Characterization of *Colletotrichum* species associated with coffee berries in northern Thailand

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

The identity of *Colletotrichum* species on *Coffea* (coffee) is not clearly understood. We report on *Colletotrichum* species associated with coffee berries in northern Thailand and compare them to species reported to cause coffee berry disease elsewhere. Morphological, cultural, biochemical and pathogenic characters, together with DNA sequence analyses resulted in the isolates clustering into three species: *Colletotrichum asianum* sp. nov., *C. fructicola* sp. nov. and *C. siamense* sp. nov. Phylogeny inferred from combined datasets of partial actin, β-tubulin (tub2), calmodulin, glutamine synthetase, glyceraldehyde-3-phosphate dehydrogenase genes and the complete rDNA ITS1-5.8S-ITS2 regions revealed groupings that are congruent with morphological characters. Biochemical and DNA sequence data from multi-genes used here provided reliable data to differentiate between *C. kahawae*, *C. gloeosporioides* and the new *Colletotrichum* species.

Key words: anthracnose, disease, endophytes, epiphytes, multigene phylogeny, new species, pathogenicity

Article Information
Received 27 October 2009
Accepted 5 November 2009
Published online 9 December 2009
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Introduction

Coffee is a tropical crop and according to FAO statistics production of green beans is around 4 million tons annually with sales of 6 to 12 billion dollars (González, 2000). Coffee is a major source of income for Thailand, being the third largest producer in Southeast Asia - after Vietnam and Indonesia (Angkasith, 2001). While there are many species of coffee in the world, only *Coffea arabica* (Arabica coffee) and *C. robusta* (robusta coffee) have major economic significance. Arabica coffee is grown in the cooler highland areas of the northern part of Thailand, while robusta coffee is grown in southern part of Thailand (Angkasith, 2001).

Despite its importance, coffee production has often been limited by a disease known as coffee berry disease. It is the major factor limiting Arabica coffee production in Kenya and other countries in Eastern Africa, especially at high altitudes (Van der Vossen *et al*., 1976; Firman and Waller, 1977; Van der Graff, 1992; Masaba and Waller, 1992; Waller and Masaba, 2006) where it may cause crop losses of 50 to 80% (Van der Vossen *et al*., 1976). Coffee berry disease is caused by the fungus *Colletotrichum kahawae* (Waller *et al*., 1993).

Several other species or strains of *Colletotrichum* have been reported from coffee including saprobic strains, and *Colletotrichum,* along with *Guignardia* and *Xylaria* are the most common genera of coffee endophytes in Puerto Rico (Masaba and Waller, 1992; Santamaría and Bayman, 2005). There is little known regarding *Colletotrichum* species associated with coffee berries in Thailand and it is not clear whether all the species that can be isolated from berries are equally pathogenic. This study characterizes species of *Colleto-*

trichum associated with apparently healthy and infected coffee berries based on morphology, biochemical tests, DNA sequence data and pathogenicity tests.

Materials and methods

Isolation of Colletotrichum species from healthy and infected coffee berries

Colletotrichum was isolated from coffee berries (*Coffea arabica*) collected at two sites in Chiang Mai, Thailand. Strains were isolated from anthracnose lesions on infected berries and from both the surface (epiphytes) and inner masses (endophytes) of healthy berries. To obtain isolates from fruit without visible sporulation a modification of the procedure described by Photita *et al*. (2005) and Than *et al*. (2008a) was used. Three 5 × 5 mm pieces of tissue were taken from the margin of infected tissues, surface sterilized by dipping in 1% sodium hypochlorite for 1 minute, immersed in 70% ethanol for 1 minute and rinsed three times with sterilized water and finally dried in sterilized tissue paper. Samples were placed on water agar and incubated at room temperature (28-30°C). The growing edges of any fungal hyphae developing from the tissues were then transferred aseptically to potato dextrose agar. The fungi were identified following sporulation. Single spore subcultures were obtained for each *Colletotrichum* isolate using the procedure described by Goh (1999). When direct examination showed that the fungus was sporulating on the berry, spore masses were picked off with a sterilized wire loop and streaked on the surface of water agar. After incubation overnight (28-30°C), single germinated spores were picked up with a sterilized needle and transferred to PDA. Pure cultures were stored in sterilized water in Eppendorf tubes at 6°C (Abang, 2003). Cultures are deposited in the culture collection of Mae Fah Luang University, BIOTEC Culture Collection (BCC), and CBS.

Morphological studies of Colletotrichum species

Mycelial discs (4 mm diam.) were taken from actively sporulating areas near the growing edge of 7 day old cultures and transferred to potato dextrose agar (PDA, Criterion®, Santa Maria, USA) and incubated at 28°C. Three replicate cultures of each isolate were investigated. After 7 days, size and shape of 20 conidia harvested from the cultures were recorded (Than *et al*., 2008a).

Colony diameter was measured daily for 7 days and growth rate was calculated as the 7 day average of mean daily growth (mm per day). After 7 days, colony size, colour of the conidial masses and zonation were recorded.

Appressoria were produced using a slide culture technique, in which 10 mm^2 squares of PDA were placed in an empty Petri dish. The edge of the agar was inoculated with spores taken from a sporulating culture and a sterile cover slip was placed over the inoculated agar (Johnston and Jones, 1997). After 3-7 days, the shape and size of the appressoria formed across the underside of the cover slip were studied.

Morphological data were analyzed using analysis of variance ($P<0.05$) with Duncan's Multiple Range Test (DMRT) by using SPSS software version 16.0 (SPSS Inc., Chicago, USA) (Kirkpatrick and Feeney, 2006).

Biochemical testing

Utilization of citrate and tartrate as a carbon source were assayed on agar plates according to the method of Bridge *et al*. (2008). Medium B with 1.2% (w/v) agar was supplemented with 1% (w/v) citric acid or ammonium tartrate and 0.005% (w/v) bromocresol purple (Waller *et al*., 1993; Bridge *et al*., 2008). Positive and negative controls containing, respectively, glucose or no additional carbon source were included for each isolate.

All media were adjusted to pH 4.5 with NaOH prior to sterilization and then autoclaved at 105°C for 20 minutes (Waller *et al*., 1993; Bridge *et al*., 2008). Test media were inoculated with agar plugs (4 mm diameter) taken from 7 day old single conidium derived cultures. Utilization was assessed by visual comparison of growth and a rise in the pH of the medium adequate to produce a dark blue to purple colour of bromocresol purple (Waller *et al*., 1993; Bridge *et al*., 2008).

Pathogenicity testing

Three representative isolates of each morphogroup were used for pathogenicity testing. Isolates tested were *Colletotrichum*

Group 1 (BML-I6, BML-I15, BPD-I2), *Colletotrichum* Group 2 (BML-I3, BML-I14, BPD-I4), and *Colletotrichum* Group 3 (BPD-I12, BPD-I16, BPD-I18).

Single spore cultures of each isolate were grown on potato dextrose agar for 7 days at room temperature (28-30°C). The spores were harvested by adding 10 ml of sterilized distilled water onto the culture, which was then gently swirled to dislodge the conidia. Spore concentration was adjusted to 10^6conidia/ml using a haemocytometer (Than *et al*., 2008a).

Coffea arabica berries were supplied by Royal Agricultural Project Coffee, Chiang Mai, Thailand. Non-infected berries were disinfected with 1% sodium hypochlorite for 5 minutes, and washed three times with distilled water. The berries were blotted dry with a sterilized tissue paper and inoculated by using wound/drop and non-wound/drop inoculation method (Lin *et al*., 2002; Than *et al*., 2008a). The wound/drop inoculation method involved pin pricking the coffee berry wall to a 1 mm depth and then placing 6 µl of conidia suspension (10^6conidia/ml) over the wound. Control fruits were inoculated with 6 µl of sterilized distilled water onto the wound. The inoculated fruits were incubated at room temperature in a closed sterile container. This experiment was arranged by using Completely Randomized Design (CRD), with three replicates per isolate. Three green and three red berries were tested per isolate. Lesion area was evaluated by measuring length, width and area of the typical anthracnose lesion developed on the berries, from 1 to 15 days after inoculation (DAI). Percentage of infected area in fruits was obtained from the lesion area divided by fruit area and multiplied by one hundred (Than *et al*., 2008a). After 15 days conidia from diseased berries were aseptically transferred onto potato dextrose agar and incubated at room temperature. The resultant cultures were checked for morphological characters to confirm Koch's postulates. All infected berries were disposed of following sterilization.

