± 1.7 × 10 ± 1.1, n = 40).

Habitat: in foliis *Hippeastrum vittati*.

Holotypus: Cultura (CSSG1), isolata e foliis morbo affectis *Hippeastrum vittatum*, Guizhou, China, 2009. Paratypus: Cultura (CSSG2), isolata e foliis morbo

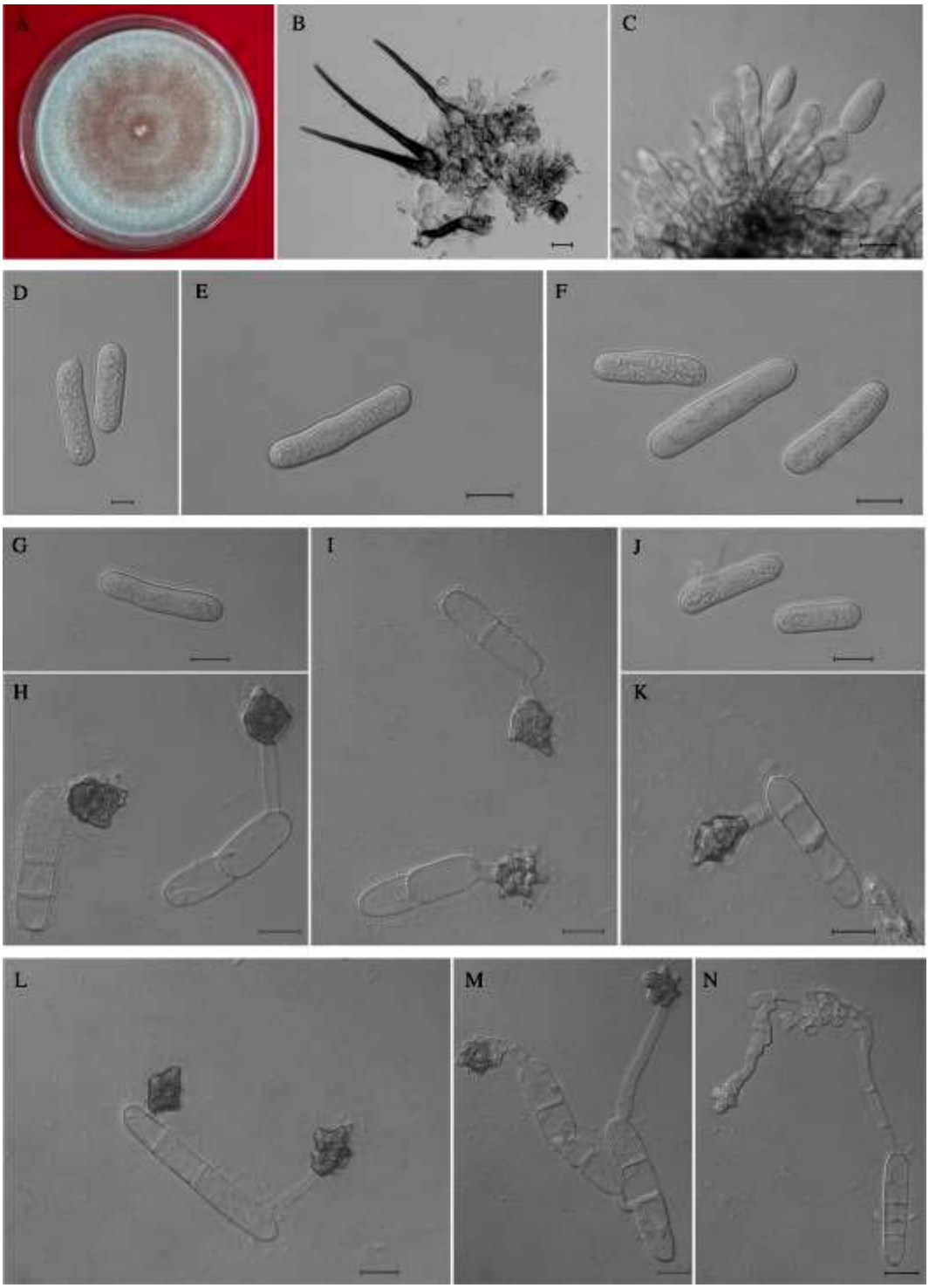

Fig. 4. *Colletotrichum hippeastri* (from holotype). **A**. Colony on PDA after 7 days. **B**, Setae. **C**. Conidiophores. **D-G** and **J**, Conidia. **H**, **I**, and **K-N**, Appressoria. Bars: **B**, **D** = 5 µm; **C, E-N** = 10 µm.

affectis *Hippeastrum vittatum*, Guizhou, China, 2009.

