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Research article

Pathogenicity and molecular phylogenetic analysis reveal *Colletotrichum siamense* as causal agent of leaf fall disease in rubber trees (*Hevea brasiliensis*) in southern Thailand

Saithong Kaewchai[a,*], Panida Paopradit[a], Chaninun Pornsuriya[b], Ruvishika Shehaki Jayawarrdena[c,d], Kevin David Hyde[c]

[a] *Faculty of Agriculture, Princess of Naradhiwas University, Narathiwat 96000, Thailand*
[b] *Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110, Thailand*
[c] *Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57240, Thailand*
[d] *School of Science, Mae Fah Luang University, Chiang Rai 57240, Thailand*

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Abstract

Importance of the work: Leaf fall disease of rubber trees (*Hevea brasiliensis*) has devastated rubber production in southern Thailand.
Objectives: To identify and characterize the pathogen responsible for leaf fall disease in rubber trees and to test the pathogenicity of the isolated pathogens in accordance with Koch's postulates.
Materials & Methods: Leaf sampling was carried out in all 13 districts of Narathiwat province, southern Thailand. Fungal isolation was performed using a tissue transplanting technique. The obtained isolates were tested for their pathogenicity that was determined using Kochs' postulates through the detached leaf technique. The causal agent was identified based on morphological and molecular evidence.
Results: In total, 160 isolates were obtained from all sampling locations. The pathogenicity tests using detached leaves demonstrated that all isolates showed the leaf spot, with three isolates (2CHI12, 1SP12-1 and 4MU33-1) producing the most severe symptoms. These three causal agents were identified as *Colletotichum siamense* 2CHI12, 1SP12-1 and 4MU33-1, based on morphological and molecular evidence. The species of the causal agent was identified using the internal transcribed spacer (ITS) region, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), actin (*ACT*), chitin synthase (*CHS-1*), beta-tubulin 2 (*TUB2*) and the Apn2-Mat1-2 intergenic spacer region, as well as the partial mating type (Mat1-2) (ApMat) genes that were amplified based on polymerase chain reaction using the primer pairs ITS1/ITS4, GDF1/GDR1, ACT-512F/ACT-783R, CHS-79F/CHS-345R, Bt2a/Bt2b, and AM-F/AM-R, respectively.
Main finding: The morphological and phylogenetic analysis confirmed that the causal agent of leaf fall disease was *Colletotichum siamense.*

* Corresponding author.
E-mail address: saithong.k@pnu.ac.th (S. Kaewchai)

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Introduction

The rubber tree (*Hevea brasiliensis* Muell.Arg., Euphorbiaceae) is a very important economic crop in several countries; however, the production of natural rubber is now concentrated in southeast Asia, especially in Thailand, Indonesia, Malaysia and Vietnam (Pornsuriya et al., 2020). These countries produce more than 97% of the global supply of natural rubber (Cao et al., 2019). As with any other crop, rubber trees are susceptible to diseases caused by fungi and fungus-like organisms. *Corynespora cassiicola* causes Corynespora leaf disease (Jinji et al., 2007; Mazlan et al., 2019), Phytophthora leaf fall disease is caused by *Phytophthora palmivora* and *P. botyosa* (Krishnan et al., 2019), *Colletotrichum acutatum* causes Colletotrichum leaf spot disease (Hunupolagama et al., 2017; Mazlan et al., 2019), powdery mildew is caused by *Erysiphe quercicola* (Liyanage et al., 2016), white rot disease is caused by *Rigidoporus microporus* (Mazlan et al., 2019) and brown rot disease is caused by *Pyrrhoderma noxium* (Sunpapao and Pornsuriya 2014; Mazlan et al., 2019). However, in late 2019, the rubber plantations in Narathiwat, Pattani and Yala provinces, which are all located in southern Thailand, were infected by a severe leaf fall disease, resulting in production losses of up to 50%. This disease was identified to be a new leaf fall disease based on the defoliation of the trees (Rojsujit et al., 2020). This leaf fall disease has also been observed in Indonesia, India, Malaysia and Sri Lanka with similar symptoms (Aliya et al., 2022). Initially, the outbreak of this disease was reported in 2016 in a rubber plantation in North Sumatra, Indonesia. From there, it spread throughout South Sumatra in 2017 and in the same year, this disease was reported to have affected rubber plantations in India and Malaysia (Aliya et al., 2022; Damiri et al., 2022). In 2019, there were reports of this disease in Sri Lanka (Aliya et al., 2022). The symptoms of this disease are observed as succulent spots on the underside of the leaves that then develop into dark brown spots; thereafter, defoliation occurs, with the level being up to 90–100%. Rojsujit et al. (2020) studied the morphology of the causal pathogen and reported that a *Colletotrichum* species was the causal agent of leaf fall disease of rubber trees in Thailand. However, the identity of the fungal species associated with leaf fall disease is still unclear. Therefore, the current research aimed to identify and characterize the pathogen responsible for leaf fall disease in rubber trees and to test the pathogenicity of the isolated pathogens in accordance with Koch's postulates.

