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Colletotrichum gloeosporioides is not a common pathogen on tropical fruits

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Abstract *Colletotrichum gloeosporioides* has been reported as one of the most important pathogens worldwide that infect at least 1000 plant species. Fruit rots (anthracnose) are often attributed to *C. gloeosporioides* and, to a lesser extent, to *C. acutatum*. These previous findings were, however, based on morphological identification or, if gene sequence data were used, comparisons were often made with wrongly applied names. *Colletotrichum gloeosporioides* was recently epitypified so that living cultures and sequence data are, for first time available for comparison with fresh collections. Analysis of sequence data of 25 isolates from eight tropical fruits are compared with the *C. gloeosporioides* epitype. Contrary to previous understanding, none of the 25 *Colletotrichum* isolates from tropical fruits was *C. gloeosporioides*. The five gene regions used in this study resolved *Colletotrichum asianum, C fructicola, C. horii, C. kahawae* and *C. gloeosporioides* in the 'gloeosporioides' complex as distinct phylogenetic lineages with high statistical support. Some other likely novel species in the "gloeosporioides" complex and *C. siamense*, however, received only moderate or low support and further studies are needed to clarify their phylogenetic affinities and taxonomic placements. Cultural, conidial and appressorial characters can be used to differentiate taxa into species complexes, but cannot separate species within a complex. This discovery will have significant impacts on many aspects of plant pathology, pathogen diagnosis, quarantine decisions, plant breeding, and plant disease management and control and these are discussed.

Keywords Anthracnose · Diagnosis · Fruit disease · Phylogeny

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Introduction

Fruit rots caused by *Colletotrichum* species are major pre- and/or post harvest diseases which seriously constrain the production, marketing and export of tropical fruits and may be of quarantine concern (Alahakoon and Brown 1994; Bailey and Jeger 1992; Hindorf 2000; Hyde et al. 2009a; Johnston and Jones 1997; Sreenivasaprasad and Talhinhas 2005). Fruit spoilage ranges from a slight loss in quality, resulting in reduced sales, to total spoilage of the fruits (Agostini et al. 1992; Bailey and Jeger 1992; Giblin et al. 2010; Hindorf 2000). The fruit rots are mainly caused by *Colletotrichum gloeosporioides*, and to a lesser extent by *C. acutatum* (Table 1). However, these two taxa are thought to be species complexes and accurate information concerning the causal species within these complexes is lacking (Hyde et al. 2009a; Johnston et al. 2008). The 'gloeosporioides' complex arose because of the artificially enlarged spore range (especially spore length) placed on *C. gloeosporioides* (von Arx 1957) to overcome the instability of spore morphology under different conditions or from different hosts. This treatment has subsequently been followed by researchers worldwide so that numerous *Colletotrichum* strains with cylindrical conidia from various studies were identified as *C. gloeosporioides* (Table 1). Molecular data used to identify strains in this complex has also been problematic, as >86% of the ITS sequences designated as *C. gloeosporioides* in GenBank were not conspecific to the *C. gloeosporioides* epitype (Cai et al. 2009; Hyde et al. 2009b). *Colletotrichum gloeosporioides,* known as one of the world's most important pathogens, is a species complex comprising morphologically indistinguishable but genetically and biologically isolated species (Cai et al. 2009; Johnston et al. 2008). Lumping taxa into species complexes is of little practical use for plant pathologists because the complexes confer little information concerning pathogenicity, host range or other features.

A milestone publication (Cannon et al. 2008) epitypified *C. gloeosporioides*, based on a collection from an orange in Italy, the original location of the type specimen cited in the prologue. Living strains and sequence data are therefore available from the epitype so that fresh isolates can be compared by DNA sequence analyses to confidently identify species, overcoming the inadequacies of traditional morphological identification (Cai et al. 2009; Hyde et al. 2009a). DNA characters are not directly influenced by environmental factors, and thus the combination of multi

Table 1 Reports of *Colletotrichum* species infecting tropical fruits

Fruit	*Colletotrichum* species	References
Avocado (*Persea americana*)	*C. acutatum, C. gloeosporioides*	Alahakoon and Brown 1994; Hindorf 2000; Hyde et al. 2009a.
Banana (*Musa* spp.)	*C. musae*	Hindorf 2000; Hyde et al. 2009b; Jelev et al. 2008; Johnston et al. 2008.
Chilli (*Capsicum annuum*)	*C. acutatum, C. capsici, C. gloeosporioides*	Johnston and Jones 1997; Kefialew and Ayalew 2008; Kim et al. 2009; Kishino and Hasegawa 1989.
Citrus spp.	*C. acutatum, C. gloeosporioides*	Alahakoon and Brown 1994; Lacap et al. 2003; Mackenzie et al. 2009.
Coffee (*Coffea arabica*)	*C. acutatum, C. asianum, C. boninense, C. capsici, C. fruticola, C. kahawae, C. gloeosporioides, C. siamense*	Giblin et al. 2010; Masyahit et al. 2009; Nei and Kumar 2000.
Dragon fruit *(Hylocereus undatus)*	*C. gloeosporioides*	Nguyen et al. 2009
Durian (*Durio zibethinus*)	*C. gloeosporioides*	Abdul Wahid 2001; Nuangmek et al. 2008
Guava (*Psidium guajava*)	*C. acutatum, C. gloeosporioides*	Alahakoon and Brown 1994; Hindorf 2000; Nuangmek et al. 2008; Peres et al. 2002; Photita et al. 2004; Pongpisutta and Sangchote 1994.
Mango (*Mangifera indica*)	*C. acutatum, C. gloeosporioides*	Abdul Wahid 2001; Hindorf 2000; Postmaster et al. 1997.
Mangosteen (*Garcinia mangostana*)	*C. gloeosporioides*	Abdul Wahid 2001.
Passion fruit (*Passiflora* spp.)	*C. acutatum, C. gloeosporioides*	Alahakoon and Brown 1994.
Papaya (*Carica papaya*)	*C. acutatum, C. capsici* and *C. gloeosporioides*	Prihastuti et al. 2009
Rose apple (*Syzygium jambos*)	*C. gloeosporioides*	Abdul Wahid 2001
Rambutan (*Nephelium lappaceum*)	*C. gloeosporioides*	Rahman et al. 2008; Saitou and Nei 1987.
Strawberry (*Fragria frageriae*)	*C. acutatum, C. gloeosporioides, C. fragariae*	Correll et al. 2000; MacKenzie et al. 2009; Schiller et al. 2006.