Description: Scape lesions circular, elliptical to striped, reddish brown (Fig. 6C). Acervuli, circular to elliptical, subepidermal, disrupting outer epidermal cell wall of host, setae sparse or absent, with buff conidia masses. Setae 70-100 × 5-7 µm ($\bar{x}$ = 88.5 ± 10.0 × 5.9 ± 0.8, n = 5), brown, smooth-walled,1-4-septate, tapered to the paler acute apex and swollen at the base (Fig. 4B). Conidiophores hyaline, 1-2 celled, not branched or branching at the base, 19-24 × 5-6µm ($\bar{x}$ = 21.5 ± 1.9 × 5.4 ± 0.4, n = 5) (Fig. 4C). Conidia 22-26 × 6.5-8 µm ($\bar{x}$ = 22.3 ±1.1 × 7 ± 0.5, n = 20), one-celled, smooth-walled, hyaline, straight, obtuse at the ends.

Colonies on PDA attaining 7.5-7.8 cm ($\bar{x}$ = 7.7 ± 1.1, n = 10) diam. in 6 days at 25°C,

growth rate 13.6-14.2 mm per day ($\bar{x}$ = 13.9 ± 0.2, n = 10); at first pale white, becoming black from the margin with age, floccose, sparse, reverse white at first, becoming black with age, with pale buff conidial masses (Fig. 4A). Sclerotia present, setose. Setae absent. Conidiophores hyaline, 20.5-39.5 (-67) × 4.5-6.5µm ($\bar{x}$ = 33.5 ± 13.0 × 5.6 ± 0.6, n = 10), 2-4-celled, branched or unbranched at the base. Conidia in white masses, 19.5-42.5 × 7-12 µm ($\bar{x}$ = 29.2 ± 5.5 × 8.8 ± 1.0, n = 100), one-celled, smooth-walled, hyaline, cylindrical, straight or slightly curved, obtuse at the ends, usually constricted near both ends or the centre (Figs 4D-G, J). Appressoria brown to dark brown, irregular, crenate or lobed, occasionally becoming complex (Figs 4H-I, K-N), 10.5-17.5 × 8-12.5 µm ($\bar{x}$ = 13 ± 1.7 × 10 ± 1.1, n = 40).

Teleomorph: not produced in culture.

Holotype: CHINA, Guizhou Province, Guiyang, on leaf of *Hippeastrum vittatum*, 23 May 2009, Y.L. Yang (GZAAS 090001; ex-holotype living culture CSSG1, CBS 125376).

Known host and distribution: *Hippeastrum vittatum*, Guiyang, China.

Additional specimens examined: China, Yunnan Province, Kunming, on scape of *Hippeastrum vittatum*, 23 May 2009, Y.L. Yang (GZAAS 090002, ex-paratype living culture CSSG2, CBS 125377).

Colletotrichum hymenocallidis Y.L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai, **sp. nov.**

(Fig. 5)

MycoBank: 515281

Etymology: *hymenocallidis,* in reference to the host *Hymenocallis americana*.

In agaro decoct tuberosum post 6 dies colonae est 6-6.4 cm diam, albo vel pallid griseae cum orbis, densus**,** reversum pallid griseae vel pallen**s** nigri, margine albo. Sclerotia absentia. setae absentia**.** Conidia in persicinum massa, 14--20 × 5-6.5 µm ($\bar{x}$ = 15.9 ± 1.1 × 5.1 ± 0.40, n = 100), aseptata, laevia, hyalina, fusiformia, recta cum unus vel duo guttulatae, interdum ad centralis anqustus, ad apicem obtusa. Appressoria atrobrunnea, ovata, interdum irregularis, margine pluries totus, interdum crenate vel lobulata, 7-11 × 5-7.5 µm ($\bar{x}$ = 8.5 ± 0.9 × 6.6 ± 0.6, n = 40).

