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A new species of *Colletotrichum* from *Cordyline fruticosa* and *Eugenia javanica* causing anthracnose disease

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Abstract — A new species *C. cordylinicola*, isolated from *Cordyline fruticosa,* is characterized by morphological and molecular characters. The species would previously have been considered as a member of the *Colletotrichum gloeosporioides* complex. Combined six gene analysis using ACT, GS, TUB2, ITS, CAL and GPDH shows that three strains of *C. cordylinicola* clustered in a distinct lineage as a sister clade to *C. kahawae*. Other reference taxa employed in the analysis include type strains of *C. asianum, C. fructicola, C. gloeosporioides, C. kahawae, C. siamense, C. simmondsii,* and authentic strains of *C. horii*. This is the first report of a *Colletotrichum* species causing disease of *Cordyline fruticosa* in Thailand. Pathogenicity testing using the strains isolated from *Cordyline fruticosa* and *Eugenia javanica* showed that two strains isolated from different hosts may represent different pathotypes.

Key words — leaf spot, plant pathogenic fungi, taxonomy

Introduction

Colletotrichum is one of the most economically important pathogenic genera causing anthracnose of fruits and leaves, affecting a wide range of hosts in the tropics and subtropics. (Freeman et al. 1998, Hindorf 2000, Damm et al. 2009, Hyde et al. 2009a,b, Shivas & Yu 2009). Both agricultural crops and fruit trees

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

Colletotrichum species	Culture collection	GenBank accession number					
		ACT	TUB-2	CAL	GS	GPDH	ITS
C. asianum	MFU 090232*	FJ 903188	FJ 907434	FJ 917501	FJ 972586	FJ 972571	FJ 972605
C. asianum	MFU 090233	FJ 907424	FJ 907439	FJ 917506	FJ 972595	FJ 972576	FJ 972612
C. asianum	MFU 090234	FJ 907421	FJ 907436	FJ 917503	FJ 972598	FJ 972573	FJ 972615
C. cordylinicola	BCC 38872	**HM470234**	**HM470249**	**HM470237**	**HM470243**	**HM470240**	**HM470246**
C. cordylinicola	BCC38864	**HM470233**	**HM470248**	**HM470236**	**HM470242**	**HM470239**	**HM470245**
C. cordylinicola	MFLU 100132	**HM470235**	**HM470250**	**HM470238**	**HM470244**	**HM470241**	**HM470247**
C. fructicola	MFU 090226*	FJ 907427	FJ 907442	FJ 917509	FJ 972592	FJ 972579	FJ 972602
C. fructicola	MFU 090227	FJ 907425	FJ 907440	FJ 917507	FJ 972594	FJ 972577	FJ 972611
C. fructicola	MFU 090228	FJ 907426	FJ 907441	FJ 917508	FJ 972593	FJ 972578	FJ 972603
C. gloeosporioides	CBS 953.97*	FJ 907430	FJ 907445	FJ 917512	FJ 972589	FJ 972582	FJ 972609
C. horii	TSG001	GU133374	GU133375	GU133376	GU133377	GQ329682	AY787483
C. horii	TSG002	GU133379	GU133380	GU133381	GU133382	GQ329680	AY791890
C. kahawae	IMI 319418*	FJ 907432	FJ 907446	FJ 917514	FJ 972588	FJ 972583	FJ 972608
C. kahawae	IMI 363578*	FJ 907433	FJ 907447	FJ 917515	FJ 972587	FJ 972584	FJ 972607
C. siamense	MFU 090230*	FJ 907423	FJ 907438	FJ 917505	FJ 972596	FJ 972575	FJ 972613
C. siamense	MFU 090231	FJ 907422	FJ 907437	FJ 917504	FJ 972597	FJ 972574	FJ 972614
C. simmondsii	BRIP 28519*	FJ 907428	FJ 907443	FJ 917510	FJ 972591	FJ 972580	FJ 972601
C. simmondsii	CBS 294.67	FJ 907429	FJ 907444	FJ 917511	FJ 972590	FJ 972581	FJ 972610
C. falcatum	CGMCC3.14187	HM171665	HM171680	HM171668	HM171674	HM171671	HM171677