Materials and Methods

Sample collection and isolation

The sample collection was carried out in Narathiwat province, southern Thailand, where the disease was first observed and a severe epidemic occurred. The samples were collected from all 13 districts (Bacho, Chanae, Cho-airong, Mueang, Ra-ngae, Rueso, Si Sakhon, Sukhirin, Su-ngai Kolok, Su-ngai Padi, Tak-Bai, Waeng and Yi-ngo) of Narathiwat province. The samples were collected between June 2021 and February 2022, during the rainy season in southern Thailand. Samples were collected more than two times in each area, with 10 samples being collected at all developmental stages of the infected leaves each time. The samples were brought to the laboratory within 24 hr of collection. The characteristics of the spot lesions of the infected leaves were observed using a stereomicroscope (S8AP0; Leica; Germany) and a light microscope (DM750; Leica; Germany).

Fungal isolation was performed based on tissue transplantation, as described by Aliya et al. (2022) with some modifications. Approximately 5 mm × 5 mm sections of infected tissues were cut and placed in 10% sodium hypochlorite (NaOCl) for 1 min. Then, the tissues were washed three times with sterile water and dried using sterile filter paper. The disinfected sections were placed on water agar and incubated at room temperature (28=30°C) for 3 d. Four sections of one lesion were placed on each plate with three replications per sample. The edges of the fungal hyphae growing on the tissues were cut and transferred to potato dextrose agar (PDA), and a pure culture was obtained using the hyphal tip method (Pornsuriya et al., 2020).

Pathogenicity tests

All obtained isolates were tested for their pathogenicity. They were grown on PDA for 7 d and conidial suspensions of test fungi were prepared with sterile distilled water at 1×10^6 conidia/mL. The pathogenicity test was carried out using the detached leaf method described by Aliya et al. (2022) with some modifications. Each rubber tree leaf was wounded using a fine sterile needle on the left and right sides of the midrib. Then, the wound was treated with a mycelial plug or conidial suspension, while an agar plug or sterile water was used for the control. Four leaves were inoculated per fungal isolate. The trials were repeated twice. The inoculated leaves were

kept in a moist chamber and maintained at room temperature for 7 d. The appearance and size of each lesion were recorded, and disease severity was calculated according to Bhunjun et al. (2021).

The three isolates that could cause severe symptoms from the detached leaf method by using an agar plug and conidial suspension were used to confirm the pathogenicity test on seedlings in the greenhouse. Seedlings of the RRIM600 clone were planted in separate plastic bags containing a soil-to-compost ratio of 1:1. The wounded and unwounded leaves were inoculated with conidial suspension (1×10^6 conidia/mL). Four rubber seedlings were inoculated per isolate, with seedlings inoculated with sterile water serving as controls. Then, the treated seedlings were covered with a plastic bag for 72 hr to maintain the humidity while the plants remained in the greenhouse for observation of symptoms for 14 d. The temperature ranges in the greenhouse during the day and at night were 30–32°C and 24–26°C, respectively. The pathogens were re-isolated from the inoculated leaves to confirm Koch's postulates. The experiment applied a completely randomized design and was repeated twice.

Morphological and molecular identifications

The three isolates of the causal pathogen were identified based on the cultural, morphological and molecular data. Cultural identification, including the texture of mycelia and the color of the tops and bottoms of the isolates, was based on observations made on the growth of the colony on the PDA medium. The size and shape of the conidia were recorded following observation under a light microscope. The colony diameter was measured daily for 9 d, from which the growth rate was calculated.

The species of fungal pathogens were confirmed using molecular techniques. The fungal isolates were cultured on PDA for 5 d. The DNA was extracted from mycelia using a mini-preparation method developed by Saitoh et al. (2006). Six loci—the internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), partial actin (*ACT*), chitin synthase (*CHS-1*), beta-tubulin (*TUB2*) and Apn2-Mat1-2 intergenic spacer, as well as the partial mating type (Mat1-2) (ApMat)—were amplified with the primer pairs ITS1/ITS4 (White et al., 1990), GDF1/GDR1(Guerber et al., 2003), ACT-512F/ACT-783R (Carbone and Kohn, 1999), CHS-79F/CHS-345R (Carbone and Kohn, 1999), Bt2a/Bt2b (Glass and Donaldson, 1995) and AM-F/AM-R (Silva et al., 2012), respectively.