Data of the anthracnose lesion area were analyzed with Duncan's Multiple Range Test (DMRT) by using SPSS software version 16.0 (SPSS Inc., Chicago, USA) (Kirkpatrick and Feeney, 2006).

DNA extraction

Isolates were grown on PDA for 7 days and mycelium was scraped from the surface. Genomic DNA was extracted using a Biospin Fungus Genomic DNA Extraction Kit (Bio-Flux®) according to the instructions of the manufacturer. DNA concentrations were estimated visually in agarose gel by comparing band intensity with a DNA ladder 100 bp (Transgen Biotech®).

PCR amplification and DNA sequencing

Partial actin (ACT), β-tubulin (TUB2), calmodulin (CAL), glutamine synthetase (GS), glyceraldehyde-3-phosphate dehydrogenase (G PDH) genes and the complete rDNA-ITS (ITS) region from 15 representative *Colletotrichum* strains were amplified by PCR reaction. The same strains of new species were used in the pathogenicity tests.

The PCR amplification for partial actin, β-tubulin (tub2) and the complete rDNA-ITS regions were amplified with primers-pair ACT512F (5'-ATGTGCAAGGCCGGTTTCG C-3') and ACT783R (5'-TACGAGTCCTTCTG GCCCAT-3') (P. Johnston, *personal communication*), Bt2a (5'-GGTAACCAAATCGGTGC TGCTTTC-3') and Bt2b (5'-ACCCTCAGTGT AGTGACCCTTGGC-3') (Glass and Donaldson, 1995), and ITS 4 (5'-TCCTCCGCTTATT GATATGC-3') and ITS 5 (5'-GGAAGTAAAA GTCGTAACAAGG-3') (White *et al*., 1990), respectively. The cycling parameters consisted of a 3 min denaturing step at 95°C followed by 34 cycles at 95°C for 1 minute, 52°C for 30 seconds, 72°C for 1 minute and a final cycle of 10 minute at 72°C.

Calmodulin was amplified using primers-pair CL1 (5'-GARTWCAAGGAGGCCTTCT C-3') and CL2 (5'-TTTTTGCATCATGAGTT GGAC-3') (P. Johnston, *personal communication*). The cycling parameters were initiated at 94°C for 2.5 minutes followed by 40 cycles at 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds and a final step at 72°C for 15 minutes.

The glutamine synthetase and glyceraldehyde 3-phosphate dehydrogenase were amplified with GSF1 (5'-ATGGCCGAGTACA TCTGG-3') and GSR1 (5'-GAACCGTCGAAG TTCCAC-3') (Guerber *et al*., 2003), and GD

F1 (5'-GCCGTCAACGACCCCTTCATTGA-3') and GDR1 (5'-GGGTGGAGTCGTACTTG AGCATGT-3') (Peres *et al*., 2008), respectively. The cycling parameters consisted of a denaturation step at 94°C for 4 minutes, followed by 34 cycles at 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 1 minute and a final cycle at 72°C for 10 minutes.

The PCR products were verified by staining with ethidium bromide on 1% agarose electrophoresis gels. DNA sequencing for partial of actin, β-tubulin-2 gene (tub2), calmodulin, glutamine synthetase, glyceraldehyde-3-phosphate dehydrogenase and the complete rDNA-ITS regions were performed by sequencing at the International Fungal Research and Development Centre, The Research Institute of Resource Insects, the Chinese Academy of Forestry, China.

Phylogenetic analysis

The accession numbers of all sequences are listed in Table 1. Sequences for each strain were aligned using Clustal X (Thompson *et al*., 1997). Alignments were manually adjusted to allow maximum alignment and maximum sequence similarity. Gaps were treated as missing data.

The analyses were similar to those used by Cai *et al*. (2006). Phylogenetic analyses were performed using PAUP* 4.0b10. Ambiguously aligned regions were excluded from all analyses. 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. Descriptive tree statistics such as tree length [TL], consistency index [CI], retention index [RI], rescaled consistency index [RC], homoplasy index [HI], and log likelihood [-ln L] (HKY model) were calculated for trees generated under different optimality criteria. Kishino-Hasegawa tests were performed in order to determine whether trees were significantly different. Clade stability of the trees resulting from the parsimony analyses were assessed by bootstrap analysis with 1000 replicates, each with 10 replicates of random stemwise addition of taxa (Felsenstein, 1985). Trees were figured in Treeview.

The model of evolution was estimated by using Mrmodeltest 2.2. 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 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 were used for calculating posterior probabilities (PP) in the majority rule consensus tree.

Results

Collection of Colletotrichum species

A summary of *Colletotrichum* isolates used in this study are listed in Table 2. Thirty-four isolates of *Colletotrichum* were collected from Pha Daeng and Mae Lod Village, which includes epiphytes, endophytes and pathogens. In addition, isolates of *C. acutatum*, *C. gloeosporioides* and *C. kahawae* were provided by BRIP, CBS and CABI Bioscience. *Colletotrichum falcatum* isolated from *Saccharum officinarum* was collected from Central Java Province, Indonesia and will be designated as the epitype for this species in a separate publication.

Morphological and cultural characterization

Differences in conidial morphology, colony characters and growth rates among the *Colletotrichum* isolates allowed them to be separated into three groups. Group 1 comprised 5 isolates; 2 epiphytes and 3 endophytes, Group 2 comprised 20 isolates; 12 epiphytes, 3 endophytes and 5 pathogens and Group 3 comprised 9 isolates; 2 epiphytes, 5 endophytes and 2 pathogens (Table 3).

Colony characters (Fig. 1): Distinct morphology on PDA was observed in each group after 7 days. Colonies produced by isolates from Group 1 varied from pale yellowish to pinkish with dense whitish-grey aerial mycelium and a few bright orange

Table 1. Sources of isolates used in this study and analysis.

Colletotrichum species	Isolates	Culture Collection	GenBank Accession Number					
			ACT	TUB-2	CAL	GS	GPDH	ITS
C. asianum	BML-I3	MFLU 090232 BCC	FJ 903188	FJ 907434	FJ 917501	FJ 972586	FJ 972571	FJ 972605
C. asianum	BPD-I4	MFLU 090233* BCC	FJ 907424	FJ 907439	FJ 917506	FJ 972595	FJ 972576	FJ 972612
C. asianum	BML-I14	MFLU 090234 BCC	FJ 907421	FJ 907436	FJ 917503	FJ 972598	FJ 972573	FJ 972615
C. siamense	BML-I6	MFLU 090229 BCC	FJ 907420	FJ 907435	FJ 917502	FJ 972599	FJ 972572	FJ 972604
C. siamense	BPD-I2	MFLU 090230* BCC	FJ 907423	FJ 907438	FJ 917505	FJ 972596	FJ 972575	FJ 972613
C. siamense	BML-I15	MFLU 090231 BCC	FJ 907422	FJ 907437	FJ 917504	FJ 972597	FJ 972574	FJ 972614
C. fructicola	BPD-I18	MFLU 090226 BCC	FJ 907427	FJ 907442	FJ 917509	FJ 972592	FJ 972579	FJ 972602
C. fructicola	BPD-I12	MFLU 090227 BCC	FJ 907425	FJ 907440	FJ 917507	FJ 972594	FJ 972577	FJ 972611
C. fructicola	BPD-I16	MFLU 090228* BCC	FJ 907426	FJ 907441	FJ 917508	FJ 972593	FJ 972578	FJ 972603
C. acutatum	BRIP 28519	BRIP 28519	FJ 907428	FJ 907443	FJ 917510	FJ 972591	FJ 972580	FJ 972601
C. acutatum	CBS 294.67	CBS 294.67	FJ 907429	FJ 907444	FJ 917511	FJ 972590	FJ 972581	FJ 972610
C. falcatum (epitype)	FAL	MFLU CBS	FJ 907431	GQ 289454	FJ 917513	FJ 972600	FJ 972585	FJ 972606
C. gloeosporioides	CBS 953.97	CBS 953.97	FJ 907430	FJ 907445	FJ 917512	FJ 972589	FJ 972582	FJ 972609
C. kahawae	IMI 319418	IMI 319418	FJ 907432	FJ 907446	FJ 917514	FJ 972588	FJ 972583	FJ 972608
C. kahawae	IMI 363578	IMI 363578	FJ 907433	FJ 907447	FJ 917515	FJ 972587	FJ 972584	FJ 972607

Note: ACT: actin; TUB-2: partial ß-tubulin (tub2); CAL: calmoudulin; GS: glutamine synthetase; GDPH: glyceraldehydes-3-phosphate dehydrogenase; ITS: complete rDNA-ITS region; MFLU: Mae Fah Luang University, Thailand; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; BRIP: Plant Pathology Herbarium, DPI&F, Queensland, Australia; IMI: CABI Europe – UK, Bakeham Lane, Egham, Surrey, UK; *holotype.

conidial masses near the inoculum point. Isolates from Group 2 produced greyish green, not when first colour is qualified as greyish colonies in the centre, with sparse white aerial mycelium, green in reverse and orange conidial masses near the inoculum point. Colonies produced by isolates from Group 3 varied from grey to dark grey with dense pale grey aerial mycelium.