gene sequence analysis along with traditional morphological identification has lead to a reliable application for identifying species of *Colletotrichum* (Cai et al. 2009; Cannon et al. 2000; Prihastuti et al. 2009; Yang et al. 2009).

The objectives of this study were to characterize the species of *Colletotrichum* associated with fruit rots (anthracnose) in Laos and Thailand. We collected diseased fruits from markets and orchards and isolated the *Colletotrichum* strains. The strains were characterized morphologically and sequenced with five gene regions, i. e. partial actin (ACT), β-tubulin-2 (TUB1, TUB2), glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes and the complete rDNA-ITS (ITS), to establish their identifications.

Materials and methods

Fungal isolation and morphological study

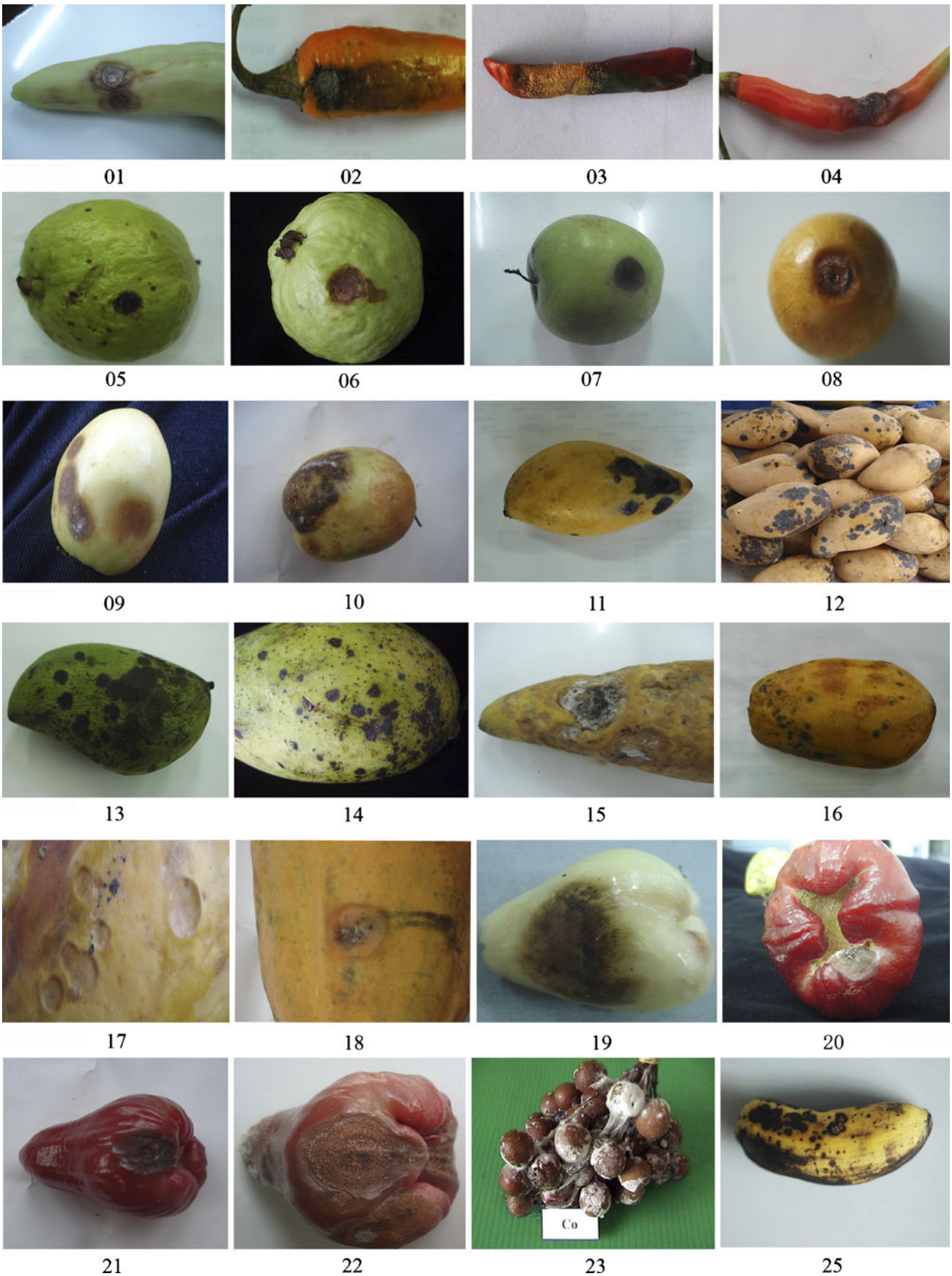
Colletotrichum strains were isolated from lesions of infected fruits from gardens, orchards and local markets in Laos and Thailand (Fig. 1). The collections were from seven locations, four in Laos (Luangprabang, Sayaboury, Vientiane and Savannaketh Provinces) and three in Thailand (Chiang Mai, Bangkok and Nakhon Si Thammarat Provinces). Eight tropical fruits were collected comprising banana (*Musa* sp.), chilli (*Capsicum* spp.), guava (*Psidium guajava*), jujube (*Zizyphus mauritiane*), longan (*Dimocarpus longan*), mango (*Mangifera indica*), papaya (*Carica papaya*) and rose apple (*Syzygium jambos*). Strains were isolated using the protocol as outlined by Cai et al. (2009). Cultures were grown on potato dextrose agar (PDA) in which three 4 mm plugs were aseptically cut from the edges of actively growing areas of 5 day old cultures and transferred to new PDA plates. Cultures were incubated at 27°C for 7 days. Three cultures of every isolate were investigated for culture colony characters. Colony diameter was recorded for 7 days and used to calculate the fungal growth rate. After 7 days, colony size and colour of the conidial masses and zonation was recorded, and conidial size and shape from 20 arbitrary conidia was measured under the microscope. Appressoria were produced and recorded using a slide culture technique as described Cai et al. (2009).