Habitat: in foliis *Hymenocallis americanis*

Holotypus: Cultura (CSSN2), isolata e foliis morbo affectis *Hymenocallis americana*, Nanning, China, 2009. Paratypus: Cultura (CSSN3), isolata e foliis morbo affectis *Hymenocallis americana*, Nanning, China, 2009.

Description: Leaf lesions elliptical or circular, reddish-brown (Fig. 6E). On host, acervuli subepidermal, disrupting outer epidermal cell wall of host, setae absent, with salmon-pink conidia masses (Fig. 5A). Conidiophores hyaline, 1-2 celled, not branching or branching at the base, 9-20 × 3-4.5 µm ($\bar{x}$ = 14.7 ± 3.1 × 3.8 ± 0.5, n = 20) (Fig.5D). Conidia 9.5-15 × 3.5-5.5 µm ($\bar{x}$ = 13.1 ± 1.4 × 4.8 ± 0.4, n = 50), one-celled, smooth-walled, hyaline, straight, sometimes slightly curved, fusiform, gradually tapered at the ends (Figs 5E, F).

Colonies on PDA attaining 6-6.4 cm in diam. ($\bar{x}$ = 6.2 ± 4.4, n = 10) in 6 days at 25°C, growth rate 8.8-11 mm per day ($\bar{x}$ = 9.9 ± 0.8, n = 10); at first white, with aging, becoming pale grey from centre with concentric zones, dense, reverse pale white at first, becoming greenish black from centre with age, margin white (Figs 5B, C). Sclerotia absent. Setae absent. Conidia formed in orange-pink conidial masses, 14-20 × 5-6.5 µm ($\bar{x}$ = 15.9 ± 1.1 × 5.1 ± 0.40, n = 100), one-celled, smooth-walled, hyaline, fusiform, straight, guttulate, occasionally constricted at centre, obtuse at the ends (Figs 5G-I). Appressoria dark brown, ovate, irregular, margin entire, or crenate to lobed, occasionally forming secondary appressorium from first appressorium, but not complex (Figs 5J-L), 7-11 × 5-7.5 µm ($\bar{x}$ = 8.5 ± 0.9 × 6.6 ± 0.6, n = 40).

Teleomorph: not produced in culture.

Holotype: CHINA, Guangxi Province, Nanning, on leaf spot of *Hymenocallis americana*, 19 June 2008, Y. L. Yang (GZAAS 080001; ex-holotype living culture CSSN2, CBS 125378).

Known host and distribution: *Hymenocallis americana*, Guangxi, China.

Additional specimens examined: CHINA, Guangxi Province, Nanning, on leaf spot of *Hymenocallis americana*, 19 June 2008, Y.L. Yang (GZAAS 080002, ex-paratype living culture CSSN3, CBS 125379).

Colletotrichum siamense Prihastuti, L. Cai & K.D. Hyde

On leaves of *Hymenocallis* sp. with anthracnose caused by *Colletotrichum siamense* occurring as brown ellipsoid spots with orange conidial masses, without setae.

Material examined: THAILAND, Chiang Rai Province, Mae Fah Luang University, on leaf of *Hymenocallis* sp., 25 November 2008, Y.L. Yang and K.D. Hyde CSST1, MFLU 09-0670, living culture MFLUCC 09-0670.