The polymerase chain reaction (PCR) amplifications were performed using a BIO-RAD T100 Thermal Cycler (BIO-RAD Laboratories; United States of America) in a 25 uL reaction volume. The PCR mixture contained 2 µL of DNA, 1 µL of forward primer, 1 µL of reverse primer 1, 5 µL of 5x HOT FIREPol® Blend Master Mix (Solis BioDyne; Estonia) and 16 µL of Rnase-free water. The thermal cycling program for the ITS region consisted of pre-denaturation at 95°C for 13 min followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 30 s, an extension at 72°C for 1 min and a final extension at 72°C for 5 min. The other loci were treated using the same conditions except for the annealing temperatures: GAPDH at 60°C, ACT at 58°C, CHS-1 at 58°C, TUB2 at 55°C and ApMat at 60°C. The PCR products were observed using gel electrophoresis. DNA sequencing was conducted using the Macrogen Sequencing Service (*Seoul*, Republic of *Korea*).

Forward and reverse DNA sequences were aligned, edited and merged using the MEGA X software (Kumar et al., 2018). The generated sequences in the current study were aligned with the known sequences in the National Center for Biotechnology Information database (Table 1) using MAFFT v. 7.0 online servers (http://mafft.cbrc.jp/alignment/server/index.html) and manually adjusted as necessary in MEGA X, except for the sequence of the ApMat gene, which was used only in the Blastn search. The phylogenetic tree estimation for each alignment was performed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). Tree with maximum parsimony were obtained using the heuristic search option with 1,000 random additions of sequences and tree bisection and reconnection as the branch-swapping algorithm of MEGA X. ML trees were constructed using MEGA X based on the TN93 + G evolutionary model. The Bayesian tree was generated using MrBayes ver. 3.2.7. (https://nbisweden.github.io/MrBayes/manual.html). Two parallel Markov chain Monte Carlo runs were performed for 1,000,000 generations and were sampled every 100 generations. The initial 1,000 generations were discarded as burn-in, with the remaining trees being used to calculate the Bayesian inference posterior probability values. The phylogenetic trees were visualized using the FigTree v1.4.0 software (http://tree.bio.ed.ac.uk/software/figtree/).

Table 1 Collection details and GenBank accession numbers of isolates (Genus *Colletotrichum*) in this study