Growth rate (Table 4): There was a statistically significant difference in growth rates among the 34 isolates. Isolates of *Colletotrichum* Group 3 (10.72 ± 0.53 mm day grew significantly faster, followed by Group 1 (9.12 ± 1.95 mm/day) and Group 2 (5.09 ± 0.38 mm/day) ($P = 0.05$).

Conidia morphology (Fig. 1): Conidia produced by isolates of *Colletotrichum* Group 1 varied from fusiform with obtuse to slightly rounded ends to sometimes oblong. *Colletotrichum* Group 2 produced cylindrical conidia with obtuse ends (oblong) with narrowing at the centre. *Colletotrichum* Group 3 produced cylindrical conidia with obtuse to slightly rounded ends. There were statistically significant differences in length and width of conidia among the three groups (Table 4).

Appressoria morphology: There was little distinction among the groups in size and shape of appressoria. Appressoria shape produced by slide cultures varied from ovoid, clavate or slightly irregular to irregular in shape (Table 4, Fig. 1).

Biochemical testing

All *Colletotrichum* species isolated from coffee berries were tested for the ability to utilize citrate or tartrate as a sole source of carbon. All isolates from Thailand grew and changed the colour of the indicator to a dark blue to purple in the tartrate test (Table 5). In the citrate test, four isolates of *Colletotrichum* Group 1, 17 of *Colletotrichum* Group 2 and six of *Colletotrichum* Group 3 were unable to grow sufficiently to produce any colour change.

Pathogenicity testing

Both non-wound/drop and wound/drop inoculation methods using green and red berries resulted in fruit infection (Table 6). Fruit symptoms from all morphogroups were similar to each other. The wound/drop inoculation method produced a higher incidence of disease than the non-wound/drop method and

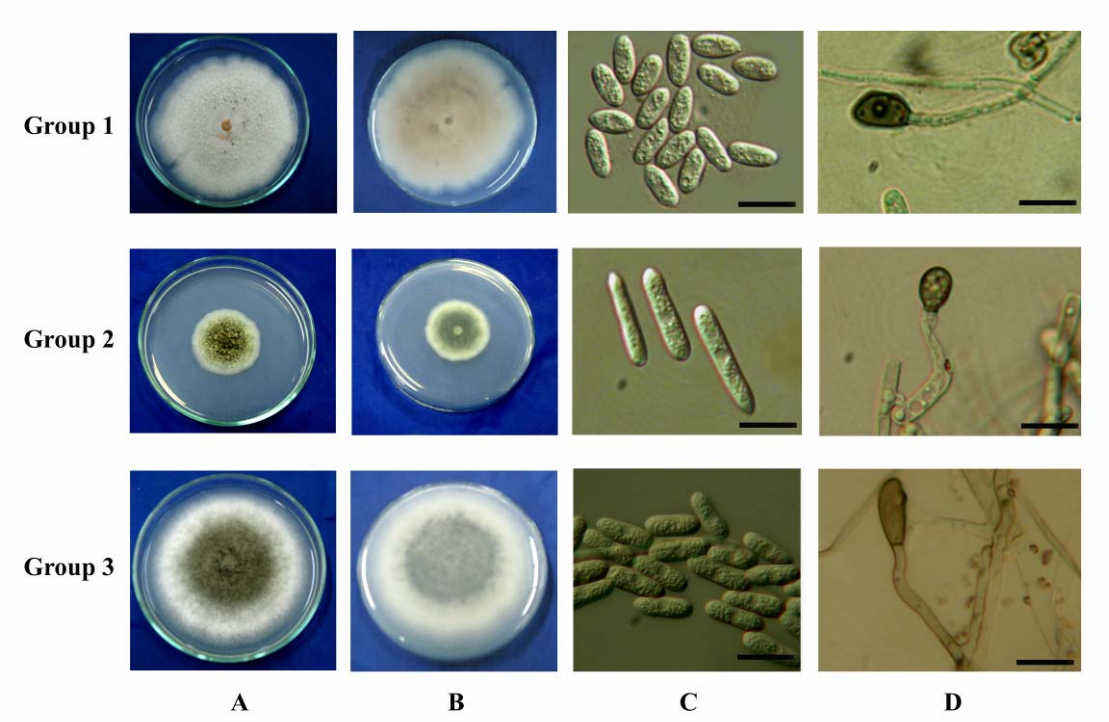

Fig. 1. Morphology and cultural characters of *Colletotrichum* isolates. Upper (**A**) and reverse (**B**) of cultures on PDA 7 days after inoculation; (**C**) Conidia; (**D**) Appressoria (Bars = 10 µm).

Table 2. Synopsis of characters of *Colletotrichum* isolates.

Species	Number of isolates	Substrate and host	Location
Colletotrichum Group 1 (***Colletotrichum siamense***)	2	Coffee berries (*Coffea arabica*)	Pha Daeng Village, Chiang Mai Thailand
Colletotrichum Group 1 (***Colletotrichum siamense***)	3	Coffee berries (*Coffea arabica*)	Mae Lod Village, Chiang Mai Thailand
Colletotrichum Group 2 (***Colletotrichum asianum***)	9	Coffee berries (*Coffea arabica*)	Pha Daeng Village, Chiang Mai Thailand
Colletotrichum Group 2 (***Colletotrichum asianum***)	11	Coffee berries (*Coffea arabica*)	Mae Lod Village, Chiang Mai Thailand
Colletotrichum Group 3 (***Colletotrichum fructicola***)	7	Coffee berries (*Coffea arabica*)	Pha Daeng Village, Chiang Mai Thailand
Colletotrichum Group 3 (***Colletotrichum fructicola***)	2	Coffee berries (*Coffea arabica*)	Mae Lod Village, Chiang Mai Thailand
C. acutatum	1	Papaya (*Carica papaya*)	Type of *C. acutatum*[#] Queensland, Australia
C. acutatum	1	Papaya (*Carica papaya*)	Ex-epitype : Queensland, Australia[#]
C. falcatum	1	Sugarcane leaves (*Saccharum officinarum*)	Epitype : Indonesia
*C. gloeosporioides**	1	Citrus leaves (*Citrus sinensis*)	Epitype of *Vermicularia gloeosporioides* Italy, Calabria, Lamezia terme
C. kahawae	2	Coffee (*Coffea arabica*)	Holotype (IMI 319418), Kenya Paratype (IMI 363578), Kenya

[#]See Shivas and Tan (2009) for recent name changes.

red berries were more susceptible than green berries. Statistically, the non-wound/drop inoculation method, when applied to green berries, did not show any difference among *Colletotrichum* groups in contrast to the red berries. In the wound/drop inoculation method, using both green and red berries, *Colletotrichum* Group 3 produced the highest incidence of disease.

Phylogenetic analyses

The actin gene comprised 494 characters after alignment, of which 58 characters are parsimony informative (11.7%). Kishino-Hasegawa (KH) test showed that eight trees generated from parsimonious analysis were not significantly different. The β-tubulin (tub2) gene comprised 506 characters after alignment, of which 95 characters were parsimony informative (18.8%). Parsimony analysis generated 140 equally parsimonious trees. The calmodulin gene comprised 838 characters after alignment, of which 193 characters were parsimony informative (23%). KH test showed that three generated trees from parsimony analysis were not significantly different. The Glutamine synthetase gene comprised 1123 characters after alignment, of which 377 characters were parsimony informative (33.6%). KH test showed that two generated trees were not significantly different. The glyceraldehyde 3-phosphate dehydrogenase gene comprised 185 characters after alignment, of which 94 characters were parsimony informative (50.8%). KH test showed that four generated trees were not significantly different. The complete rDNA-ITS region comprised 630 characters after alignment, of which 40 characters were parsimony informative (6.3%). KH test showed that 225 generated trees were not significantly different. The above single gene phylogenetic trees give essentially similar topologies and species groupings but the parsimony bootstrap and posterior probabilities are relatively low (trees not shown). Among these, the trees generated from GS and CAL are better resolved as compared to other gene trees possibly because these two dataset contain more parsimony informative characters than other datasets.