Note: ACT: actin; TUB-2: partial ß-tubulin; CAL: calmoudulin; GS: glutamine synthetase; GDPH: glyceraldehydes-3-phosphate dehydrogenase; ITS: complete rDNA-ITS region. The newly generated sequence in this study are shown in bold. CGMCC: China General Microbial Culture Collection. MFU: Mae Fah Luang University, Thailand; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; BRIP: Plant Pathology Herbarium, Department of Primary Industries, Queensland, Australia; IMI: CABI Europe – UK, Egham, Surrey TW209TY, UK; *: ex-type cultures

can be affected by *Colletotrichum* anthracnose, resulting in reduction in yield quantity or quality. *Colletotrichum* species are cosmopolitan with either multiple species occurring on a single host or a single species on multiple hosts (Cai et al. 2009, Crouch & Beirn 2009, Hyde et al. 2009b). Fungus/host relationships are broad, imprecise and often overlapping. *Colletotrichum* species can infect many hosts and may adapt to new environments (Sanders & Korsten 2003a), leading to serious cross infection problems in plant production. The study of pathogenic variability of *Colletotrichum* species is therefore important and the understanding of the host range of a particular pathogen may help in efficient disease control and management (Whitelaw-Weckert et al. 2007).

Artificial inoculation methods in vitro are commonly used to test the pathogenicity of a fungal species, as it is easy to control environmental conditions. Common inoculation methods for pathogenicity testing include drop inoculation and wound/drop inoculation (Cai et al. 2009, Kanchana-udomkan et al. 2004, Lin et al. 2002, Sharma et al. 2005, Than et al. 2008a).

Colletotrichum gloeosporioides sensu lato has previously been listed as causing disease of a very wide range of fruits and infecting leaves of many hosts in Thailand (and Laos) (Ratanacherdchai et al. 2007, Than et al. 2008b, Yang et al. 2009). This species has recently been epitypified with a living strain that has been sequenced with sequence data deposited in GenBank (Cannon et al. 2008). This has enabled researchers to compare their isolates of *Colletotrichum* with the *C. gloeosporioides* epitype. This has resulted in the description of several new species in the *C. gloeosporioides* species complex (Prihastuti et al. 2009, Yang et al. 2009). With the introduction of several new species it is important to establish whether they are host-specific or have a wide host range, as this will have important implication in disease control and management. The objective of this paper is to introduce a new *Colletotrichum* species causing leaf disease of *Cordyline fruticosa* in Laos and Thailand. It is characterized morphologically and phylogenetically in this paper and its ability to infect several hosts is established.

Material and methods

Isolation and morphological examination

The methods of isolation used by Cai et al. (2009), Prihastuti et al. (2009) and Yang et al. (2009) were followed. Two strains of *Colletotrichum* were isolated from anthracnose of infected leaves of *Cordyline fruticosa* from a garden in Chiang Mai, Thailand and one from leaves of rose apple in a garden in Vientiane, Laos. The growth rate was measured for 7-day old colonies on PDA. Herbarium material is deposited in MFLU while extype cultures are deposited at MFLUCC and *BIOTEC* Culture Collection (*BCC*), with some duplicate strains deposited in China General Microbial Culture Collection (CGMCC) under material transfer agreement 7/2552.

DNA extraction

Isolates were grown on PDA and incubated at 27°C for 7 days. 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.

PCR amplification and DNA sequencing

Partial actin (ACT), β-tubulin (TUB2), calmodulin (CAL), glutamine synthetase (GS), glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes and the complete rDNA-ITS (ITS) region from three strains were amplified by PCR reactions. The primers, reaction system and thermo cycles were the same as used by Prihastuti et al. (2009).

PCR products were verified by staining with ethidium bromide on 1% agarose electrophoresis. PCR products were then purified using the GFX PCR Purification Kit (27-9602-01; Amersham Biosciences) according to the manufacturer's protocol. Sequencing was carried out at the SinoGenoMax Company Limited, Beijing.

Phylogenetic analyses

Sequences of *Colletotrichum* isolates (Table 1) from different hosts were aligned with ClustalX (Thompson et al. 1997) and optimized manually to allow maximum alignment and maximum sequence similarity. Gaps were treated as missing data. Phylogenetic analysis was carried out based on the aligned dataset by PAUP* 4.0b10 (Swofford 2000). 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 (Kishino & Hasegawa 1989) were performed in order to determine whether trees were significantly different. Clade stability of the tree resulting from maximum parsimony analysis was assessed by bootstrap analysis with 1000 replicates, each with 10 replicates of random stemwise addition of taxa (Felsenstein 1985). Trees were figured in TreeView (Page 1996).

The model of evolution was estimated by using Mrmodeltest 2.2 (Nylander 2004). Posterior probabilities (PP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001). Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100 generations (resulting in 10,000 total trees). The first 2000 trees, which represented the burn-in phase of the analyses, were discarded and the remaining 8000 trees were used for calculating posterior probabilities (PP) in the majority rule consensus tree.