Species	Culture accession No.	Host/Substrate	Location	GenBank accession					
				ITS	GAPDH	ACT	CHS-1	TUB2	ApMat
C. aenigma	ICMP 18608*	*Persea americana*	Israel	JX010244	JX010044	JX009443	JX009774	JX010389	KM360143
	ICMP 18686	*Pyrus pyrifolia*	Japan	JX010243	JX009913	JX009519	JX009789	JX010390	-
C. aeschynomenes	ICMP 17673 = ATCC 201874	*Aeschynomene virginica*	USA	JX010176	JX009930	JX009483	JX009799	JX010392	-
C. alatae	CBS 304.67 = ICMP 17919	*Dioscorea alata*	India	JX010190	JX009990	JX009471	JX009837	JX010383	
	ICMP 18122	*Dioscorea alata*	Nigeria	JX010191	JX010011	JX009470	JX009846	JX010449	KC888932
C. alienum	ICMP 12071*	*Malus domestica*	New Zealand	JX010251	JX010028	JX009572	JX009882	JX010411	-
	ICMP 18621	*Persea americana*	New Zealand	JX010246	JX009959	JX009552	JX009755	JX010386	-
C. asianum	ICMP 18648 = CBS 124960	*Mangifera indica*	Panama	JX010193	JX010017	JX009546	JX009871	-	-
	ICMP:18580 = CBS 130418*	*Coffea arabica*	Thailand	JX010196	JX010053	JX009584	JX009867	JX010406	-
C. boninense	MAFF 305972 = CBS 123755	*Crinum asiaticum* var. *sinicum*	Japan	MH863323	JQ005240	JQ005501	JQ005327	JQ005588	-
C. endophytica	LC 0324 = MFLU13-0418*	*Pennisetum purpureum*	Thailand	KC633854	KC832854	KF306258	-	MZ673954	-
	OBP5	*Piper nigrum*	India	KJ947310	KJ947279	KJ947187	KJ947233	KJ947210	-
C. fructicola	ICMP 18581 = CBS 130416	*Coffea arabica*	Thailand	JX010165	JX010033	JX009501	JX009866	JX010405	-
	ICMP 18645 = CBS 125395	*Theobroma cacao*	Panama	JX010172	JX009992	JX009543	JX009873	JX010408	-
C. fructicola (syn. *C. ignotum*)	ICMP 18646 = CBS 125397	*Tetragastris panamensis*	Panama	JX010173	JX010032	JX009581	JX009874	JX010409	-
C. gloeosporioides	IMI 356878 = CBS 112999 = ICMP 17821*	*Citrus sinensis*	Italy	JX010152	JX010056	JX009531	JX009818	JX010445	-
	CBS 119204 = ICMP 18678	*Pueraria montana* var. *lobata*	USA	JX010150	JX010013	JX009502	JX009790	-	-
C. gloeosporioides (syn. *Gloeosporium pedemontanum*)	CBS 273.51(*) = ICMP 19121	*Citrus limon*	Italy	JX010148	JX010054	JX009558	JX009903	-	-
C. grevilleae	CBS 132879*= CPC 15481	*Grevillea* sp.	Italy	KC297078	KC297010	KC296941	KC296987	-	-
C. horii	ICMP 10492 = NBRC 7478	*Diospyros kaki*	Japan	GQ329690	JX009964	JX009438	JX009752	JX010450	-
	ICMP 12942	*Diospyros kaki*	New Zealand	GQ329687	JX010001	JX009533	JX009748	JX010375	JQ899280
C. ledongense	LD1680	*Hevea brasiliensis*	China	MG242008	MG242016	MG242014	MG242018	MG242010	-
	LD1683	*Hevea brasiliensis*	China	MG242009	MG242017	MG242015	MG242019	MG242011	-
C. musae	CBS 116870	*Musa* sp.	USA	JX010146	JX010050	JX009433	JX009896	JX010413	-
	ICMP 17817 = IMI 52264	*Musa* sp.	Kenya	JX010142	JX010015	JX009432	JX009815	JX010395	-
C. nupharicola	CBS 469.96 = ICMP 17938	*Nuphar luteum* subsp. *polysepalum*	USA	JX010189	JX009936	JX009486	JX009834	JX010397	-
	CBS 470.96* = ICMP 18187	*Nuphar luteum* subsp. *polysepalum*	USA	JX010187	JX009972	JX009437	JX009835	JX010398	-
	CBS 472.96 = ICMP 17940	*Nuphar luteum subsp. polysepalum*	USA	JX010188	JX010031	JX009582	JX009836	JX010399	-
C. proteae	CBS 132882 = CPC 14859*	*Protea* sp.	South Africa	KC297079	KC297009	KC296940	KC296986	-	-
	CBS 134301	*Protea* sp.	South Africa	KC842385	KC842379	KC842373	KC842377	KC842387	-
C. queenslandicum	ICMP 1778*	*Carica papaya*	Australia	JX010276	JX009934	JX009447	JX009899	JX010414	KC888928
	ICMP 18705	*Coffea* sp.	Fiji	JX010185	JX010036	JX009490	JX009890	JX010412	-
C. salsolae	ICMP 19051*	*Salsola tragus*	Hungary	JX010242	JX009916	JX009562	JX009863	JX010403	KC888925
	CBS 119296	*Glycine max* (inoculated)	Hungary	JX010241	JX009917	JX009559	JX009791	-	-

Table 1 Continued

Species	Culture accession No.	Host/Substrate	Location	GenBank accession					
				ITS	GAPDH	ACT	CHS-1	TUB2	ApMat
C. siamense	ICMP 18578* = CBS 130417	*Coffea arabica*	Thailand	JX010171	JX009924	JX009518	JX009865	JX010404	-
	ICMP 12567	*Persea americana*	Australia	JX010250	JX009940	JX009541	JX009761	JX010387	-
	ICMP 17795	*Malus domestica*	USA	JX010162	JX010051	JX009506	JX009805	JX010393	-
	1 SP12-1	*Hevea brasiliensis*	Thailand	LC746228	LC746231	LC746234	LC746237	LC746240	LC746243
	2 CHI 12	*Hevea brasiliensis*	Thailand	LC746229	LC746232	LC746235	LC746238	LC746241	LC746244
	4 MU33-1	*Hevea brasiliensis*	Thailand	LC746230	LC746233	LC746236	LC746239	LC746242	-
C. siamense (syn. *C. hymenocallidis*)	CBS 125378	*Hymenocallis americana*	China	JX010278	JX010019	JX009441	JX009865	JX010404	JQ899289
C. theobromicola	CBS 124945*	*Theobroma cacao*	Panama	JX010294	JX010006	JX009444	JX009869	JX010447	-
	ICMP 17958 = MUCL 42295	*Stylosanthes guianensis*	Australia	JX010291	JX009948	JX009498	JX009822	JX010381	-
C. tropicale	CBS 124949* = ICMP 18653	*Theobroma cacao*	Panama	JX010264	JX010007	JX009489	JX009870	JX010407	-
	CBS 124943	*Annona muricata*	Panama	KP703360	JX010014	JX009570	JX009868	KP703431	KP703766
C. xanthorrhoeae	BRIP 45094* = ICMP 17903 = CBS 127831	*Xanthorrhoea preissii*	Australia	JX010261	JX009927	JX009478	JX009823	JX010448	-
	ICMP 17820 = IMI 350817a	*Xanthorrhoea* sp.	Australia	JX010260	JX010008	JX009479	JX009814	-	-