DNA extraction, PCR amplification and DNA sequencing

Isolates were grown on PDA and incubated for 7 days at 27°C. Mycelia were scraped down from the surface of agar. Genomic DNA was extracted by using a Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) according to the manufacturer's protocol. Quality and quantity of DNA were estimated visually by staining with ethidium bromide on 1% agarose gel electrophoresis. Partial actin (ACT), β-tubulin (TUB2), glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes and the complete rDNA-ITS (ITS) region from *Colletotrichum* strains were amplified by PCR as previously described (Prihastuti et al. 2009). β-tubulin (TUB1) region was amplified by using primer Bt1a & Bt1b (Glass and Donaldson 1995). PCR products were verified by staining with ethidium bromide on 1% agarose electrophoresis and purified using the GFX PCR Purification Kit (27-9602-01; Amersham Biosciences) according to the manufacturer's protocol. Sequencing was carried out at the Shanghai Sangon Biological Engineering Technology & Services Co using forward and reverse primers and the results were manually checked for errors. Sequences used in the phylogenetic analysis are listed in Table 2.

Phylogenetic analyses

Sequences of *Colletotrichum* isolates from different hosts were aligned in Mega 4.1 (Tamura et al. 2007) and optimized manually to assure positional homology. Gaps were treated as missing data. Phylogenetic analyses were performed using Mega 4.1 (Tamura et al. 2007). Maximum parsimony (MP) trees were obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar 2000) with search level 1, in which the initial trees were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset. Clade stability of the tree resulting from maximum parsimony analysis was assessed by bootstrap analysis with 1000 replicates (Kishino and Hasegawa 1989). The minimum evolution (ME) trees were also created and evaluated with 1,000 bootstrap replicates. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (30) at a search level of 1. The Neighbor-joining algorithm (Saitou and Nei 1987) was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset. The model of evolution was estimated by using Mrmodeltest 2.2. Posterior probabilities (PP) (Bridge et al. 2008) were determined by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes 3.0b4 (Nuangmek et al. 2008). Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100th generation (resulting in 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.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◀ **Fig. 1** Anthracnose and fruit rot symptoms on tropical fruits

Results

Strain isolation

Twenty-five *Colletotrichum* isolates from fruit rots of eight fruit hosts in Loas and Thailand were used in the phylogenetic analysis. The fruit rots varied from brown to black spots, and dark sunken lesions to light brown sunken areas (Fig. 1). All isolates are deposited in the culture collection of Mae Fah Luang Culture collection with some duplicates in the BIOTEC culture collection (BCC) and IFRDC (under MTA C01/2010). Hebarium material is deposited in Mae Fah Luang University (MFLU).