Pathogenicity testing

The protocol followed the methods outlined by Cai et al. (2009) and Yang et al. (2009), modified as follows. Pathogenicity testing used one isolate from *Cordyline fruticosa* and one from *Eugenia javanica*. Each was inoculated onto three fruits of chilli

(*Capsicum* sp.), guava (*Psidium guajava*), mango (*Mangifera indica*), papaya (*Carica papaya*), orange (*Citrus* sp.), and rose apple *(Eugenia javanica)* and onto three detached leaves of *Cordyline fruticosa.* Incubation duration was dependent on the nature of lesion development and anthracnose symptoms were scored as a + or –.

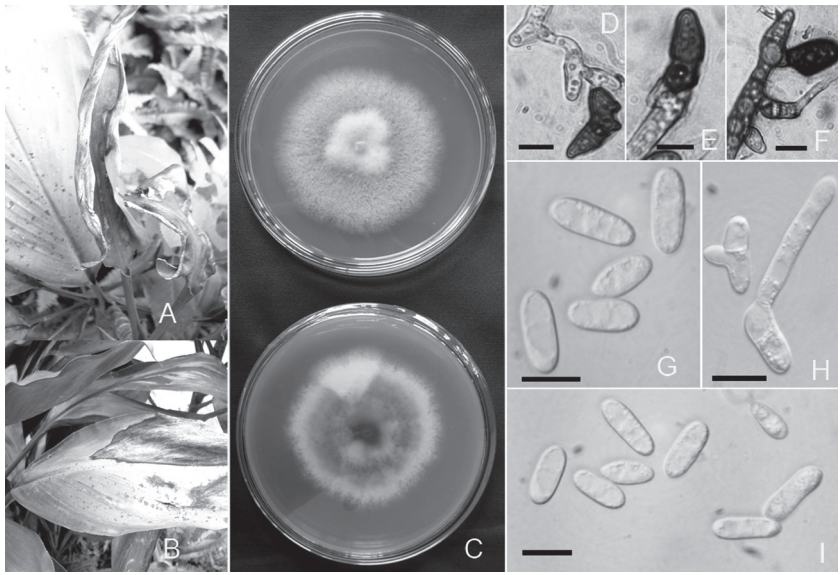

Figure 1. *Colletotrichum cordylinicola* (from BCC 38872, holotype) (A, B) symptoms on *Cordyline fruticosa*. (C) upper and lower view of cultures on PDA after 7 days growth; (D, E,F) appressoria; (G, I) conidia; (H) conidia germination (Bars: D–I = 10 µm).

Results

Taxonomy

Colletotrichum cordylinicola Phoulivong, L. Cai & K.D. Hyde, **sp. nov.** Figure 1

MycoBank 518577

Coloniae crescentes post 7 dies in PDA ad 27°C 75 mm diam. Conidiogenae producentia in acervulis, tubulosa. Conidia 11–20 × 4–5 µm, unicellularae, hyalinae, cylindrici, laeviaad apicem obtuse. Appressoria 13–13.4 × 7.2–7.3 µm, brunnea vel atro-brunnea, irregulariter ovoidea vel clavati.

Holotype: Thailand, Chiang Mai Province, San Sai District, Maejo Village, on *Cordyline fruticosa* (L.) A. Chev. (*Agavaceae*), 15 March 2009, Sitthisack Phoulivong, MFLU10 0289; extype living culture MFUCC 090551, BCC 38872 and CGMCC.

Etymology: Referring to the host genus *Cordyline.*

Colonies on PDA attaining 75 mm diam. in 7 days at 27°C, growth rate 10.8–11.6 mm/day (mean= 11.2, n = 5), white, sparse, with grey-orange visible conidial masses and with floccose aerial mycelia in centre, reverse slightly greenish.

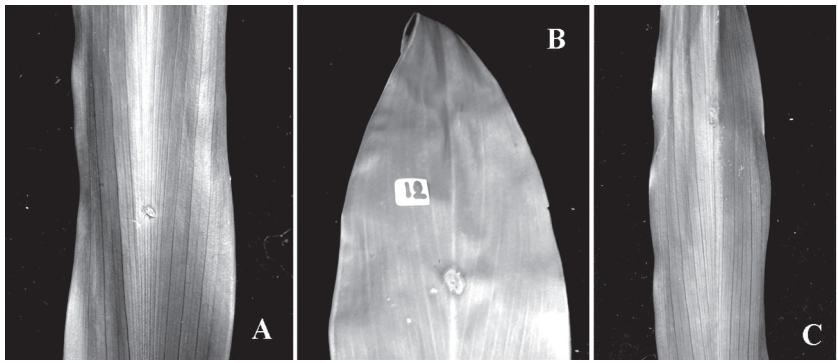

Figure 2. Anthracnose symptom on *Cordyline fruticosa* 7 days after inoculation.

