Article

Discovery of Three Novel *Cytospora* Species in Thailand and Their Antagonistic Potential

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Abstract: During an ongoing research survey of saprobic fungi in Thailand, four coelomycetous strains were isolated from decaying leaves in Chiang Mai and Phitsanulok Provinces. Morphological characteristics demonstrated that these taxa are typical of *Cytospora* in forming multi-loculate, entostromatic conidiomata, branched or unbranched conidiophores, with enteroblastic, phialidic conidiogenous cells and hyaline, allantoid, aseptate conidia. Multiloci phylogeny of ITS, LSU, ACT, RPB2, TEF1- α and TUB2 confirmed these taxa are distinct new species in *Cytospora* in Cytosporaceae (Diaporthales, Sordariomycetes), viz., *Cytospora chiangmaiensis*, *C. phitsanulokensis* and *C. shoreae*. *Cytospora chiangmaiensis* has a close phylogenetic relationship with *C. shoreae*, while *C. phitsanulokensis* is sister to *C. acaciae*. These three novel species were also preliminary screened for their antagonistic activity against five plant pathogenic fungi: *Colletotrichum fructicola*, *Co. siamense*, *Co. artocarpicola*, *Co. viniferum* and *Fusarium sambucinum*. *Cytospora shoreae* and *C. phitsanulokensis* showed >60% inhibition against *Co. viniferum* and *F. sambucinum*, while *C. chiangmaiensis* had moderate inhibition activity against all pathogens.

Keywords: antifungal activity; coelomycetous fungi; Cytosporaceae; multiloci phylogeny; saprobes; taxonomy

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1. Introduction

Cytospora Ehrenb. is mainly characterized by the coelomycetous asexual morph producing single or labyrinthine, loculate stromata, filamentous conidiophores, with enteroblastic, phialidic conidiogenous cells and hyaline, allantoid, aseptate conidia [1–3]. The sexual morph of *Cytospora* is characterized as solitary, immersed to erumpent ascostromata, composed of prosenchymatous or pseudoparenchymatous cells of valsoid or diatrypelloid configurations, embedded in ectostromatic disc, present or absent paraphyses, J-, ellipsoid to clavate asci with refractive apical ring, and hyaline, ellipsoid to allantoid, aseptate ascospores [1,4,5]. Ehrenberg [6] initially introduced the genus *Cytospora* to accommodate four species, *C. betulina*, *C. epimyces*, *C. resinae* and *C. ribis*. The type species, *C. chrysosperma*, was introduced by Donk [7] as the asexual morph of *Valsa sordida* [8]. *Cytospora* was previously treated as the asexual morph of *Valsa* and other related genera, *Leucocytophora*, *Leucostoma*, *Valsella* and *Valseutypella* [4,8,9]. Based on the International Code of Nomenclature for Algae, Fungi, and Plants (ICN) [10], Rossman et al. [8] recommended to use *Cytospora* instead of *Valsa* as *Cytospora* was prior introduced and thus *Valsa* was treated as

a synonym of *Cytospora*, together with the genera *Leucocytospora*, *Leucostoma*, *Valsella* and *Valseutypella* [4,8].

Species identification in *Cytospora* was problematic in the past due to overlapping morphological characteristics and wide host ranges [11–15]. A polyphasic approach including host association, geographical distribution, morphological characteristics and multiloci phylogeny has been widely accepted for identifying *Cytospora* species [1–3,5,9,16–18]. To date, 671 species epithets are listed in Index Fungorum [19], but less than a quarter of taxa in this genus are clarified with phylogenetic placement. In recent years, about 117 species have been accepted based on morpho-molecular approaches [20], and these species have primarily been described from China, Italy, Russia and Thailand [1–3,5,9,16–18,21].

Cytospora is a cosmopolitan genus with diverse ecological niches, including plant pathogens, endophytes and saprobes, colonizing decaying wood substrates [1,2,17,18]. *Cytospora* species are important causal agents of diebacks and stem cankers in numerous woody plants such as *Betulaceae*, *Pinophyta*, *Fabaceae*, *Juglandaceae*, *Rosaceae*, *Salicaceae*, *Tiliaceae* and *Ulmaceae* [1,3,16–18,22,23]. Furthermore, *Cytospora* has also been reported to produce potential secondary metabolites with various biological activities, such as antibacterial, antifungal and antiviral activities [24–26]. Investigation of the endophytic fungus *Cytospora* sp. from Costa Rica yielded novel antibiotic compounds: cytoskyrins and cytosporones [24,25]. Upon screening novel secondary metabolites produced by the endophytic fungus *Cytospora* sp. from Chinese mangrove, new bicyclic sesquiterpene and seiricardine D were isolated and they showed effective inhibition of human and plant pathogens [27].