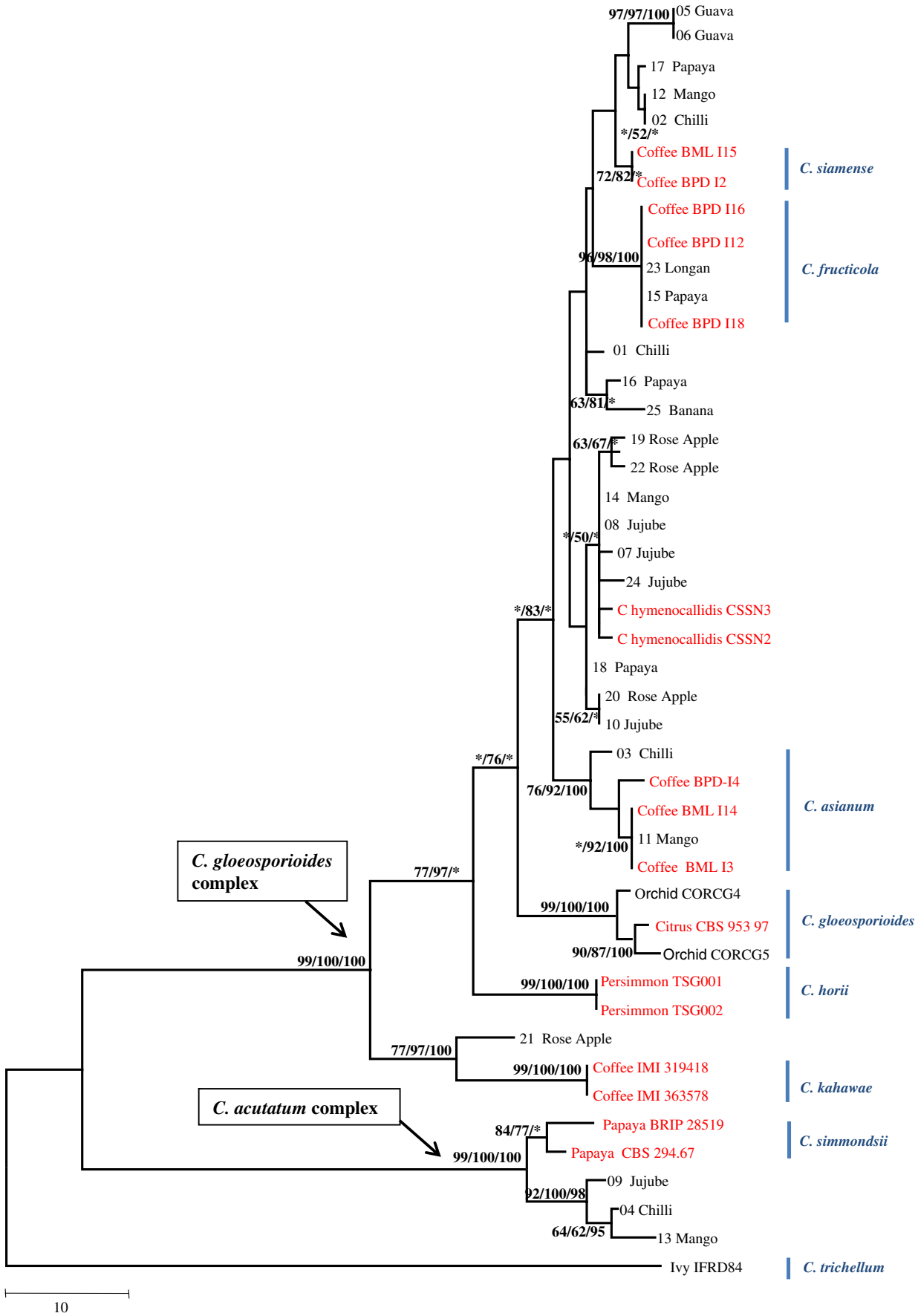
Molecular analysis

Sequence data from five gene fragments were analysed and the phylogenetic relationships of the 25 strains were inferred using the Maximum Parsimony (MP), Minimum Evolution (ME) and Bayesian analysis methods. One of the 72 equally parsimonious trees is shown in Fig. 2. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches. The ME and Baysian tree is essentially similar to the MP tree and therefore not shown. MP bootstrap, ME bootstrap and Bayesian Posterior Probabilities are provided for the branches. The *Colletotrichum* isolates clustered into several different lineages, which included named species and undetermined taxa. The phylogram in Fig. 2 comprises two species complexes, i.e. 'acutatum' complex (with five fusiform-spored strains), and the 'gloeosporioides' complex (with 39 cylindrical-spored

Table 2 *Colletotrichum* strains causing fruit rot in Laos and Thailand and their phenotypic characters