The combined datasets of partial ACT, TUB2, CAL, GS, GPDH and the complete rDNA-ITS region comprised 2362 characters after alignment, of which 462 characters were

Table 3. Summary of the life mode of *Colletotrichum* isolates.

Morphogroup	Isolates	Life Mode
Colletotrichum Group 1	BPD-I2	endophyte
(5 isolates)	BPD-I13	epiphyte
	BML-I6	endophyte
(*Colletotrichum siamense*)	BML-I13	epiphyte
	BML-I15	endophyte
Colletotrichum Group 2	BPD-I3	epiphyte
(20 isolates)	BPD-I4	endophyte
	BPD-I6	epiphyte
(*Colletotrichum asianum*)	BPD-I7	epiphyte
	BPD-I8	epiphyte
	BPD-I9	epiphyte
	BPD-I10	epiphyte
	BPD-I11	epiphyte
	BPD-I14	endophyte
	BML-I1	epiphyte
	BML-I2	epiphyte
	BML-I3	pathogen
	BML-I4	epiphyte
	BML-I5	epiphyte
	BML-I8	epiphyte
	BML-I9	pathogen
	BML-I10	pathogen
	BML-I11	pathogen
	BML-I14	endophyte
	BML-I16	pathogen
Colletotrichum Group 3	BPD-I1	epiphyte
(9 isolates)	BPD-I5	epiphyte
	BPD-I12	endophyte
(*Colletotrichum fructicola*)	BPD-I15	endophyte
	BPD-I16	endophyte
	BPD-I17	endophyte
	BPD-I18	pathogen
	BML-I7	endophyte
	BML-I12	pathogen

parsimony informative. Pasimony analysis generated only one tree (TL = 1680, CI = 0.889, RI = 0.884, RC = 0.751, HI = 0.111) shown in Fig. 2. The phylogram constructed using combined dataset showed that coffee berry isolates represented in Groups 1, 2 and 3 clustered into three distinct clades with high bootstrap support (>90%). Groups 1, 2 and 3 were distinct clades from the type species of *C. acutatum*, *C. gloeosporioides* and *C. kahawae*, which indicate that they are new species. Furthermore, the combination of these genes revealed that *C. gloeosporioides* and *C. kahawae* are distinct species supported by high bootstrap confidence (100%).

Taxonomy

Colletotrichum asianum Prihastuti, L. Cai & K.D. Hyde, **sp. nov.** (Figs 3a-j) MycoBank: 515408.

Coloniae albae-viridis vel griseo-viridis. Conidia 8.7-20.3 × 3-4.7 μm, unicellularae, laevae, hyalinae, cylindrici, ad apicem obtuse. Appressoria 4.7-10.7 × 3.3-6.7 μm, brunnea vel atro-brunnea, irregulariter ovoidea, clavate.

Etymology: *asianum*, in reference to the region where the species was found.

Description: Colonies on PDA at first greenish white and becoming greyish green to dark green at the centre with age, reverse dark green at the centre, max. of 42 mm diam. in 7 days at 28°C, growth rate 4.67-5.5 mm/day ($\bar{x}$ = 5.20 ± 0.31, n = 9) (Figs 3a,b). Aerial mycelium in small tufts, white, sparse, with orange to dark orange conidial masses. *Sclerotia* absent. *Acervuli* absent in culture. *Setae* absent. *Conidia* 8.7-20.3 × 3-4.7 μm ($\bar{x}$ = 12.87 ± 2.52 × 3.40 ± 0.46, n = 180), common both in mycelium and conidial masses, one-celled, smooth-walled, guttulate, hyaline, cylindrical with obtuse ends (oblong) with slight narrowing at the center (Figs 3c,d,f,g,i,j). *Appressoria* 4.7-10.7 × 3.3-6.7 μm ($\bar{x}$ = 7.67 ± 1.72 × 5.07 ± 0.96, n = 30) in slide cultures, mostly formed from conidia, brown to dark brown, ovoid, clavate to irregular in shape and often becoming complex with age (Figs 3e,h).

Teleomorph: not produced in culture.

Holotype: THAILAND, Chiang Mai Province, Mae Taeng District, Mae Lod Village, Royal Agricultural Project Coffee, on berry of *Coffea arabica*, 16 January 2008, H. Prihastuti (MFLU 090233); extype living culture BCC.

Known distribution: Chiang Mai Province, Thailand.

Additional specimens examined: THAILAND, Chiang Mai Province, Mae Taeng District, Pha Daeng Village, near Mushroom Research Centre, on berry of *Coffea arabica*, 12 December 2007, H. Prihastuti (**Paratype** in MFLU 090234, extype living culture BCC); THAILAND, Chiang Mai Province, Mae Taeng District, Mae Lod Village, Royal Agricultural Project Coffee, on berry of *Coffea arabica*, 16 January 2008, H. Prihastuti (**Paratype** in MFLU 090232, extype living culture BCC).

Colletotrichum fructicola Prihastuti, L. Cai & K.D. Hyde, **sp. nov.** (Figs 4a-n) MycoBank: 515409.

Table 4. Summary of morphological data of *Colletotrichum* isolates.

Species	Colony characters	Conidia			Appresoria		Growth rate (mm/day)
		Length (µm)	Width (µm)	Shape	Length (µm)	Width (µm)	
Colletotrichum Group 1 **(*C. siamense*)**	Cottony, dense greyish white aerial mycelium, pale yellowish to pinkish colony	10.17±1.49 a (7-18.3)	3.60±0.48 c (3-6)	Fusiform	6.67±1.89 a (4-15.3)	4.26±0.45 a (3.5-5.3)	9.12±1.95 b (6.5-11.5)
Colletotrichum Group 2 **(*C. asianum*)**	Tufted, sparse white aerial mycelium, greyish green, slow growing	12.26±1.66 c (7-20.3)	3.38±0.36 a (3-5.7)	Cylindrical	6.72±1.34 a (4.3-10.7)	4.72±0.85 b (3.3-8)	5.09±0.38 a (4.08-5.67)
Colletotrichum Group 3 **(*C. fructicola*)**	Cottony, dense pale grey aerial mycelium, grey to dark grey colony, fast growing	11.37±0.96 b (9.7-14)	3.54±0.35 b (3-4.3)	Cylindrical	7.54±1.55 b (4.3-11.7)	4.35±0.85 a (3-7.3)	10.72±0.53 c (9.67-11.5)

*The mean difference is significant at the 0.05 level; values with same letter based on Duncan's multiple range tests in a column do not differ significantly

Table 5. Summary of carbohydrate utilization by *Colletotrichum* isolates.

Species	Citrate		Tartrate	
	Positive	Negative	Positive	Negative
Colletotrichum Group 1 (5 isolates) **(*C. siamense*)**	1	4	5	0
Colletotrichum Group 2 (20 isolates) **(*C. asianum*)**	3	17	20	0
Colletotrichum Group 3 (9 isolates) **(*C. fructicola*)**	3	6	9	0

Note: Number of tests showing positive/negative utilization of organic acids as a sole carbon source. (Positive: able to grow sufficiently on citrate or tartrate to give a dark blue or purple colour; negative: unable to grow sufficiently on citrate or tartrate to produce any colour change).

Table 6. Summary of percentage of infected area of fruits caused by *Colletotrichum* isolates.

Species	Isolate	Non-Wound/drop		Wound/drop	
		Green	Red	Green	Red
Colletotrichum	BML-I6	0	25.57	0	42.33
Group 1	BML-I15	19.10	60.27	54.57	54.43
(***C. siamense***)	BPD-I2	11.13	51.23	46.37	53.80
	Mean	**10.08 ± 15.50a**	**45.69 ± 19.52b**	**33.64 ± 25.52a**	**50.19 ± 8.10a**
Colletotrichum	BML-I3	26.30	8.93	17.37	42.57
Group 2	BML-I14	0	25.47	43.30	46.60
(***C. asianum***)	BPD-I14	0	41.10	0	100
	Mean	**8.77 ± 17.45a**	**25.17 ± 16.52a**	**20.22 ± 24.41a**	**63.06 ± 29.28a**
Colletotrichum	BPD-I12	16.03	51.27	51.10	100
Group 3	BPD-I16	0	100	22.97	100
(***C. fructicola***)	BPD-I18	0	85.47	52.13	69.50
	Mean	**5.34 ± 16.03a**	**78.91 ± 25.06c**	**42.07 ± 17.50a**	**89.93 ± 20.17b**

*The mean difference is significant at the 0.05 level; values with same letter based on Duncan's multiple range tests in a column do not differ significantly.