Results

Sample collection and isolation

The diseased samples were collected from different locations in Narathiwat province, Thailand and different symptoms were observed. These symptoms manifested at different stages of the infection, indicating the progression of the disease. The beginning stage of the disease was observed as a small dark spot in the lower and upper parts of the leaf, which became dark and light brown (Fig. 1A). In severe cases, there were many lesions on the leaf and the leaf would then turn orange-red before falling off the tree (Fig. 1B). As the disease progressed, it led to defoliation of the tree (Figs. 1C–1D). The symptoms were observed under the stereomicroscope and showed as dark and light brown spots (Figs. 2A, 2B). These spots had an irregular shape with a light brown center and dark brown edge (Fig. 2C). A pink stripe could be observed separating the light brown center and dark brown edge (Fig. 2D). Many acervuli were observed on the light brown spots (Fig. 2E). Observations using a light microscope determined the presence of pycnidia and conidia (Figs. 2F–2H).

In total, 160 fungal isolates were obtained from the sampling locations. Based on morphological characteristics, such as the texture of the mycelium and color on the PDA and the shape and size of conidia, all isolates were identified as *Colletotrichum* sp.

Fig. 1 Disease symptoms of leaf fall disease on rubber trees: (A) development of leaf spots on rubber leaves; (B) leaf spots on rubber tree leaves; (C) and (D) defoliation of rubber trees

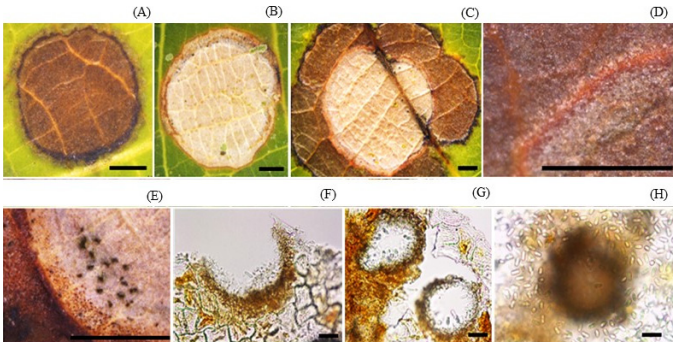


Fig. 2 Disease symptoms observed under a stereo microscope and a compound light microscope: (A) dark brown spot; (B) light brown spots; (C) light brown spots surrounded by dark brown regions; (D) pink stripe between light brown and dark spots; (E) acervuli; (F)–(H) pycnidia and conidia, where scalebars (A)–(E) = 1 mm and (F)–(H) = 20 µm

Pathogenicity tests

All *Colletotrichum* isolates were tested for pathogenicity using the detached leaf technique, with all isolates producing a light brown spot that was similar to that observed in the field. No symptoms were observed in the control. The three isolates that showed the most severe leaf spots were 2CHI12, 1SP12-1 and 4MU33-1 (Fig. 3). The pathogenicity test on the seedlings showed that all three of these isolates caused symptoms and leaf fall similar to the observations made in the field (Figs. 4A–4C). However, the mycelial plug or conidial suspension of the *Colletotrichum* isolates did not infect any of the non-wounded leaves during the pathogenicity test.

Morphological and molecular identification

The causal pathogen isolates 2CHI12, 1SP12-1 and 4MU33-1, which produced the most disease severity, were used for morphological and molecular study. The cultural



Fig. 3 Pathogenicity testing, based on a potato dextrose agar plug or sterile water, showing leaf spot caused by *Colletotrichum siamense* isolates: (A) isolate 2CHI12; (B) isolate 1SP12-1; (C) isolate 4MU33-1, where inoculated leaves with agar plug in upper row and with spore suspension in lower row and control leaves with no lesions in left column of each subfigure inoculated

colony of the causal pathogen isolate 2CHI12 produced white, cottony, fluffy colonies that later turned greyish pale to grey yellowish with orange conidial masses at the inoculation point. The conidia were cylindrical and hyaline with rounded ends. The length of the conidium was 3.42–3.71 µm and the width was 13.19–15.70 µm, with an average size of 3.61×14.67 µm (Fig. 5A). The average growth rate of the colony was 9.18 mm/d.