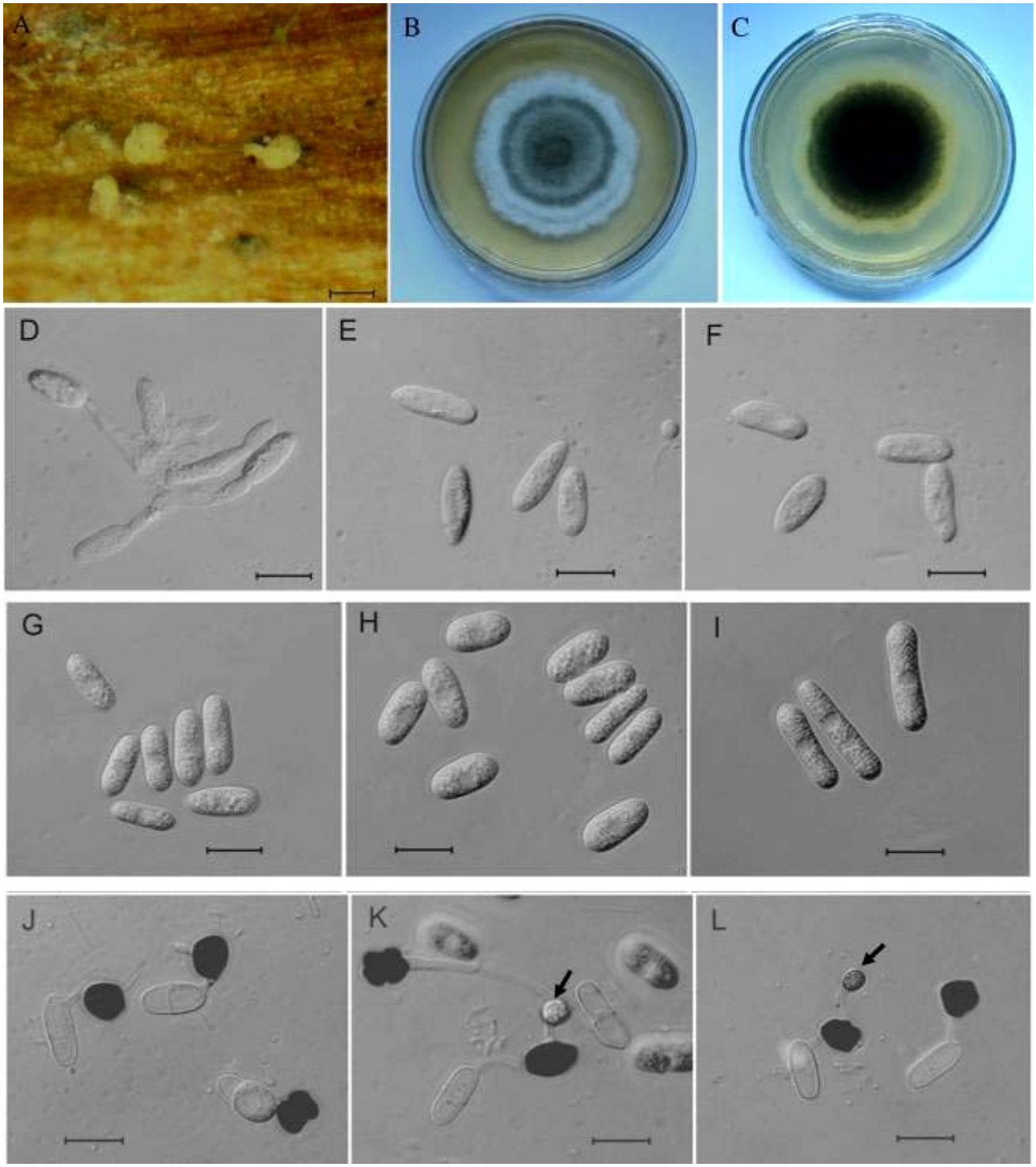

Fig. 5. *Colletotrichum hymenocallidis* (from holotype). **A**. Acervuli on leaf of *Hymenocallis americana*. **B**, **C**. Colony on PDA after 7 days, upper B and reverse C. **D**. Conidiophores. **E** and **F**. Conidia from conidial masses on leaf of *H. americana*. **G-I**. Conidia from conidial masses from culture. **J-L**. Appressoria (arrows indicate secondary appressoria). Bars: **A** = 200 μm; **D-L** = 10 μm.

Colletotrichum spaethianum (Allescher) Damm, P.F. Cannon & Crous

Symptoms of anthracnose of *Hymenocallis americana* caused by this species are similar to that caused by *C. truncatum*. The acervuli of *C. truncatum* were larger than *C. spaethianum* and the setae are longer.

Material examined: CHINA, Yunnan province, Jinghong, on leaf of *Hymenocallis americana*, 1 August 2008, Y.L. Yang. (GZAAS 080003, ex-living culture CSSX3; GZAAS 080004, ex-living culture CSSX5; GZAAS 080012, ex-living culture, CSSX10).