Coloniae albae vel griseo. Conidia 9.7-14 × 3-4.3 μm, unicellularae, laevae, hyalinae, cylindrici, ad apicem obtuse. Appressoria 4.3-9.7 × 3.7-7.3 μm, brunnea vel atro-brunnea, irregulariter ovoidea, clavate. *Ascomata* 312-385 × 354-490 μm, atro-brunnea vel brunnea, globosa vel subglobosa. *Asci* 30-55 × 6.5-8.5 μm, unitunicati, tenuitunicata, clavate vel cymbiformis. *Ascospores* 9–14 × 3–4 μm, unicellularae, hyalinae, leniter curvatae vel curvatae.

Etymology: *fructicola*, in reference to the substrate on which the type was found.

Description: Colonies on PDA at first white, becoming grey to dark grey at the centre with age, in reverse greyish green with white halo max. of 83 mm diam. in 7 days at 28°C, growth rate 10.58-11.5 mm/day ($\bar{x}$ = 11 ± 0.25, n = 9) (Figs 4a,b). Aerial mycelium pale grey, dense, cottony, without visible conidial masses. *Sclerotia* absent. *Acervuli* absent in culture. *Setae* absent. *Conidia* 9.7-14 × 3-4.3 μm ($\bar{x}$ = 11.53 ± 1.03 × 3.55 ± 0.32, n = 180), common in mycelium, one-celled, smooth-walled with a large guttule at the centre and surrounded by smaller guttules, hyaline, cylindrical with obtuse to slightly rounded ends, sometimes oblong (Figs 4c-f). *Appressoria* 4.3-9.7 × 3.7-7.3 μm ($\bar{x}$ = 7.35 ± 1.28 × 4.49 ± 0.91, n = 30) in slide cultures, mostly formed from mycelia, brown to dark brown, ovoid, clavate and slightly irregular to irregular in shape and often becoming complex with age (Fig. 4g).

Teleomorph: *Glomerella* sp.

Ascomata 312-385 × 354-490 μm ($\bar{x}$ = 345.67 ± 36.83 × 431.33 ± 69.89, n = 10), light brown to brown, globose to subglobose, with hairs, semi-immersed or completely immersed in PDA. Peridium of *textura angularis*, thick-walled (Fig. 4n). *Asci* 30-55 × 6.5-8.5 μm ($\bar{x}$ = 41.22 ± 7.02 × 7.61 ± 0.58, n = 25), unitunicate, thin-walled, 6-8 spored, clavate or cymbiform (Figs 4h-k). *Ascospores* 9–14 × 3–4 μm ($\bar{x}$ = 11.91 ± 1.38 × 3.32 ± 0.35, n = 25), one-celled, hyaline, large guttulate at the centre and surrounded by small guttules, slightly curved to curved with obtuse to slightly rounded ends (Fig. 4l,m). Many formed within 3 months.

Holotype: THAILAND, Chiang Mai Province, Mae Taeng District, Pha Daeng Village, near Mushroom Research Centre, on berry of *Coffea arabica*, 12 December 2007, H. Prihastuti (MFLU 090228); extype living culture BCC.

Known distribution: Chiang Mai Province, Thailand.

Additional specimens examined: THAILAND, Chiang Mai Province, Mae Taeng District, Pha Daeng Village, near Mushroom Research Centre, on berry of *Coffea arabica*, 12 December 2007, H. Prihastuti (**Paratype** in MFLU 090227, extype living culture BCC); THAILAND, Chiang Mai Province, Mae Taeng District, Pha Daeng Village, near Mushroom Research Centre, on berry of *Coffea arabica*, 12 December 2007, H. Prihastuti (**Paratype** in MFLU 090226, extype living culture BCC).

Colletotrichum siamense Prihastuti, L. Cai & K.D. Hyde, **sp. nov.** (Fig. 5)
MycoBank: 515410.

Coloniae albae, vel pallide brunnea. Conidia 7-18.3 × 3–4.3 μm, unicellularae, laevae, hyalinae, fusiformes, ad apicem obtuse. Appressoria 4.7-8.3 × 3.5-5 μm, brunnea, irregulariter ovoidea vel clavate.

Etymology: *siamense*, in reference to the region where the type was found.

Description: Colonies on PDA at first white and becoming pale brownish to pinkish, reverse pale yellowish to pinkish, max. of 82 mm diam. in 7 days at 28°C, growth rate 6.58–11.5 mm/day ($\bar{x}$ = 9.30 ± 1.93, n = 9) (Figs 5a,b). Aerial mycelium greyish white, dense, cottony, with visible conidial masses at the inoculum point. *Sclerotia* present in some culture. *Setae* absent. *Conidiomata* brown to dark brown, conspicuous for their brown setae. *Conidia* 7-18.3 × 3–4.3 µm ($\bar{x}$ = 10.18 ± 1.74 × 3.46 ± 0.36, n = 180), common in mycelium, one-celled, smooth-walled, guttulate, hyaline, fusiform with obtuse to slightly rounded ends, sometimes oblong (Figs 4e-i). *Appressoria* 4.7-8.3 × 3.5-5 µm ($\bar{x}$ = 6.67 ± 1.05 × 4.13 ± 0.44, n = 30) in slide cultures, mostly formed from mycelia, brown, ovoid, sometimes clavate and often becoming complex with age (Fig. 4d).

Teleomorph: not produced in culture.

Holotype: THAILAND, Chiang Mai Province, Mae Taeng District, Mae Lod Village, Royal Agricultural Project Coffee, on berry of *Coffea arabica*, 16 January 2008, H. Prihastuti (MFLU 090230); extype living culture BCC.

Known distribution: Chiang Mai Province, Thailand.

Additional specimens examined: THAILAND, Chiang Mai Province, Mae Taeng District, Pha Daeng Village, near Mushroom Research Centre, on berry of *Coffea arabica*, 12 December 2007, H. Prihastuti (**Paratype** in MFLU 090229, extype living culture BCC) ;