During a survey of saprobic fungi in Thailand, three *Cytospora* species were collected and isolated. Based on morphological characteristics and phylogenetic analyses, our strains were identified as new species of *Cytospora*. In addition, these fungi were tested in vitro as the first step for screening new biocontrol agents against pathogenic fungi.

2. Materials and Methods

2.1. Collection and Isolation of Fungi

Decaying leaves were collected from disturbed forests in Chiang Mai and Phitsanulok Provinces in Thailand. The forests were disturbed due to the utilization of bioresources by the local communities. Samples were kept in plastic bags with labels of location, date, host and collector details before being taken to laboratory for morphological observation. We followed Senanayake et al. [28] for single spore isolation by using potato dextrose agar (PDA) and incubating at room temperature (28 °C). Pure cultures were deposited in Mae Fah Luang University Culture Collection (MFLUCC) and type specimens were deposited in the Herbarium of Mae Fah Luang University (MFLU). New taxa were registered in Faces of Fungi [29] and Index Fungorum databases [19].

2.2. Morphological Observation

Conidiomata on host surface were examined using a Motic SMZ 168 Series stereo microscope (Motic Incorporation Ltd., Hong Kong). Hand-sectioning of conidiomata was carried out and the sections were mounted on a slide with a drop of distilled water. Morphological characteristics including structure and size of stromata, ectostromatic disc, ostioles as well as shape and size of conidiogenous cells, conidiophores and conidia were observed and photographed using a Nikon ECLIPSE 80i compound microscope equipped with a Canon EOS 600D digital camera. Microscopic elements were measured using the Tarosoft (R) Image Frame Work program. The measurements of each structure were represented as minimum value–maximum value ($\bar{x}$ = sum of all measurements/ n , n = number of measurements). The figures were processed using Adobe Photoshop CS6 Extended version 10.0 software (Adobe Systems, San Jose, CA, USA).

2.3. DNA Extraction, PCR Amplification and Sequencing

Genomic DNA was extracted from fresh mycelium which was grown on PDA for 1–2 weeks using the Biospin Fungus Genomic DNA Extraction Kit (BioFlux®, Hangzhou, China) following the manufacturer's protocol. The amplification of specific ribosomal DNA regions was carried out using two gene regions, including the internal transcribed spacers region of ribosomal DNA (ITS) [30] and the partial 28S large subunit nuclear ribosomal DNA (LSU) [31], and four protein coding gene regions: the RNA polymerase II second largest subunit (RPB2) [32], α -actin (ACT) [33], the translation elongation factor 1- α (TEF1- α) [33] and beta-tubulin (TUB2) [34]. The final volume of PCR mixtures was 25 μ L, including 8.5 μ L ddH₂O, 12.5 μ L 2 $\times$ PCR MasterMix (TIANGEN Co., Beijing, China), 2 μ L DNA template and 1 μ L of each forward and reverse primer. The PCR primers and conditions for each gene regions are described in Table 1. The purification and sequencing of PCR products were conducted by TsingKe Company (Kunming, China).

Table 1. Partial marker regions, primers and PCR conditions used in this study.

Genes (Primer Pair)	References	PCR Condition
ITS (ITS5/ITS4)	[30]	An initial denaturation step of 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 50 s at 55 °C and 90 s at 72 °C, and a final extension step of 10 min at 72 °C
LSU (LROR/LR5)	[31]	
TEF1- α (728F/986R)	[33]	
TUB2 (Bt2a/Bt2b)	[34]	
ACT (512F/783R)	[33]	An initial denaturation step of 5 min at 96 °C, followed by 35 cycles of 40 s at 95 °C, 30 s at 58 °C and 1 min at 72 °C, and a final extension step of 5 min at 72 °C
RPB2 (fRPB2-5F/fRPB2-7cR)	[32]	An initial denaturation step of 5 min at 95 °C, followed by 40 cycles of 1 min at 95 °C, 1 min at 52 °C and 90 s at 72 °C, and a final extension step of 10 min at 72 °C

2.4. Phylogenetic Analyses

Sequences generated in this study were analyzed with other sequences retrieved from the GenBank (www.ncbi.nlm.nih.gov/blast/, accessed on 1 August 2021) and recent publications [1,2,17] (Table 2). Alignments of the individual locus were aligned with MAFFT v.7 (<http://mafft.cbrc.jp/alignment/server/index.html>, accessed on 3 August 2021; [35]) and automatically trimmed using TrimAl (<http://trimal.cgenomics.org> accessed on 1 August 2021; [36]). The single-locus datasets of ITS, LSU, ACT, RPB2, TEF1- α and TUB2 regions were initially analyzed by maximum likelihood (ML) criterion with 120, 72, 69, 50, 55 and 41 sequences, respectively. The tree topologies obtained from single-locus analyses were checked for incongruence and the results did not show any topological conflicts. Phylogenetic analyses of the final concatenated ITS, LSU, ACT, RPB2, TEF1- α and TUB2 sequence datasets were performed via maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI).