Sclerotia absent. Setae absent. Conidiophores congregative, straight or geniculate, produced in the acervuli. Conidia 11–20 × 4–5 µm (mean = 15.37 ± 0.6 × 4.5 ± 0.56, n = 30), one-celled, hyaline, cylindrical with round ends, smooth-walled, guttulate. Spore germination on PDA mostly observed near the apex of the conidia, sometimes from the centre. Appressoria in slide culture 13–13.4 × 7.2–7.3 µm (mean = 13.20 ± 0.94 × 7.25 ± 0.61, n = 10), mostly formed from conidia, brown to dark brown, variable in shape including ovoid, clavate or slightly irregular, often becoming complex with age.

Teleomorph — not produced in culture.

Known Distribution — Thailand and Laos.

Additional Specimens Examined: **Laos**, Vientiane, Pakngum District, Don-ngaeng Village, on rose apple fruit (*Eugenia javanica* Lam. (*Myrtaceae*)), 26 September 2007, Sitthisack Phoulivong, MFLU10 0290, living culture MFLU 09 0636, IFRD 2149, BCC38864, CGMCC 3.14199. **Thailand**, Chiang Rai, Doi Tung, on *Cordyline fruticosa*, Noireung Parinn, MFLU10 0291, living cultures MFLU 100132, CGMCC 3.14200.

Phylogenetic study

The dataset of six combined genes comprised 2506 characters after alignment, of which 545 characters were parsimony informative (21.7%). The KH test showed that the two trees inferred from parsimonious analysis were not significantly different. One of the most parsimonious trees (TL = 1377, CI = 0.895, RI = 0.881, RC = 0.798, HI = 0.105) generated from dataset of six combined gene regions is shown in Figure 3 The phylograms inferred from single genes ACT, GS, TUB2, ITS, CAL and GPDH show similar topology as that from combined datasets but with much lower statistical support for branches (results not shown). In the phylogenetic tree, three strains of *C. cordylinicola* clustered in a distinct lineage and appeared as a sister clade to *C. kahawae* (100% bootstrap and posterior probability). Other reference taxa employed in the analysis include type strains

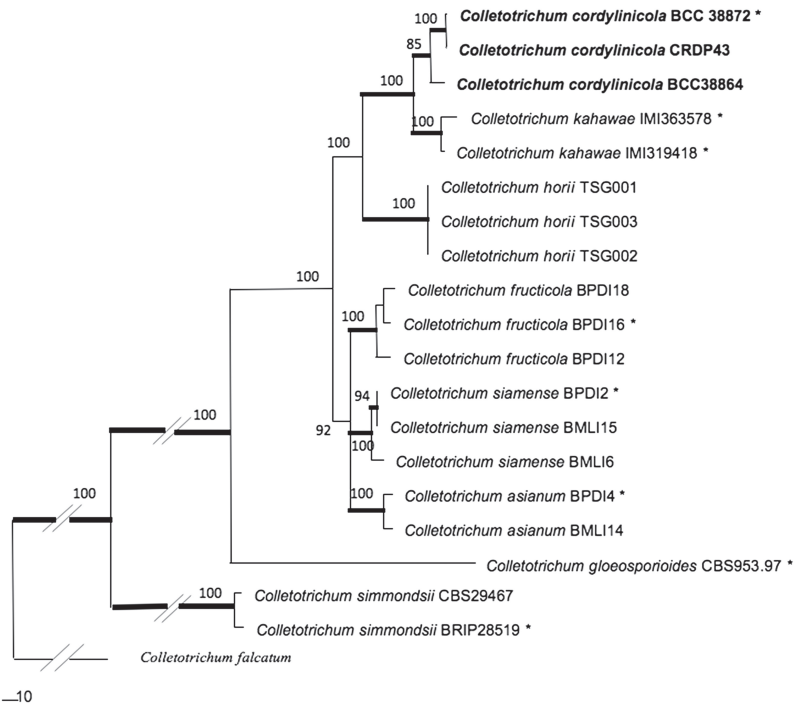

Figure 3. Maximum parsimony phylogram showing phylogenetic relationships among isolates of *Colletotrichum cordylinicola* and closely related taxa based on combined ACT, TUB2, CAL, GS, ITS, and GPDH sequences. Data were analysed with random addition sequence, unweighted parsimony, and treating gaps as 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*. * indicates sequences from type specimens.

of *C. simmondsii, C. asianum, C. fructicola, C. gloeosporioides, C. kahawae, C. siamense* and authentic strains of *C. horii.*

Pathogenicity testing

Two isolates of *C. cordylinicola* were tested for their pathogenicity and potential for cross infection. In inoculation tests, the strain isolated from *Cordyline fruticosa* infected *Cordyline fruticosa* leaves (Figure 2.) and papaya fruit but did not infect the other fruits tested. The *C. cordylinicola* isolate from rose apple infected rose apple as well as citrus, chilli, guava, mango and papaya fruits but not *Cordyline fruticosa* leaves. The qualitative comparison of symptom development on different hosts is shown in Table 2.