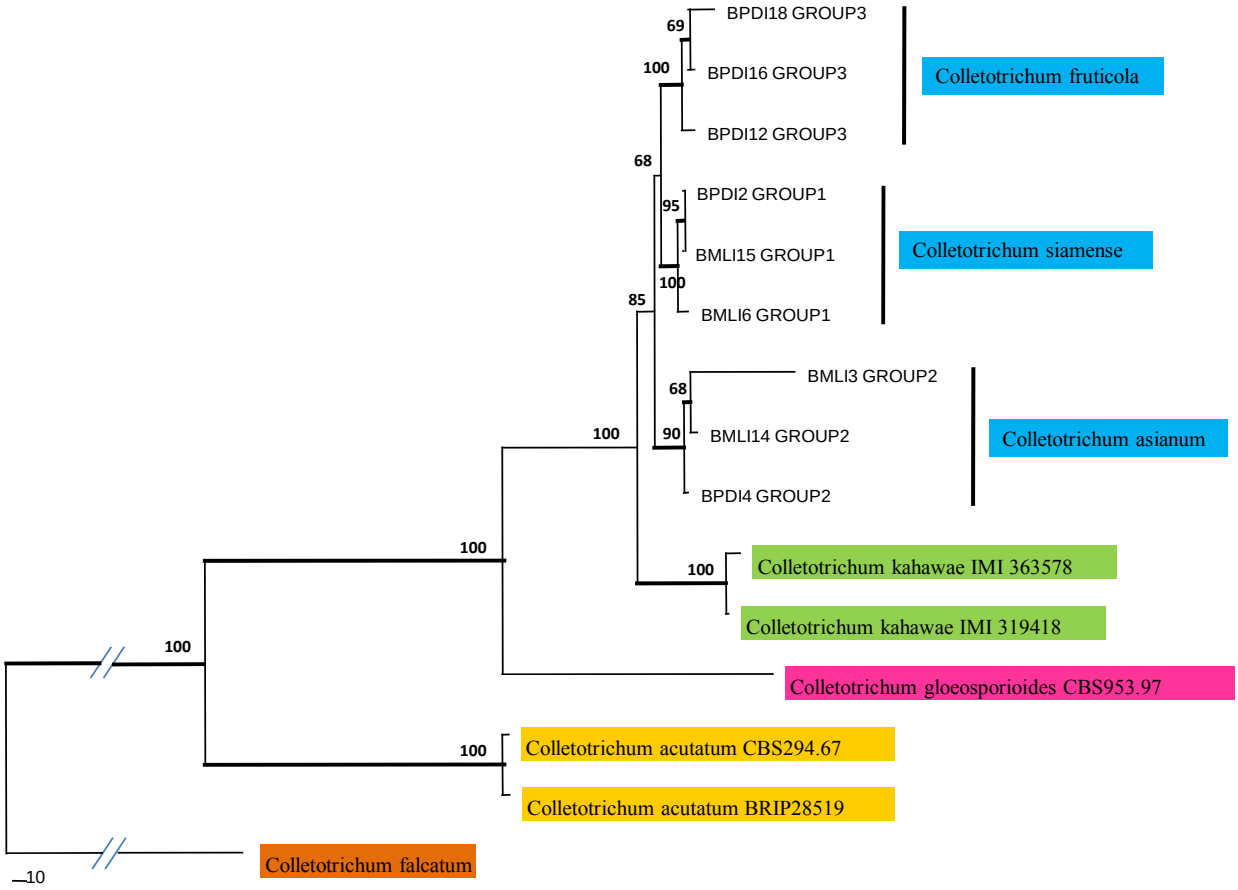

Fig. 2. Maximum parsimony phylogram showing phylogenetic relationships among isolates of *Colletotrichum* from coffee berries in Chiang Mai, Thailand and selected type species of *Colletotrichum* based on combined ACT, TUB2, CAL, GS, ITS and GPDH sequences**.** Data were analysed with random addition 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 *Colletotrichum falcatum*.

Fig. 3. *Colletotrichum asianum* (from holotype). Upper (a) and reverse (b) sides of cultures on PDA 7 days after inoculation; (c, d, f, g, i, j) conidia; (e and h) appressoria; (g) conidiogenous cell (Bars = 10 µm).

THAILAND, Chiang Mai Province, Mae Taeng District, Mae Lod Village, Royal Agricultural Project Coffee, on berry of *Coffea arabica*, 16 January 2008, H. Prihastuti (**Paratype** in MFLU 090231, ex-type living culture BCC).

A synopsis of species of *Colletotrichum* presently known from coffee is listed in Table 7. *Colletotrichum asianum* is similar to *C. kahawae* in growth rate and colony colour. The conidial shape of these species however is distinctly different (straight in *C. kahawae* versus cylindrical with narrowing centre in *C. asianum*) (Table 7). Although these species are culturally similar, they differ both morphologically and genetically. *Colletotrichum fructicola* produces overlapping, but overall smaller conidia as compared to *C. kahawae* (7.5-17 × 3.5-5 µm in *C. kahawae* versus 9.7-14 × 3-4.3 µm in *C. fructicola* (Table 7). *Colletotrichum fructicola* differs from *C. kahawae* in morphology, physiology and genetically. *Colletotrichum siamense* resembles *C. acutatum* in cultural characters and conidial shape. The conidia of *C. siamense* are fusiform, sometimes with obtuse slightly rounded ends, while *C. acutatum* has pinkish colonies and fusiform conidia. *Colletotrichum acutatum* has been also recorded associated with coffee berries, but it clustered in a different clade to *C. siamense*.

Discussion

The main objectives of this study were to characterize the species of *Colletotrichum*

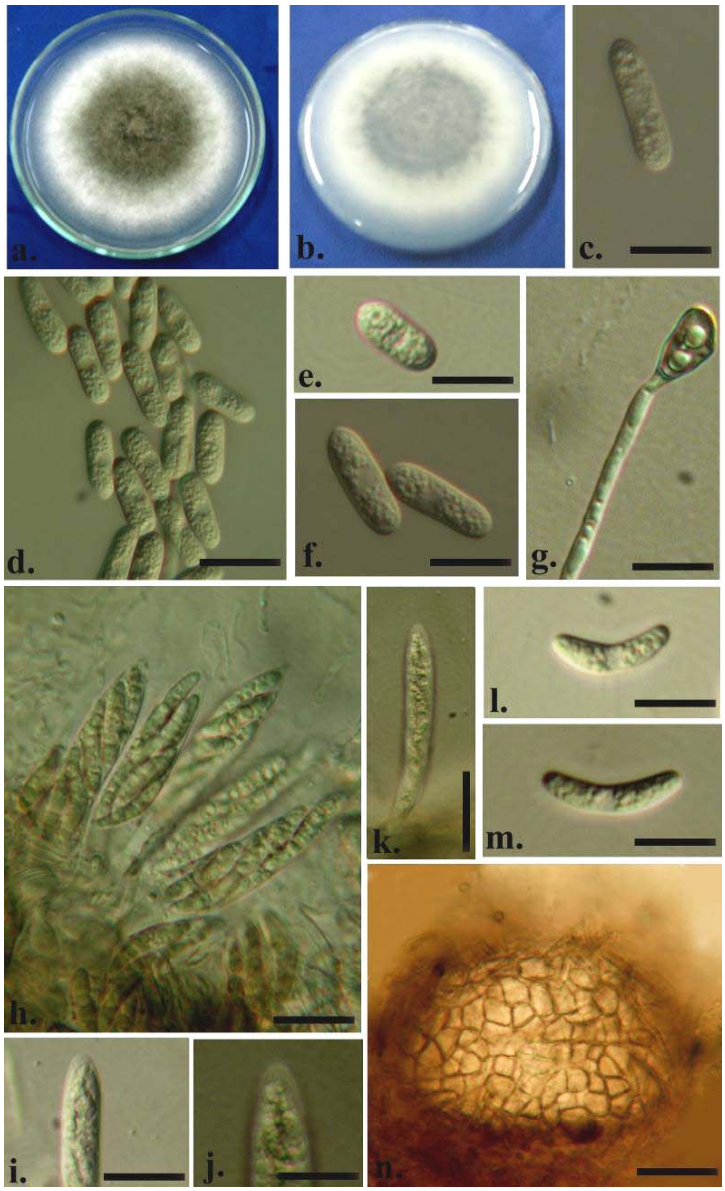

Fig. 4. *Colletotrichum fructicola* (from holotype). Upper (a) and reverse (b) sides of cultures on PDA 7 days after inoculation; (c-f) conidia; (g) appressorium; (h-k) asci; (l, m) ascospores; (n) peridium (Bars: a-m = 10 µm, n = 100 µm).

associated with coffee berries in northern Thailand and to determine whether multi-gene phylogenies were congruent with morphological characters. Phylogenies inferred from gene sequences support a close relationship of isolates with similar morphological characters in Groups 1, 2 and 3. This shows that DNA sequence analyses were useful for delimiting species of the genus *Colletotrichum*. DNA sequence analyses have been suggested by various authors to overcome the inadequacies of morphological criteria (Sreenivasaprasad *et al*., 1996; Abang *et al*., 2002; Moriwaki *et al*., 2002; Peres *et al*., 2002; Guerber *et al*., 2003; Photita *et al*., 2005; Du *et al*., 2005; Shenoy *et al*., 2007; Whitelaw-Weckert *et al*., 2007; Peres *et al*., 2008; Than *et al*., 2008a,b; Crouch *et al*., 2009).

Usefulness of characters used in Colletotrichum identification

Based on the shape and size of conidia, the 34 isolates clustered into three morphogroups. The differences of conidial size were statistically significant, both in length and width of conidia. Differentiation among these *Colletotrichum* morphogroups therefore appears to be reliable based on traditional methods such as conidial shape and size. Similar results have been reported by Simmonds (1965), Sutton (1962, 1965, 1966, 1968, 1980) and von Arx (1981).