The causal pathogen isolate 1SP12-1 produced white, cottony, fluffy colonies that later turned grey with orange conidial masses at the inoculation point. The conidia were cylindrical and hyaline with rounded ends. The length of the conidium was 3.74–5.00 µm and the width was 12.28–14.61 µm, with an average size of 4.20×13.98 µm (Fig. 5B). The average growth rate of the colony was 9.33 mm/d.



Fig. 4 Pathogenicity testing of *Colletotrichum siamense* isolates on seedling of rubber tree clone RRIM600: (A) isolate 2CHI12; (B) isolate 1SP12-1; (C) isolate 4MU33-1

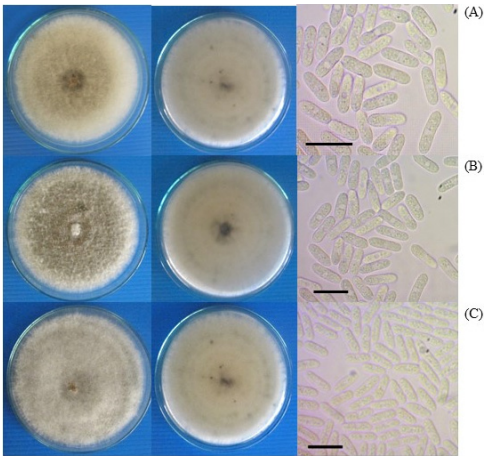


Fig. 5 Morphology and cultural characteristics of *Colletotrichum siamense* isolates: (A) isolate 2CHI12; (B) isolate 1SP12-1; (C) isolate 4MU33-1, where left and middle columns are upper and reverse of cultures on potato dextrose agar age 9 d, right column is conidia and scale bars = 20 µm

The causal pathogen isolate 4MU33-1 produced white, cottony, fluffy colonies that later turned pale grey with orange conidial masses at the inoculation point. The conidia were cylindrical and hyaline with rounded ends. The length of the conidium was 3.79–5.29 µm and the width was 14.13–16.00 µm, with an average size of 4.22×15.02 µm (Fig. 5C). The average growth rate of the colony was 9.11 mm/d. The characteristics of the *C. siamense* in the current study and in other studies are shown in Table 2.

The three isolates 1SP12-1, 2CHI12 and 4MU33-1, were identified through their molecular properties. The BLAST search results for these sequences of the three identified isolates showed that they belonged to the *C. gloeosporioides* species complex, with the *GAPDH*, CHS-*1, TUB* and *ApMat* genes closely related to *C. siamense* at 99–100% similarity, while the ITS region and *ACT* gene were related to *C. aenigma*, *C. fructicola*, *C. gloeosporioides* and *C. siamense* at 100% identity. Following Jayawardena et al. (2021) a 5-locus concatenated dataset (ITS, *GAPDH*, *ACT*, *CHS-1* and *TUB2*) contained 44 reference sequences with *C. boninense* (CBS 123755) as an outgroup and 1,879 characters including gaps. The gene boundaries in the alignment were: ITS: 1–491, *GAPDH*: 492–736, *ACT*: 737–985, *CHS-1*: 986–1201 and *TUB2*: 1,202–1879. The ML tree confirmed the tree topology and posterior probabilities of the Bayesian consensus tree. The phylogenetic tree demonstrated that the three isolates of the causal agent of leaf fall disease in rubber trees were clustered in *C. siamense* group with the Bayesian inference posterior probability (BIPP) value of 0.99 support (Fig. 6). These results supported the observed morphological characteristics and confirmed that the isolates 2CHI12, 1SP12-1 and 4MU33-1, belonged to *C. siamense.*