Table 2: Pathogenicity and potential of cross infection of *Colletotrichum cordylinicola* on a range of hosts.

Isolate number	Hosts	Infection on inoculated fruits*						Infection on inoculated leaves of *C. fruticosa*
		Chilli	Guava	Mango	Orange	Papaya	Rose apple	
BCC 38872	*Cordyline fruticosa*	-	-	-	-	+	-	+
BCC 38864	*Eugenia javanica*	+	+	+	-	+	+	-

Discussion

Colletotrichum cordylines Pollacci (from Italy) is the only species of *Colletotrichum* described from *Cordyline* (*C. indivisa*). Conidial sizes were not provided in the protologue (Pollacci 1899: 44; Saccardo & Sydow 1899: 1017) and the name has not recently been used (Hyde et al. 2009b). It is impossible to establish whether our collections have any relationship to the type of *C. cordylines,* as there are no living extype cultures and it is presently impossible to isolate DNA from such an old type specimen. It is, therefore, prudent to introduce our collections as a new species.

Colletotrichum cordylinicola is morphologically similar to several species in the *C. gloeosporioides* complex. Species in this complex are difficult to differentiate based solely on morphology. Phylogenetic analysis using ITS sequences could not confidently resolve its systematic placement but showed that this fungus is well clustered in the *C. gloeosporioides* complex (details not shown). A multi-locus phylogeny based polyphasic approach was therefore employed to infer interspecific relationships in this group of fungi (Cai et al. 2009). In the six-gene combined phylogeny, the species relationships are well defined with all the major clades supported by parsimony bootstrap support and Bayesian posterior probabilities (Figure 3). The conidial morphology of *C. cordylinicola* is similar to that of *C. siamense*. However, *C. cordylinicola* can be distinguished from this species by its appressoria, which are irregular in shape (Figure 1). In the phylogenetic tree, *C. cordylinicola* does not group with *C. siamense*, but clusters as a sister clade to *C. kahawae* (Figure 3). Although similar in conidial morphology, *C. cordylinicola* can be differentiated from *C. kahawae* by its significantly larger appressoria (13–13.4 × 7.2–7.3 vs 4.5–10 × 4–7 µm) and smaller conidia. This is the first report of *Colletotrichum* species causing anthracnose on *Cordyline fruticosa* in Thailand.

Identification of species within the *C. gloeosporioides* complex has been a difficult issue as these species are morphologically very similar (Bailey & Jeger 1992, Sutton 1992). Morphology of conidia and appressoria, colony characters, host association, growth rate, and biochemical data should be used in conjunction

with a multilocus phylogeny to identify a *Colletotrichum* species accurately (Cai et al. 2009; Prihastuti et al. 2009). In this study, a phylogenetically well-defined lineage is associated with distinct morphological and other phenotypic characters. It is therefore given species rank and described as a new species.

The strain of *C. cordylinicola* isolated from rose apple failed to infect *Cordyline fruticosa*, while that from *Cordyline fruticosa* failed to infect rose apple. In morphology, the two strains are essentially similar except the one from rose apple produced conidia that are slightly acute at one end, while the conidia in the strain from *Cordyline fruticosa* are rounded at both ends. The strains are, however, shown to be related based on multigene phylogenetic analysis with 100% support (Figure 3). The strain from rose apple infected more fruits than that from *Cordyline fruticosa*. This finding supports the statement of Johnston (2000) that "there are no general rules concerning host relationships within *Colletotrichum* . . . the group so recognized cannot be assumed genetically equivalent, even when appearing to be biologically similar". It will be interesting to establish whether these strains represent two pathotypes in nature (Bailey & Jeger 1992). Pathogenicity may be affected by several environmental factors such as variety and condition of the fruit, humidity and temperature, and the concentration of inoculum (Simmonds 1965, Freeman et al. 1998). The result reported here may not accurately reflect the true virulence potential. Future research should attempt to determine the pathogenicity of these strains according to natural infections rather than artificial inoculations. On the other hand, if more phenotypic divergence of these two strains could be identified following further collections or study, the systematic relationship between the two strains may need a re-evaluation.

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