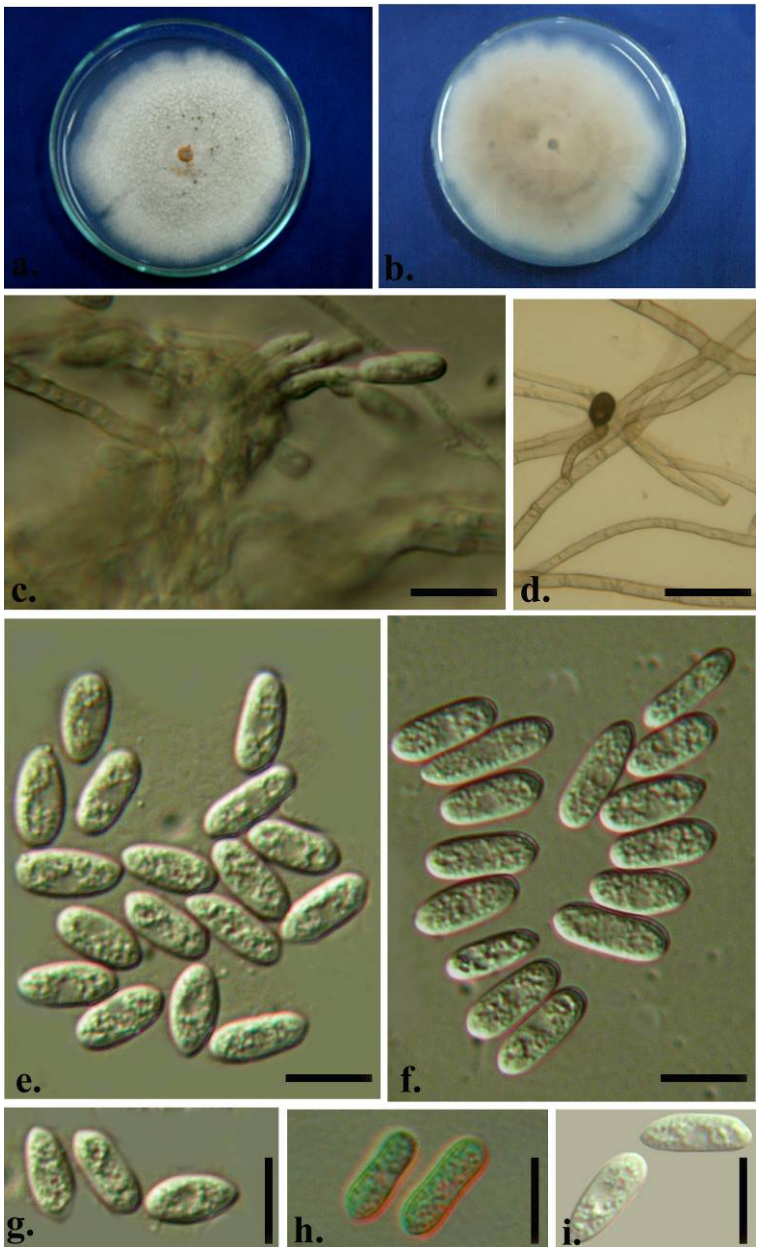

Fig. 5. *Colletotrichum siamense* (from holotype). Upper (a) and reverse (b) sides of cultures on PDA 7 days after inoculation; (c) conidiogenous cell; (d) appressorium; (e-i) conidia (Bars = 10 µm).

However, appressorial size and shape showed little distinction among the three morphogroups. Sanders and Korsten (2003) and Than *et al*. (2008a) found that appressorial morphology was uninformative in distinguishing *Colletotrichum* species. However, appressorial characters were thought to warrant a more detailed examination (Simmonds, 1965) and were useful in differentiating grass species in association with host (Crouch *et al*., 2009).

Cultural characters and colony growth rates *in vitro* were important characteristics for distinguishing the three *Colletotrichum* morphogroups. Similar results have been reported for *Colletotrichum* species from chilli (Than *et al*., 2008a).

It has previously been shown that the two isolates of *C. kahawae* used in this study were unable to grow on either citrate or tartrate sufficiently to produce any colour change (Waller *et al*., 1993; Bridge *et al*. (2008). The inability of *C. kahawae* to utilize either citrate or tartrate as a sole carbon source provides a useful test to distinguish *C. kahawae* from the other closely related taxa of coffee. This inability may be indirectly related to pathogenicity, but directly related to a reduced saprobic capability (Waller *et al*., 1993; Derso and Waller, 2003; Bridge *et al*., 2008).

Pathogenicity testing using isolates from the three morphogroups of *Colletotrichum* showed that all isolates were pathogenic to

both green and red berries. Waller *et al*. (1993) showed that *C. kahawae* and *C. gloeosporioides* can be distinguished on the basis of pathogenicity to green berries. The two strains used in this study have been shown to be pathogenic to seedlings of coffee and cause a serious disease of coffee berries in Africa (Waller *et al*., 1993). Lesion development may vary considerably with the interplay of factors such as variety and condition of the fruit, humidity and temperature, and the concentration of inoculum (Simmonds, 1965; Freeman *et al*., 1998). This result may not accurately reflect the true virulence potential. Some of the Thai isolates were also isolated as epiphytes and endophytes and may be opportunistic pathogens. Future research should attempt to determine the pathogenicity testing of *Colletotrichum* species according to natural infections rather than artificial inoculations.

The phylogenetic placement of *Colletotrichum* isolates from coffee berries based on a single gene resulted in unstable topology with very low branch support (data not shown). This shows that a single gene may be inadequate to infer the relationships in a *Colletotrichum* species complex. However, the phylogenetic grouping based on the combined datasets differentiated the coffee berry isolates into three highly-supported phylogenetic lineages which appear to be congruent with their morphological characters. Similar results have been reported from the *Colletotrichum* species complexes associated with grasses (Crouch *et al*., 2006). Sreenivasaprasad *et al*. (1993) showed that *C. kahawae* is very close to *C. gloeosporioides* on the basis of rDNA sequences. However, this was based on a single base difference in rDNA sequence data used to discriminate related taxa. Due to the limited number of informative sites identified, the underlying difference is very small. Crouch *et al*. (2006) used data from multiple genes to estimate the limits of gene flow within and between populations of *Colletotrichum* species associated with grasses. Cannon *et al*. (2008) showed that *C. kahawae* is closely related to *C. gloeosporioides* aggregate based on rDNA-ITS sequence analysis. The results presented here confer with Cannon *et al*. (2008) and demonstrate that multi-gene phylogenies can distinguish *C. kahawae* and *C. gloeosporioides* as distinct species Previously, *C. kahawae* was differentiated from *C. gloeosporioides* by morphological, biochemical and pathogenic characteristics (Waller *et al*., 1993; Derso and Waller, 2003).

The traditional taxonomy of most taxa within *Colletotrichum* has been based primarily upon variation in conidial size and shape, appressoria, colony characters and host association (Bailey and Jeger, 1992; Sutton, 1992). These characters may not be informative for species identification within *Colletotrichum*, which is vital for developing and implementing disease management, resistance breeding and pathogen control (Freeman *et al*., 1998). A combination of molecular diagnostic tools with traditional morphological techniques is an appropriate and reliable approach for studying *Colletotrichum* species complexes (Cannon *et al*., 2000; Abang, 2003; Than *et al*., 2008a).

There has been controversy as to whether *Colletotrichum kahawae* should be a valid species or a sub-population of *C. gloeosporioides*, as *C. kahawae* can only be distinguished from *C. gloeosporioides* and other close relatives by biochemical and physiological characters (Correll *et al*., 2000; Cannon *et al*., 2000). We support the legitimacy of the name *C. kahawae* because 1) it is phylogenetically distant to *C. gloeosporioides* and other close relatives (Fig. 2), and 2) there are significant phenotypic differences including physiological and biochemical characters between this and other species. Here an important implication is that the phenotypic characters of a *Colletotrichum* species should be expanded to a polyphasic sense as discussed by Cai *et al*. (2009). Species reorganization solely based on morphology has long been shown to be taxonomically inadequate and there is a great need to apply more characters to the taxonomy of *Colletotrichum* (Sutton, 1962; 1965; 1980; 1992; Du *et al*., 2005; Crouch *et al*., 2009; Cai *et al*., 2009). The relationship between *C. kahawae* and *C. gloeosporioides* is a good example as they cannot be distinguished by morphology alone, but bear sufficient distinctions in other characters and DNA sequence data. The above justifications can also be used to support the establishment of the three new species from coffee described in this

paper, as they are phylogenetically and phenotypically distinct. Similar practices have been successfully applied in other economically important groups such as *Aspergillus*, *Lasiodiplodia, Penicillium* and *Fusarium* (Frisvad and Samson, 2004; Samson and Varga, 2007; Alves *et al*., 2008; Kvas *et al*., 2009).