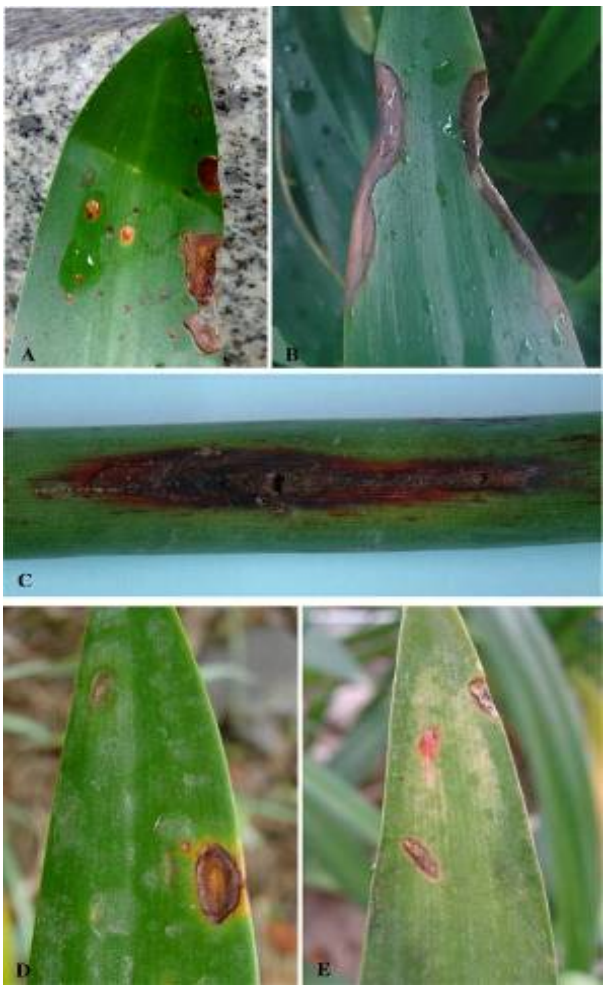

Fig. 6. Anthracnose symptoms on leaves of amaryllids (*Amaryllidaceae*). **A**. *Colletotrichum boninense* on leaf of *Crinum asiaticum*. **B**. *Colletotrichum truncatum* on leaf of *Hymenocallis americana*. **C**. *Colletotrichum hippeastri* on scape of *Hippeastrum vittatum*. **D**. *Colletotrichum fructicola* on leaf of *Crinum asiaticum*. **E**. *Colletotrichum hymenocallidis* on leaf of *Hymenocallis americana*.

Colletotrichum truncatum (Schwein.) Andrus & W.D. Moore

This taxon causes brown stripes or spots on leaves with pale yellow conidial masses, setae are long and dense (Fig. 6B).

Material examined: CHINA, Guangxi Province, Nanning, on leaf of *Crinum asiaticum* (GZAAS 080007, ex-living culture CSSX2) and *Hymenocallis americana* (GZAAS 080010, ex-living culture CSSX9; GZAAS 080014, ex-living culture CSSX4), 19 June 2008, Y.L. Yang; THAILAND, Chiang Rai Province, Mae Fah Luang University, on leaf of *Hymenocallis* sp., 25 November 2008, Y.L. Yang and K.D. Hyde, CSST3, MFLU 09-0671, living culture MFLUCC 09-0671.

Pathogenicity testing and host range

The three new species are not host-specific (Table 6). Isolate CSSN2 (*Colletotrichum hymenocallidis*) from *Hymenocallis americana* can infect fruits of *Capsicum annuum* and *Lycopersicon esculentum* by wound/drop inoculation forming salmon-pink conidial masses, without setae. Both non-wound/drop and wound/drop inoculation resulted in leaf infection of *Hymenocallis americana*, with similar symptoms to the field infection. Isolate CSSG1 (*Colletotrichum hippeastri*) from *Hippeastrum vittatum* caused disease on the leaves of *Crinum asiaticum* by wound/drop inoculation. Reddish brown spots formed with acervuli and few setae on *Crinum asiaticum* leaves. Isolate CSSK4 (*Colletotrichum cliviae*) can infect *Bletilla striata by* wound/drop inoculation resulting in numerous pale yellow conidial masses on the leaves. In addition, CSSK4 can infect *Crinum asiaticum* by non-wound/drop and wound/drop inoculation forming a similar symptom as that in field.