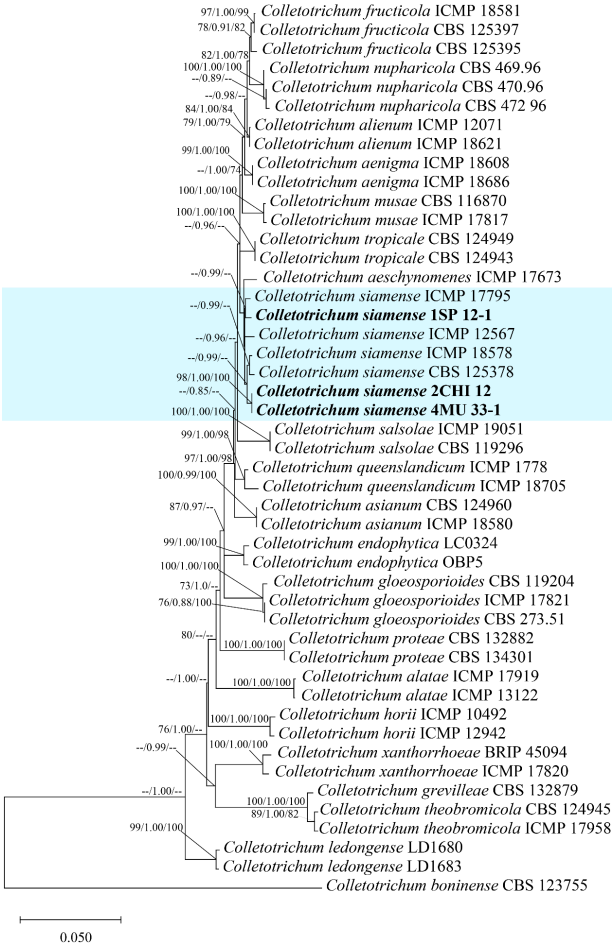


Fig. 6 Phylogenetic tree generated using maximum likelihood analysis based on combined internal transcribed spacer, GAPDH, ACT, CHS-1 and TUB2 gene sequences, where tree displays phylogenetic relationships between *Colletotrichum* species in *Colletotrichum gloeosporioides* species complex (with *C. siamense* in blue highlighted area and bold indicates three isolates of this study, bootstrap supports values above 70% and Bayesian posterior values above 0.70 are shown at each node (maximum likelihood/ Bayesian inference posterior probability /maximum parsimony) and *Colletotrichum boninense* CBS 123755 is used as outgroup

Table 2 Morphological characteristic of *Colletotrichum siamense* isolates

Colony character	Conidia width (µm)	Conidia Length (µm)	Conidia shape	Growth rate (mm/day)	Reference
Cottony dense greyish-white aerial mycelium pale yellowish to a pinkish colony	3.0–6.0 (3.60±0.48)	7.0–18.3 (10.17±1.49)	Fusiform	9.12±1.95 (6.5–11.5)	Prihastuti et al. (2009)
White colony later turning grayish-pale yellowish to orange grayish white and cottony mycelium	4–7.0 (5.7±0.7)	9.0–16.0 (13.3±1.6)	Cylindrical with slightly rounded ends	5.0–12.5	Dwarka et al. (2016)
Cottony, pale yellowish to pinkish mycelium	3.0–6.0 (3.6±0.5)	7.0–18.3 (10.2±1.49)	Fusiform to cylindrical	9.1	Sharma et al. (2015)
White with dense cottony aerial mycelium; a few acervuli at the point of inoculation with orange conidial masses; reverse grey to pale brown; covers the entire plate	(3) 3.5–5.0 (5)	(12) 13.5–17.5 (18)	-	-	James et al. (2014)
White, cottony and fluffy, which later turned pale grey or pale yellow with orange conidial masses at the inoculum point	3.47–5.29 (4.02±0.52)	12.28–16.00 (14.55±0.74)	Cylindrical with round ends	9.18	This study

Discussion

The causal pathogen of new leaf fall disease in the current study was identified as *Colletotrichum siamense* based on morphological and molecular approaches. The analyses of the 160 isolates confirmed that *Colletotrichum* was the pathogen of leaf fall using Koch's postulates (data not shown). The results from the *in vitro* pathogenicity test indicated that the wounded leaves inoculated with either the spore suspension or mycelial plug of *C. siamense* produced lesions similar to those observed on rubber leaves in the field. The pathogen was unable to infect unwounded leaves using the pathogenicity test techniques. These findings were similar to those of Aliya et al. (2022) and Pornsuriya et al. (2020), as they reported that none of the unwounded leaves produced any lesions. It can be assumed that the rubber leaves were infected by the pathogen only in the presence of wounds, which was probably caused by other microorganisms, insects, poor soil conditions, lack of nutrition, wind, heavy rainfall or human activities (Aliya et al., 2022). In Narathiwat province, where the leaf fall disease was first observed, severe occurrences have been noted in the monsoon season from October to December.