Species of Colletotrichum known from coffee

The taxonomy of *Colletotrichum* is confused, both for the anamorphic species and its teleomorph *Glomerella*. Therefore, by establishing the accurate identification of *Colletotrichum* species associated with coffee berries in northern Thailand, this study has introduced three new species which can be identified based on morphological, cultural and biochemical characters, pathogenicity testing and phylogenetic analyses. A synopsis of species of *Colletotrichum* presently known from coffee is listed in Table 7. The following new species are introduced and justified in this paper.

1. ***Colletotrichum asianum*** is similar to *C. kahawae* in growth rate and colony colour. The conidial shape of these species however is distinctly different (straight in *C. kahawae* versus cylindrical with narrowing centre in *C. asianum*) (Table 7). Although these species are culturally similar, they differ both morphologically and genetically.

2. ***Colletotrichum fructicola*** produces overlapping, but overall smaller conidia as compared to *C. kahawae* (7.5-17 × 3.5-5 µm µm in *C. kahawae* versus 9.7-14 × 3-4.3 µm in *C. fructicola*) (Table 7). *Colletotrichum fructicola* differs from *C. kahawae* in morphology, physiology and genetically, thus this species is introduced as a new species.

3. ***Colletotrichum siamense*** resembles *C. acutatum* in cultural characters and conidial shape. The conidial of *C. siamense* are fusiform, sometimes with obtuse slightly rounded ends. Generally, fusiform conidia are recognized in *C. acutatum* which have pinkish colonies. The conidial shape of these species however is slightly different (fusiform in *C. acutatum* versus fusiform with obtuse slightly rounded ends in *C. siamense*) (Table 7). *Colletotrichum acutatum* has been recorded associated with coffee berries, but *Colletotrichum siamense* is introduced as a new species because it clustered in a different clade from the type of *C. acutatum*.

Phylogeny

Colletotrichum asianum, *C. fructicola* and *C. siamense* appear to be closely related to *C. kahawae*, perhaps due to the fact that they were isolated from the same host. This suggests that host-specificity may be relevant to taxonomic placement of *Colletotrichum* species. Similar results have been reported for *C. gloeosporioides* from almond in Israel (Freeman *et al*., 2000). The combined datasets of partial ACT, TUB2, CAL, GS, GPDH and ITS region of the three new species show the distinct difference from other species of *Colletotrichum*, and this is congruent with their morphological and physiological characters.

Life mode

The three new species from coffee each comprised isolates from lesions (pathogens), apparently healthy berry tissues (endophytes) and the surface of berries (epiphytes). This indicates the each species is possibly able to survive in any of the three life modes and are opportunistic pathogens. Several studies have shown that *Colletotrichum* species occur in epiphytic and endophytic stages from different plant hosts (Photita *et al*., 2004; Santamaría and Bayman, 2005; Promputtha *et al*., 2007; Huang *et al*., 2008, 2009; Tao *et al*., 2008). Furthermore, pathogenicity testing showed that all the isolates were pathogenic. However, this experiment was conducted under controlled laboratory conditions. In contrast, Photita *et al*. (2004) have shown that *C. gloeosporioides* which occurred as endophytes on *Musa acuminata* did not produce any disease symptoms. On the other hand, *C. magna* has been investigated to elucidate the fungal symbiotic lifestyle based on the molecular genetics (Rodriguez and Redman, 2000). Rodriguez and Redman (2000) have shown that the mutation of a single genetic locus appears to be responsible for symbiotic lifestyle alteration in *C. magna*. The contribution of the genetic basis of *Colletotrichum* lifestyle on varied hosts and sites will need further investigation. On coffee, all of the new species were able to penetrate and infect non-wounded berries.

Table 7. Synopsis of species of *Colletotrichum* known from coffee.

Characters	***Colletotrichum coffeanum***	***Colletotrichum coffaeophilum***	***Colletotrichum kahawae***	***Colletotrichum gloeosporioides***	***Colletotrichum asianum***	***Colletotrichum siamense***	***Colletotrichum fructicola***
Colony	Undescribed	Undescribed	Grey, becoming grey to dark, olivaceous grey, dark greenish in reverse, max. 32 mm diam. in 7 days at 28°C.	Grey, becoming dark grey to black, black circular zones in reverse, max. 83 mm diam. in 7 days at 28°C.	Greenish white, becoming greyish green to dark green at the centre with age, dark green at the centre in reverse, max. 42 mm diam. in 7 days at 28°C.	White, becoming pale brownish to pinkish, pale yellowish to pinkish colonies in reverse, max. 82 mm diam. in 7 days at 28°C.	White, becoming grey to dark grey at the centre with age, dark circular around the growing margin at the centre in reverse, max. 83 mm diam. in 7 days at 28°C.
Conidia	Oblong, often curved, hyaline (12-18 × 4-5 μm).	Ellipsoidal, 1-guttulate, hyaline (13-15 × 6-8 μm).	Straight, cylindrical, guttulate, obtuse at the apex (7.5-17 × 3.5-5 μm).	Cylindrical, obtuse end and slightly tapered, sometimes slightly rounded ends to oblong (8-11 × 3-4.5 μm).	Cylindrical with obtuse ends (oblong) with slight narrowing at the center, guttulate, hyaline (8.7-20.3 × 3–4.7 μm).	Fusiform, sometimes with obtuse to slightly rounded ends, sometimes oblong, guttulate, hyaline (7-18.3 × 3–4.3 μm).	Cylindrical with obtuse to slightly rounded ends, sometimes oblong, large guttule at centre surrounded by small guttule, hyaline (9.7-14 × 3-4.3 μm).
Appressoria (in slide cultures)	Undescribed	Undescribed	Circular to slightly irregular, pale to medium brown, often becoming complex (4.5-10 × 4-7.5 μm).	Circular to slightly irregular, pale to medium brown, often becoming complex (4.5-10 × 4-7.5 μm).	Circular to slightly irregular, medium brown to brown, (3-5.1 × 2.1-3.3 μm)	Ovoid, clavate and slightly irregular to irregular, often becoming complex with age, brown to dark brown, mostly formed from conidia (4.7-10.7 × 3.3-6.7 μm).	Ovoid, sometimes clavate, often becoming complex with age, brown, mostly formed from (4.7-8.3 × 3.5-5 μm).
Teleomorph	Unknown	*Glomerella cingulata*	Unknown	*Glomerella cingulata*	Unknown	Unknown	*Glomerella* sp.
Substrate	Twigs, leaves	Undescribed	Berries	Leaves	Berries	Berries	Berries
Host	*Coffea arabica*	*Coffea* sp.	*Coffea arabica*	*Citrus sinensis*	*Coffea arabica*	*Coffea arabica*	*Coffea arabica*
Origin Country	Brazil	Costa Rica	Kenya	Italy	Thailand	Thailand	Thailand
Material examined	Noack (1901)	Spegazzini (1919)	IMI 319418, IMI 363578	CBS 953.97	MFLU 090232	MFLU 090229	MFLU 090226

The future

We have a long way to go before we can establish how many species of *Colletotrichum* there actually are, however, this study has shown that multigene sequence data can help to reveal cryptic species. We now need to look more closely at *Colletotrichum* species complexes and species relationships with hosts. This is already happening for other genera such as *Fusarium*; for instance the *Fusarium oxysporum* complex has been shown to comprise at least 50 distinct species (Kvas *et al*., 2009). Several other important plant pathogenic genera, e.g. *Botryosphaeria* and anamorphs (Phillips *et al*., 2007; Alves *et al*., 2008), *Mycosphaerella* and its anamorphs (Braun and Crous, 2007; Burgess *et al*., 2007; Crous *et al*., 2007), *Phoma* (Aveskamp *et al*., 2008), *Phomopsis* (Santos *et al*., 2009), *Pestalotiopsis* (Jeewon *et al*., 2002, 2003; Hu *et al*., 2007), and *Phyllosticta* (Wulanderi *et al*., 2009) all need to be studied in this way.

Acknowledgements

Double Degree Program under Biro Perencanaan dan Kerjasama Luar Negeri-Departemen Pendidikan Nasional Indonesia Fund is thanked for providing a masters scholarship. Part of this research was funded by research grants 51101010029 and 52101010002 awarded by Mae Fah Luang University. We are grateful to the Research Institute of Resource Insects for providing equipment and laboratory facilities for the molecular study. This research was also funded by AF Grant and the Grant for Essential Scientific Research of National Non-profit Institute (no. CAFYBB2007002) from Chinese Academy of Forestry.

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