Fruit	MFU, IFRDC no.	Fungal species or undetermined group (isolate no. as in Figs.1, 2)	Conidial shape, mean length and width of conidia, and mean length and width of appressoria (μm)	Growth rate mm day^{-1}
Chilli[1]	2091, 09 0616	*C. fruticola* (01)	Cylindrical, 17.26 ^{Ha}, 5.73 ^{D}, 10.35 ^{D}, 7.85 ^{A}	10.4 ^{F}
Chilli[2]	2092, 09 0617	Undetermined (02)	Cylindrical, 14.26 ^{I}, 4.8 ^{J}, 10 ^{D}, 5.41 ^{D}	11 ^{F}
Chilli[3]	2096, 09 0618	*C. asianum* (03)	Cylindrical, 15.86 ^{I}, 5.4 ^{F}, 10.69 ^{C}, 7.36 ^{B}	7.73 ^{F}
Chilli[4]	0210, 09 0619	Undetermined (04)	Fusiform, 11.73 ^{J}, 4.13 ^{L}, 7.49 ^{H}, 6.53 ^{D}	7.53 ^{H}
Guava[2]	2105, 09 0620	Undetermined (05)	Cylindrical, 15.86 ^{I}, 5.2 ^{G}, 9.72 ^{D}, 7.77 ^{A}	11.4 ^{E}
Guava[5]	2107, 09 0621	Undetermined (06)	Cylindrical, 11.33 ^{J}, 4.5 ^{K}, 7.64 ^{H}, 5.41 ^{D}	6.13 ^{I}
Jujube[1]	2110, 09 0622	Undetermined (07)	Cylindrical, 17.2 ^{H}, 5.46 ^{F}, 9.73 ^{D}, 6.06 ^{D}	10.93 ^{F}
Jujube[5]	2112, 09 0623	Undetermined (08)	Cylindrical, 15.33 ^{I}, 5.13 ^{H}, 9.3 ^{G}, 6.11 ^{D}	10.6 ^{F}
Jujube[3]	2115, 09 0624	Undetermined (09)	Fusiform, 11.83 ^{J}, 5 ^{I}, 6.39 ^{H}, 5.83 ^{D}	4.26 ^{J}
Jujube[4]	2116, 09 0625	Undetermined (10)	Cylindrical, 17.60 ^{G}, 5.26 ^{G}, 10.2 ^{D}, 6.46 ^{D}	12 ^{C}
Jujube[4]	2155, 09 0639	Undetermined (24)	Cylindrical, 15.6 ^{I}, 5 ^{I}, 8.47 ^{G}., 5.9 ^{D}	9.73 ^{F}
Mango[2]	2120, 09 0626	*C. asianum* (11)	Cylindrical, 18 ^{G}, 5.53 ^{E}, 9.58 ^{E}, 6.67 ^{D}	10.4 ^{F}
Mango[5]	0211, 09 0627	Undetermined (12)	Cylindrical, 22.26 ^{A}, 5.2 ^{G}, 8.61 ^{G}, 6.67 ^{D}	7.33 ^{H}
Mango[3]	2124, 09 0628	Undetermined (13)	Fusiform, 14.6 ^{I}, 4.33 ^{L}, 8.47 ^{G}, 6.59 ^{D}	5.33 ^{J}
Mango[6]	0212, 09 0629	Undetermined (14)	Cylindrical, 20.6 ^{B}, 5.53 ^{E}, 10.13 ^{D}, 7.36 ^{B}	11.2 ^{F}
Papaya[1]	2131, 09 0630	*C. fruticola* (15)	Cylindrical, 15.06 ^{I}, 5 ^{I}, 9.2 ^{G}, 5.4 ^{D}	10.06 ^{F}
Papaya[2]	2133, 09 0631	Undetermined (16)	Cylindrical, 15.2 ^{I}, 4.7 ^{J}, 9.16 ^{G}, 6.25 ^{D}	11.1 ^{F}
Papaya[7]	0213, 09 0632	Undetermined (17)	Cylindrical, 17.73 ^{G}, 6.4 ^{A}, 9.37 ^{F}, 7.22 ^{C}	12.6 ^{A}
Papaya[4]	2138, 09 0633	Undetermined (18)	Cylindrical, 20.2 ^{C}, 5 ^{I}, 11.53 ^{B}, 7.^{77} ^{A}	9.86 ^{F}
Rose apple[1]	2142, 09 0634	Undetermined (19)	Cylindrical, 18.8 ^{F}, 6.13 ^{B}, 9.44 ^{F}, 6.66 ^{D}	10.73 ^{F}
Rose apple[5]	0214, 09 0635	Undetermined (20)	Cylindrical, 17.4 ^{G}, 6.26 ^{B}, 12.22 ^{A}, 6.87 ^{C}	9.66 ^{F}
Rose apple[4]	2149, 09 0636	Undetermined (21)	Cylindrical, 19.46 ^{D}, 5.53 ^{E}, 8.47 ^{G}, 5.9 ^{D}	12.53 ^{B}
Rose apple[6]	2151, 09 0637	Undetermined (22)	Cylindrical, 14.33 ^{I}, 4.66 ^{J}, 9.72 ^{D}, 7.22 ^{C}	11.4 ^{E}
Longan[1]	2154, 09 0638	*C. fruticola* (23)	Cylindrical, 19.2 ^{E}, 6 ^{C}, 8.61 ^{G}, 6.25 ^{D}	11.6 ^{D}
Banana[2]	2156, 09 0640	Undetermined (25)	Cylindrical, 19.2 ^{E}, 6 ^{C}, 7.29 ^{H}, 6.87 ^{C}	12 ^{C}
			LSD (between group) 1.35, 0.3, 1.68, 1.18	

[1] =Chiang Mai, Thailand; [2] =Bangkok, Thailand; [3] =Luangprabang, Laos; [4] =Vientiane, Laos; [5] =Nakornsrithammarath, Thailand; [6] =Savannakhet, Laos; [7] =Sayaboury, Laos. [a] Least Significant Difference (LSD) means with the same letter in each column are not significantly different from each other based on DMRT test in Sirichai statistics version 6.


97/97/100
05 Guava
06 Guava
17 Papaya
12 Mango
02 Chilli
/52/
Coffee BML I15
72/82/*
Coffee BPD I2
C. siamense
Coffee BPD I16
Coffee BPD I12
96/98/100
23 Longan
15 Papaya
Coffee BPD I18
C. fructicola
01 Chilli
16 Papaya
63/81/*
25 Banana
63/67/*
19 Rose Apple
22 Rose Apple
14 Mango
08 Jujube
/50/
07 Jujube
24 Jujube
/83/
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C hymenocallidis CSSN2
18 Papaya
20 Rose Apple
55/62/*
10 Jujube
/76/
03 Chilli
76/92/100
Coffee BPD-I4
Coffee BML I14
*/92/100
11 Mango
Coffee BML I3
C. asianum
C. gloeosporioides complex
77/97/*
Orchid CORCG4
99/100/100
Citrus CBS 953 97
90/87/100
Orchid CORCG5
C. gloeosporioides
99/100/100
99/100/100
Persimmon TSG001
Persimmon TSG002
C. horii
77/97/100
21 Rose Apple
99/100/100
Coffee IMI 319418
Coffee IMI 363578
C. kahawae
C. acutatum complex
84/77/*
Papaya BRIP 28519
99/100/100
Papaya CBS 294.67
C. simmondsii
92/100/98
09 Jujube
04 Chilli
64/62/95
13 Mango
Ivy IFRD84
C. trichellum
10