Discussion

The main objective of this study was to identify and characterize the *Colletotrichum* species associated with anthracnose of *Amaryllidaceae* in southwest China and Chiang Rai, Thailand based on morphological characters and DNA sequence data. A taxonomic strategy for studying *Colletotrichum* species has been reviewed by Cai *et al*. (2009), in which a polyphasic approach is suggested since single phenotypic character has been proven to be inadequate to differentiate *Colletotrichum* species. It was emphasized that multiple-loci phylogeny should be employed as the basis for establishing species relationships. In the current study, twenty strains of *Colletotrichum* were separated into six morph-types using morphological characters, while ITS sequence data (Fig. 1) delineated eight phylogenetic lineages but with low support for 2 clades. Combined multi-gene datasets (Fig. 2) delineated eight, well-supported, distinct phylogenetic lineages (bootstrap > 95%) representing distinct taxa and these met the standard required for the rank of phylogenetic species (Taylor *et al*., 2000). Four of the species had type or epitype data in GeneBank, which was used for comparison. Three species are described as new species, and each taxon is uniquely characterized by the

Table 6. Results of inoculation tests with *Colletotrichum* isolates from *Amaryllidaceae*.

***Colletotrichum* strain** **Plant**		**C. *hymenocallidis*** **(CSSN2)**	**C. *hippeastri*** **(CSSG1)**	***C. cliviae*** **(CSSK4)**
Clivia miniata	W	-	-	+
	UW	-	-	+
Crinum asiaticum	W	-/-	+	-
	UW	-	-	-
Hippeastrum vittatum	W	-	+	-
	UW	-	+	-
Hymenocallis americana	W	+	-	-
	UW	+	-	-
Agapanthus africanus	W	-	-	-
	UW	-	-	-
Bletilla striata	W	-	-	+
	UW	-	-	-
Capsicum annuum	W	+	-	-
	UW	-	-	-
Cymbidium hookerianum	W	-	-	-
	UW	-	-	-
Lycopersicon esculentum	W	+	-	-
	UW	-	-	-
Phaseolus vulgaris	W	-	-	-
	UW	-	-	-
Polygonatum odoratum	W	-	-	-
	UW	-	-	-
Vitis vinifera	W	-	-	-
	UW	-	-	-

Note: W, wound inoculation; NW, non-wound inoculation; +, symptom observed; -, no symptom observed.

molecular identities at the ITS, GPDH, β-tubulin, CAL, Actin and CHS I loci.

Species of Colletotrichum on Amaryllidaceae

Colletotrichum boninense (MAFF 3060 94, CBS 119185, CBS 241.78), *C. capsici* (IMI 233426; synonymy, *C. truncatum* (Hyde *et al*., 2009)), *C. crassipes* (IMI 165604, IMI 304052,IMI 259839) and *C. dematium* (IMI 208403, IMI 124329) have been previously recorded from *Amaryllidaceae* anthracnose (Sutton, 1980; Moriwaki *et al*., 2003; http://www.cbs.knaw.nl/fungi/BioloMICS.aspx, http://194.203.77.76/herbIMI/Name.asp). *Colletotrichum boninense* was reported as the causal agent of anthracnose on *Dracaena sanderiana* and *Euonymus japonica* (Lee *et al*., 2005; Farr *et al*., 2006), and an endophyte on a number of hosts worldwide (Lu *et al*., 2004). Recent indications are that *C. boninense* may be a species complex (P.W. Crous and R.G. Shivas, pers. comm.). *Colletotrichum capsici* and *C. dematium* also cause disease of a number of host plants from tropical to temperate regions (Sutton, 1992; Hyde *et al*., 2009; Damm *et al*., 2009). In this study, *C. dematium* and *C. crassipes* were not encountered. Six other taxa, *Colletotrichum cliviae*, C. *fructicola*, *C. hippeastri*, *C. hymenocallidis*, *C. siamense* and *C. spaethianum*, were recorded for the first

Table 7. Synopsis of morphological characters of *Colletotrichum hippeastri* and similar taxa.