The mycelia of *C. siamense* on the PDA medium were white, cottony and fluffy, later turning light pale grey and pale yellow with orange conidial masses at the inoculation point. The character of the colony on PDA and spore size were similar to those reported by James et al. (2014). The causal pathogen *C. siamense* was determined according to the molecular properties and phylogenetic tree inferred from the combined datasets of the ITS region, *GAPDH*, *ACT*, *CHS-1* and *TUB2* gene sequences. Identification of the different *Colletotrichum* species is difficult due to their limited morphological characters and single gene phylogenetic analyses cannot be used to successfully delineate the species (Hyde et al., 2009). Hence, multi loci, such as *ACT*, ApMat, *CAL*, *CHS-1*, *GPDH*, *GS* and *TUB2*, have been used for *Colletotrichum* species identification (Khodadadi et al., 2020; Alhudaib et al., 2023).

Rojsujit et al. (2020) reported that the causal pathogen of the new leaf fall disease in rubber trees in Thailand was *Colletotrichum* sp.; however, Pornsuriya et al. (2020) reported that *Neopestalotiopsis cubana* and *N. formicarum* were the causal pathogens of new leaf fall disease. However, the causal pathogen of the new leaf fall disease in the current study was the same fungus as reported by Aliya et al. (2022), who determined that *C. siamense* was the primary pathogen of leaf fall disease and *Pestalotiopsis jesteri* was the secondary pathogen in Malaysia.

Leaf fall disease in rubber plantations is reported to be caused by a complex of pathogens. For instance, in Gabon, secondary leaf fall disease in rubber trees was caused by *Colletotrichum gloeosporioides* (Guyot et al., 2001). In India, *Corynespora cassicola* and *C. gloeosporioides* were the main causes of leaf disease, with *Corynespora* leaf fall disease occurring in the dry season, whereas *Colletotrichum* leaf disease was prominent in the rainy season when the high humidity caused severe outbreaks of the disease, resulting in trees devoid of leaves (Manju et al., 2014). In Indonesia, pathogens of leaf fall disease in rubber plants include *C. gloeosporioides*, *Oidium heveae*, *C. cassiicola* and *Pestalotiopsis microspora*; however, *P. microspora* is the main pathogen of new leaf fall disease in rubber trees there (Alchemi and Jamin, 2022; Damiri et al., 2022). Nyaka-Ngobisa et al. (2017) reported that *P. microspora* caused leaf blight in rubber trees in Cameroon.

Colletotrichum species can cause many diseases in rubber trees, as *Colletotrichum acutatum* and *C. gloeosporioides* are causal agents of *Colletotrichum* leaf disease (CLD) in Sri Lanka (Thambugala and Deshappriya, 2009) and *Colletotrichum* leaf spot disease in India (Manju et al., 2014). *Colletotrichum siamense* is the causal agent of CLD in rubber trees in China (Wang et al., 2022). Other studies have shown that *Colletotrichum* spp. can cause leaf disease in rubber trees, including *C. australisinense, C. bannanense*, *C. fructicola*, *C. ledongense*, *C. siamense* and *C. wanningense*. Among them, *C. australisinense* and *C. siamense* have been recognized as major causal agents of leaf disease in rubber trees (Liu et al., 2018; Cao et al., 2019).

Originally, *Colletotrichum siamense* was known to be associated with coffee berry disease in Thailand (Prihastuti et al., 2009). It can infect many plants and cause many diseases (Weir et al., 2012), such as crown rot in strawberries (Luo et al., 2021), as well as anthracnose disease and leaf disease in many plants, including *Citrus* spp., *Piper nigrum*, *Artocarpus heterophyllus*, *Artocarpus sericicarpus*, *Coffea arabica*, *Coffea canephora*, *Eriobotrya japonica*, *Ficus carica*, *Mentha* sp., *Rosmarinus officinalis*, *Theobroma cacao*, *Jasminum sambac*, *Pongamia pinnata*, *Bauhinia forficata* subsp. *pruinose* and *Macadamia integrifolia*, (Wikee et al., 2011; James et al., 2014; Larran et al., 2015; Dwarka et al., 2016; Qiu et al., 2020; Jian et al., 2021; Perez-Mora et al., 2021; Wang et al., 2021). Casas et al. (2021a, b) reported that *C. siamense* can be endophytic and is used as a biological control in anthracnose disease.

In conclusion, the current study clearly showed that *C. siamense* caused the new leaf fall disease in rubber trees in southern Thailand. This study attempted to identify and characterize the *Colletotrichum siamense* fungi causing leaf fall disease of rubber tree in southern Thailand using both morphological and molecular analyses. However, the potential impacts of the disease on the production of rubber latex and on disease control still need to be verified in the near future.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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