◀ **Fig. 2** Phylogram of tree generated maximum parsimony analysis based on combined ACT, TUB1, GPDH, ITS and TUB2 sequences. Clade stabilities were calculated from maximum pasimony (≥50%), minimum evolution (≥50%) and Baysian posterior probability (≥ 95%). The tree is rooted with *Colletotrichum trichellum.* The ex-type or verified strains are shown in red

strains). Each of these complexes has high bootstrap and posterior probability support.

The 'gloeosporioides' complex consisted of 22 strains isolated from fruits in this study, and 17 reference strains that are either ex-types or voucher strains with confirmed identities. The latter includes *C. asianum*, *C. fructicola, C. kahawae, C. gloeosporioides* and *C. siamense* represented by ex-holotype, ex-paratype or ex-epitype strains, and *C. horii* represented by voucher strains (Fig. 2). *C. gloeosporioides sensu stricto* is represented by the ex-epitype (IMI 356878=CBS 953.97) and two verified strains isolated in a different study from *Vanda* sp. from China. None of the 22 strains from Asian fruits clustered with the ex-epitype of *C. gloeosporioides* and therefore should belong to other species. *C. asianum*, *C. fructicola, C. kahawae, C. gloeosporioides* and *C. horii* are well resolved in the phylogenetic tree with high bootstrap and posterior probabilities support, while *C. siamense* received only moderate support (Fig. 2). Among the 22 strains from fruit, four strains could be confidently classified as *C. fructicola* (No. 15 and 23) or *C. asianum* (No. 3 and 11), while all other 18 strains could not be confidently identified and some may represent novel species (Fig. 2).

The 'acutatum' complex comprised the ex-holotype culture of *C. simmondsii* (BRIP 28519) from *Carica papaya* in Australia and three undetermined strains which occurred on chilli, jujube and mango (Fig. 2). The three isolates had similar cultural characteristics, relatively short fusiform ascospores (11.7–14.6×4.1–5 μm) and a slow growth rate (6.4–8.5 mm per day) and is not conspecific with *C. acutatum* (Guerber and Correll 2001; Vinnere et al. 2002).

Morphology, culture characteristics and growth rate

Morphological features were determined and correlated with the species or complexes determined in the phylogenetic tree. Isolates in the 'gloeosporioides' complex produced two types of conidia: those with cylindrical conidia with obtuse ends (oblong) and narrowing at the centre, and those with obtuse to slightly rounded ends and not narrowed. Isolates in the 'acutatum' complex produced conidia that were fusiform with obtuse to slightly rounded ends. There were statistically significant differences in length and width of conidia among some strains (Table 3), however, conidial morphology could not be confidently used alone to determine species within a complex. Similarly, appressoria were produced by all isolates in slide cultures and varied from ovoid, clavate, to irregular (Table 3). The size and shape of appressoria also failed to distinguish amongst species.

Based on similarities in colony characteristics following growth on PDA for 7 days at 27°C, the isolates could be grouped into seven morphotypes (Lacap et al. 2003). The grouping of morphotypes had little relationship to the *Colletotrichum* species. There were differences in growth rates of individual strains, but it was not possible to differentiate between species and undetermined taxa based on growth rates.

Discussion

Although *C. gloeosporioides*, and to a lesser extent *C. acutatum*, has previously been shown to be the causal agent of tropical fruit rots, the most striking discovery of this study is that none of the 25 strains isolated from Laos and Thai fruits was either of these species. Previous understanding that anthracnose of most tropical fruits is caused by *C. gloeosporioides* or *C. acutatum* should therefore be reconsidered. Phylogenetic analysis however, showed that most of the strains included in this study belong to the 'gloeosporioides' complex. The 23 strains in the 'gloeosporioides' complex clustered into several different clades, some of which were statistically well-supported monophyletic groups. Only four of these strains could be assigned to known species, i.e. *C. asianum* and *C. fructicola*, based on a conjunction of phenotypic characters and genealogical concordance species recognition (Cai et al. 2009). The remaining 19 strains may represent undescribed species that cannot be placed in any known species with the current data.

Strains isolated from the same host may contain more than one phylogenetic species. For example, four strains isolated from papaya clustered in four different phylogenetic clades; four strains isolated from mango also appeared in four different phylogenetic clades; five strains from jujube clustered in three different clades; four strains from rose apple were in three clades, while four from chilli scattered in four different clades. These results provide further evidence that one plant may often host more than one pathogenic *Colletotrichum* species and using host as a taxonomic criterion may result in significant misidentification and confusion.