Taxa	Colony characteristics and growth rate	Conidial shape and size	Conidial appressorium, shape and size	Referrence
[1]*Colletotrichum dracaenophilum*	Pale pink, margin pale pink, sparse aerial mycelium; rosy buff to saffron in reverse; 6–6.7 cm in 6 days	Broadly clavate to cylindrical, frequently slightly curved, 22–34 × 6.5–9.5 µm, $\overline{x}$ = 29 × 8.5µm	No data	Farr *et al*., 2006
[1]*Colletotrichum hippeastri*	Pale white to black, floccose, sparse, reverse pale white black; 6.8-7.1 cm in 6 days	Cylindrical, straight, occasionally slightly curved, obtuse at the ends, usually constricted near each end or in the centre, becoming 2-4-celled, 19.5-42.5 × 7-12, $\overline{x}$ = 29.2 × 8.8 µm	Medium to dark brown, irregular, crenate or lobed, occasionally becoming complex, 10.5-17.5 × 8-12.5, $\overline{x}$ = 13 × 10 µm	This paper
[2]*Colletotrichum nupharicola*	Flattened, wet and slimy toward centre, at first yellowish to orange with whitish margins, darkening from centre outwards; covering plate (but never reaching perimeter) in ca 3 weeks	Cylindrical to clavate, becoming 2-celled, 14-53 × 5-10 µm	Blackish, ovoid to clavate, 6-10 × 7-9 µm	Johnson *et al*., 1997
[3]*Colletotrichum sansevieriae*	Greyish white and partly cream to pink, felted with aerial mycelium, reverse grey to dark olivaceous grey and partly cream to pink; Growth rate: no data	Straight, cylindrical, obtuse at apex, and slightly acute at base, base with truncate attachment point, 12.5-32.5 × 3.8-8.8µm, $\overline{x}$ = 18.4 × 6.4 µm	No data	Nakamura *et al*., 2006

Incubation: [1]On PDA in alternating 12 hours near UV/12 hours dark at 25ºC; [2]On PDA at 20° under 12 hours near UV/12 hours; [3]On PDA in the dark at 25°C.

time as the cause of anthracnose on *Amaryllidaceae*. *Colletotrichum fructicola*, *C. siamense* and *C. spaethianum* have been also recorded on coffee berries, jujube, papaya, lilium and hosta (Damm *et al*., 2009; Prihastuti *et al*., 2009; Phoulivong *et al*., pers. comm.).

Justification of new species

Colletotrichum cliviae

Colletotrichum cliviae is similar to *C. boninense*, *C. crassipes* and *C. orbiculare* in conidial width. However, conidial shape is different; conidia of *C. crassipes* are truncate at the base while those of *C. boninense* have a hilum-like low protuberance at the base (Sutton, 1980; Moriwaki *et al*., 2003). In addition, *Colletotrichum cliviae* grows faster in culture than *C. orbiculare* and *C. boninense* (15.2-16 mm/day in *C. cliviae* vs 10.4-12.2 mm/day in *C. boninense* and 5.1-5.3 mm/day in *C. orbiculare*) (Sutton, 1980; Moriwaki *et al*., 2003; Asakura *et al*., 2009). In the phylogram derived from single and combined multi-gene dataset, three *C. cliviae* isolates clustered together and phylogenetically separated from above morphologically similar species (Figs 1, 2).

Colletotrichum hippeastri

Conidia of *Colletotrichum hippeastri* overlap in size with those of *C. dracaenophilum* D.F. Farr & M.E. Palm, *C. sansevieriae* M. Nakamura & M. Ohzono and *C. nupharicola* D.A. Johnson, Carris & J.D. Rogers (Johnson *et al*., 1997; Farr *et al*., 2006; Nakamura *et al*., 2006). *Colletotrichum hippea-*

stri differs in several other characters (Table 7). In *C. hippeastri* the conidia are distinct in being usually narrower near each end or at the centre, while the conidia of *C. dracaenophilum* and *C. nupharicola* do not narrow (Johnson *et al*. 1997; Farr *et al*., 2006). Germinating conidia of *C. hippeastri* form 2-4 cells (Fig. 4), a character distinct from other species of *Colletotrichum*. The conidia of *C. nupharicola* become 2-celled and secondary conidia form from germ tubes (Johnson *et al*., 1997). The appressoria of *C. hippeastri* are larger than those of *C. nupharicola* (10.5-17.5 × 8-12.5 µm vs. 6-10 × 7-9 µm), and they are irregular, crenate or lobed (Figs 4H, I, K-N), whereas those of *C. nupharicola* are ovoid to clavate. The conidia of *C. hippeastri* are longer than those of *C. sansevieriae* (mean 29.2 × 8.8 µm vs 18.4 × 6.4 µm). In addition, *C. sansevieriae* is highly host-specific to *Sansevieria* (Nakamura *et al*., 2006). Colonies of *C. hippeastri* grow faster than those of *C. dracaenophilum*, *C. sansevieriae* or *C. nupharicola*, and other colony characters are also different (Johnson *et al*., 1997; Farr *et al*., 2006; Nakamura *et al*., 2006) (Table 7). Because the ITS sequence of *C. sansevieriae* is very short (159 bases pairs, in GeneBank), we did not include these sequences in our ITS phylogenetic analysis. The ITS2 of *C. hippeastri* and of *C. sansevieriae* (AB212991, strain:Sa-1-2; MAFF239721, ex-holotype) have nineteen base differences. Finally, *C. hippeastri* clustered separately from *C. dracaenophilum* in phylograms inferred from ITS sequences (Fig. 1).