ITS sequences of the three strains excluded from the 'gloeosporioides' complex were incorporated into the backbone tree (Cai et al. 2009) and it was found that they are most closely related to *C. simmondsii* in the 'acutatum' complex. Multigene phylogeny showed that these three strains clustered in a sister clade to *C. simmondsii* with strong statistical support and probably represent a novel species. The holotype of *C. acutatum* was described from

Table 3 Sequences used in the phylogenetic analysis

Isolate No.	Strain numbers	Species	GenBank accession number				
			ACT	TUB1	GPDH	ITS	TUB2
01	MFU090616	*Colletotrichum sp.*	HM038263	HM038269	HM038319	HM038348	HM038310
02	MFU090617	*Colletotrichum sp.*	HM038250	HM038270	HM038315	HM038354	HM038294
03	MFU090618	*C. asianum*	HM038242	HM038267	HM038313	HM038342	HM038292
04	MFU090619	*Colletotrichum sp.*	HM038264	HM038291	HM038335	HM038360	–
05	MFU090620	*Colletotrichum sp.*	HM038244	HM038285	HM038333	HM038353	HM038305
06	MFU090621	*Colletotrichum sp.*	HM038246	HM038286	HM038316	HM038341	HM038297
07	MFU090622	*Colletotrichum sp.*	HM038253	HM038276	HM038331	HM038340	HM038303
08	MFU090623	*Colletotrichum sp.*	HM038255	HM038283	HM038324	HM038338	HM038307
09	MFU090624	*Colletotrichum sp.*	HM038265	HM038289	HM038337	HM038362	
10	MFU090625	*Colletotrichum sp.*	HM038258	HM038277	HM038327	HM038339	HM038306
11	MFU090626	*C. asianum*	HM038243	HM038268	HM038314	HM038343	HM038293
12	MFU090627	*Colletotrichum sp.*	HM038245	HM038282	HM038317	HM038351	HM038308
13	MFU090628	*Colletotrichum sp.*	HM038266	HM038290	HM038336	HM038361	–
14	MFU090629	*Colletotrichum sp.*	HM038249	HM038271	HM038328	HM038346	HM038304
15	MFU090630	*C. fructicola*	HM038251	HM038272	HM038321	HM038356	HM038302
16	MFU090631	*Colletotrichum sp.*	HM038261	HM038280	HM038322	HM038355	HM038295
17	MFU090632	*Colletotrichum sp.*	HM038254	HM038279	HM038332	HM038352	HM038296
18	MFU090633	*Colletotrichum sp.*	HM038256	HM038274	HM038325	HM038349	HM038299
19	MFU090634	*Colletotrichum sp.*	HM038247	HM038281	HM038323	HM038344	–
20	MFU090635	*Colletotrichum sp.*	HM038259	HM038287	HM038318	HM038345	HM038300
21	MFU090636	*Colletotrichum sp.*	HM038260	HM038288	HM038334	HM038359	HM038301
22	MFU090637	*Colletotrichum sp.*	HM038248	HM038284	HM038329	HM038347	HM038311
23	MFU090638	*C. fructicola*	HM038252	HM038273	HM038320	HM038357	HM038298
24	MFU090639	*Colletotrichum sp.*	HM038257	HM038275	HM038330	HM038350	HM038309
25	MFU090640	*Colletotrichum sp.*	HM038262	HM038278	HM038326	HM038358	HM038312
	CBS953.97[b]	*C. gloeosporioides*	FJ907430	–	FJ972582	FJ972609	FJ907445
	CORCG4[a]	*C. gloeosporioides*	HM034800	–	HM034806	HM034808	HM034810
	CORCG5[a]	*C. gloeosporioides*	HM034801	–	HM034807	HM034809	HM034811

ACT actin, *TUB-1* partial β-tubulin (tub1), *TUB-2* partial β-tubulin (tub2), *CAL* calmoudulin, *GS* glutamine synthetase, *GDPH* glyceraldehydes-3-phosphate dehydrogenase, *ITS* complete rDNA-ITS region

[a] sequences obtained from GenBank

[b] ex-epitype of *Colletotrichum gloeosporioides*

papaya in Queensland (Than et al. 2008a, b) and *C. acutatum sensu stricto* was not found on fruits in this study. Although *C. acutatum* has been reported to cause anthracnose of chilli fruits in Thailand (Than et al. 2008a, b, c) this was later revealed to be *C. simmondsii* following further molecular analysis (Shivas and Tan 2009). Therefore it is unclear if *C. acutatum* occurs in Laos or Thailand.

The discovery of new species within the 'gloeosporioides' complex is not surprising. *Colletotrichum* species on coffee berries have been extensively studied and numerous strains in the 'gloeosporioides' complex have been isolated. Before 1993, *C. gloeosporioides* was initially thought to be the cause of all coffee berry disease, but a new species, *C. kahawae*, for the highly pathogenic strain causing coffee berry disease in Africa was established based on differences in growth rate and biochemical characters (Waller et al. 1993). *C. kahawae* however, could not be differentiated from *C. gloeosporioides* using morphological characters. The validity of *C. kahawae* was therefore questioned for many years (Cannon et al. 2000; Correll et al. 2000; Varzea et al. 2002) and only recently has it been confidently supported in various molecular phylogenetic studies (Bridge et al. 2008; Cai et al. 2009; Prihastuti et al. 2009). Previous studies of anthracnose on coffee berries in northern Thailand (Prihastuti et al. 2009) established three new species that bear both phenotypic distinctions and evolutionary distances from *C. gloeosporioides* and two of these species (*C. asianum* and *C. fructicola*) are resolved with strong support in Fig. 2. These

studies of *Colletotrichum* on coffee berries provide a good example of how *C. gloeosporioides* has previously been misunderstood.