Colletotrichum hymenocallidis

The conidial size and shape of *Colletotrichum hymenocallidis* is similar to that of *C. siamense*, however, their colony characters is different; reverse of *C. siamense* is yellowish white while that of *C. hymenocallidis* is greenish black. The conidial and mycelial appressorium in *C. hymenocallidis* are larger than those of *C. siamense* (Prihastuti *et al*., 2009) (Table 5). Based on the phylograms deduced from CHS I and combined datasets of six genes, they occur in two different clades (Fig. 2). Although the conidial size and shape in *C. gloeosporioides* is similar to *C. hymenocallidis*, the latter grows slower than *C. gloeosporioides* under the same conditions (10 mm/d in *C. hymenocallidis* versus 26.5 mm/d in *C. gloeosporioides*) (Cannon *et al*., 2008). In the single and combined gene trees, *C. hymenocallidis* formed a distinct lineage separated from *C. gloeosporioides* (Figs. 1, 2).

Comparison of conidial appressoria with mycelial appressoria

Appressorium shape and size are important characters for species identification in *Colletotrichum* (von Arx, 1957; Johnston and Jones, 1997; Crouch *et al*., 2009b;), and both conidial and mycelial appressoria have been induced and characterized in various studies (Sutton, 1980, 1992; Johnston and Jones, 1997; Johnson *et al*., 1997; Chaky *et al*., 2001; Crouch *et al*., 2009 b). In the present study, we tried inducing both conidial and mycelial appressoria but mycelial appressoria often failed to develop. Conidial appressoria were usually smaller and had less variation in shape than mycelial appressoria (Table 5). In addition, induction of mycelial appressorium, especially for cylindrical-spored species, often took more than 10 days (Cannon *et al*., 2008; Crouch *et al*., 2009b). It is therefore easier to characterize conidial appressoria and this has been suggested by Cai *et al*. (2009).

Pathogenicity analysis

With the exception of the three new species, five other species identified in this study have been previously reported from several other hosts (Sutton, 1980; Shenoy *et al*., 2007b; Moriwaki *et al*., 2003; Damm *et al*., 2009; Prihastuti *et al*., 2009; Phoulivong *et al*., pers. comm.). We therefore selected the three new species for pathogenicity testing *in vitro* against 12 plants belonging to 6 families. The results showed that *C. cliviae*, *C. hippeastri* and *C. hymenocallidis* are not host-specific as they are able to infect two or three different hosts (Table 6). The virulence potential of *Colletotrichum* species has been shown to be dependent on interplay of various factors such as plant varieties, humidity, temperature and the concentration of inoculum (Simmonds, 1965; Freeman *et al*., 1998). The pathogenicity and virulence of these new species therefore needs testing *in vivo*.

Future study

The *Amaryllidaceae* comprises 51 genera, with over 800 species and a worldwide distribution (Meerow *et al*., 1998, 2000). A high diversity of *Colletotrichum* species, including many undocumented new species, could be expected if more hosts are studied.

Acknowledgements

We thank Dr. Cheng from the Research Institute of Resources Insects (RIRI) of the Chinese Academy of Forestry, for supplying several reference strains. The project is supported by Scientific and Technological Foundation of Guizhou Province (QianKeHeJZiG [2008] 700120) and Scientific Foundation of Guizhou Academy of Agricultural Science (QianNoKeYuanZhuanXian [2008] 023), China and Mae Fah Luang University, research funding.

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