The new *Colletotrichum* species isolated from coffee in Thailand (Prihastuti et al. 2009) were epiphytes, endophytes, or pathogens. In the present study, two of these species, *C. asianum* and *C fructicola* were found to infect other fruits (Fig. 2). However, *C. siamense* from coffee (Prihastuti et al. 2009) was not isolated in the present study, although Yang et al. (2009) isolated it from *Hymenocallis* sp. It therefore appears that some species of *Colletotrichum* such as *C. horii* (on persimmon) and *C. kahawae* (on coffee) may be restricted to certain hosts or families, while others have a wide host range. This is particular true of *C. fructicola* which has now been isolated from coffee, *Crinum asiaticum*, longan and papaya (Prihastuti et al. 2009; Yang et al. 2009; Fig. 2). *Colletotrichum gloeosporioides* was not isolated from any of the fruits used in this study, but it is not restricted to *Citrus* as two strains used in Fig. 2 were isolated from *Vanda* sp.

The strains used in this study cluster into two species complexes ('acutatum' and 'gloeosporioides') with high bootstrap support (Fig. 2) and confirm that the taxa in these complexes are distinct. The 'gloeosporioides' complex presently comprises *C. asianum, C. fructicola, C. horii, C. hymenocallidis, C. kahawae, C. gloeosporioides* and *C. siamense* (Cai et al. 2009; Johnston et al. 2008; Prihastuti et al. 2009; Yang et al. 2009), plus several further species that need epitypifying (e.g. *C. musae*). This study has revealed that several novel species appear to occur on tropical fruits in Laos and Thailand and therefore the number of novel species worldwide may be large. The 'acutatum' complex currently comprises *C. simmondsii, C. fioriniae, C. lupinii* and *C. simmondsii* (Shivas and Tan 2009), plus the potential new species isolated in this study (Fig. 2). There is a lot more work needed before we can fully understand the genus.

Analysis of the usefulness of morphological characters and growth rate in distinguishing *Colletotrichum* species was carried out. Cultural characters, conidia and appressoria morphology and growth rate have previously been used in combination to differentiate species (Prihastuti et al. 2009; Yang et al. 2009). The combination of phenotypic characters is important in distinguishing genetically isolated but morphologically similar species, for example, *Colletotrichum gloeosporioides* and *C. kahawae*. Further study is needed to correlate the phenotypic characters with the phylogenetic lineages for those unclassified strains in this study.

ITS sequence data which has been generally adopted for barcoding fungi (Seifert 2009), is inadequate for this genus as previously noted (Cai et al. 2009; Prihastuti et al. 2009; Yang et al. 2009). ITS sequence data is useful for placing species in a species complex and also providing an idea which species in a complex to which it can be assigned. However, other genes are needed to infer robust phylogenetic relationships and provide high confidence for species clusters. In this study, we used five gene fragments, and this has provided a relatively robust support for species within the 'gloeosporioides' complex. *Colletotrichum asianum, C fructicola, C. horii, C. kahawae, C. gloeosporioides* all receive high support as distinct phylogenetic lineages. Some strains that failed to cluster in any of above may contain novel species and further study is needed to confidently infer relationships. It is not the purpose of this paper to introduce new species but to show that our previous knowledge concerning infection of tropical fruits by *C. acutatum* and *C. gloeosporioides* in Laos and Thailand and probably elsewhere is incorrect. We will carry out further work concerning the resolution and designation of new species.

Correct identification of fungal pathogens is essential for quarantine decisions, in plant breeding, and in pathogen management and control (Cai et al. 2009). The systematic scheme for *Colletotrichum* species is now in a state of transition. Previously applied concepts using host and morphological characters to define species have been shown to be artificial and do not agree with evolutionary relationships. Numerous studies have shown that one strain can often infect several hosts, whilst morphological characters are highly dependent on environmental factors (von Arx 1957; Sutton 1966). The discovery in the current study is a reflection that host groups do not correlate with the phylogenetic groups. It has been proposed that species concepts in *Colletotrichum* should be based on multiple gene phylogeny and correlation between the genotype and phenotype (Cai et al. 2009). This is a sensible trend for future studies.

The findings presented here will have significant impact on many aspects of plant pathology, in particular, species identification, quarantine, plant breeding, and disease management and control. For example, the 'gloeosporioides' complex on coffee berries has been well characterized and several distinct genetic and phenotypic species have been established (Prihastuti et al. 2009; Waller et al. 1993). Among these *C. kahawae* is a strongly aggressive pathogen specific to coffee in Africa (Waller et al. 1993) and should not be allowed to 'enter' into other continents by continuing with strict quarantine protocols. On the other hand, *C. asianum*, *C. fructicola* and *C. siamense* are opportunistic pathogens of coffee berries (Prihastuti et al. 2009) and appear to have a wide host range, thus are not of quarantine significance. The current study also indicates that morphologically similar isolates from chilli, mango, papaya, rose apple and jujube may also comprise more than one distinct species, and these species are currently poorly

defined and characterized. Epitypification combined with analysis and comparison of sequence data will fundamentally change the understanding of species relationships, not only in *Colletotrichum*, but also in many other important plant pathogenic genera (e.g. *Botryosphaeria, Fusarium*, *Pestalotiopsis, Phomopsis* and *Phyllosticta*). Researchers must therefore transfer new knowledge to quarantine authorities so they can rapidly establish new quarantine protocols.

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