Table 2. Taxa used in the phylogenetic analyses and their corresponding GenBank accession numbers. The newly generated sequences are indicated in red bold. The ex-type strains are bold. N/A = unavailable sequence data.

Species Name	Culture Accession No.	Genbank Accession No.					
		ITS	LSU	ACT	RPB2	TEF1- α	TUB2
<i>Cytospora abyssinica</i>	CMW 10181	AY347353	N/A	N/A	N/A	N/A	N/A
<i>C. abyssinica</i>	CMW 10178	AY347354	N/A	N/A	N/A	N/A	N/A
<i>C. abyssinica</i>	CMW 10179	AY347352	N/A	N/A	N/A	N/A	N/A
<i>C. abyssinica</i>	CBS 117004	KY051833	KX965299	KX964737	KX965495	KX965098	N/A
<i>C. acaciae</i>	CBS 468.69	DQ243804	N/A	N/A	N/A	N/A	N/A

Table 2. Cont.

Species Name	Culture Accession No.	Genbank Accession No.					
		ITS	LSU	ACT	RPB2	TEF1- α	TUB2
<i>C. acaciae</i>	Fi2427	MG253918	N/A	N/A	N/A	N/A	N/A
<i>C. acaciae</i>	CPC 28392	KY051980	KX965438	KX964858	KX965579	KX965220	KX965027
<i>C. acaciae</i>	CBS 468.69	KY051937	KX965394	KX964817	N/A	KX965181	KX964990
<i>C. acaciae</i>	CBS 362.93	KY051929	KX965386	KX964811	KX965547	KX965173	KX964983
<i>C. acaciae</i>	CBS 112156	KY051776	KX965241	KX964688	N/A	N/A	KX964888
<i>C. amygdali</i>	CBS 144233	MG971853	N/A	MG972002	N/A	MG971659	N/A
<i>C. amygdali</i>	LH356	MG971852	N/A	MG972001	N/A	MG971658	N/A
<i>C. brevispora</i>	CBS 116829	AF192321	N/A	N/A	N/A	N/A	N/A
<i>C. brevispora</i>	CBS 116811	AF192315	N/A	N/A	N/A	N/A	N/A
<i>C. brevispora</i>	CBS 116829	KY051803	KX965267	KX964709	KX965477	KX965073	KX964909
<i>C. brevispora</i>	CBS 116813	KY051788	KX965252	KX964698	KX965470	KX965061	KX964898
<i>C. californica</i>	CBS 144234	MG971935	N/A	MG972083	N/A	MG971645	N/A
<i>C. californica</i>	KARE1106	MG971948	N/A	MG972094	N/A	MG971647	N/A
<i>C. californica</i>	KARE93	MG971930	N/A	MG972079	N/A	MG971640	N/A
<i>C. cedri</i>	CBS 196.50	AF192311	N/A	N/A	N/A	N/A	N/A
<i>C. cedri</i>	CBS 196.50	KY051905	KX965364	KX964790	KX965534	KX965153	N/A
<i>C. ceratosperma</i>	CBS 116.21	AY347335	N/A	N/A	N/A	N/A	N/A
<i>C. ceratosperma</i>	CFCC 89625	KR045646	KR045725	N/A	KU710977	KP310861	KR045687
<i>C. ceratospermopsis</i>	CFCC 89626	KR045647	KR045726	KU711011	KU710978	KU710934	KR045688
<i>C. ceratospermopsis</i>	CFCC 89627	KR045648	KR045727	KU711012	KU710979	KU710935	KR045689
<i>C. chiangmaiensis</i>	MFLUCC 21-0049	MZ356514	MZ356518	MZ451157	MZ451165	MZ451161	MZ451169
<i>C. curvata</i>	MFLUCC 15-0865	KY417728	N/A	KY417694	N/A	N/A	N/A
<i>C. davidiana</i>	CXY 1350	KM034870	N/A	N/A	N/A	N/A	KM034902
<i>C. davidiana</i>	CXY 1374	KM034869	N/A	N/A	N/A	N/A	KM034901
<i>C. diopuiensis</i>	MFLUCC 18-1419	MK912137	MK571765	MN685819	N/A	N/A	N/A
<i>C. diopuiensis</i>	MFLUCC 18-0598	MT215491	MT215540	N/A	MT212203	N/A	N/A
<i>C. erumpens</i>	MFLUCC 16-0580	KY417733	KY417767	KY417699	KY417801	N/A	N/A
<i>C. erumpens</i>	CFCC 53163	MK673059	MK673089	MK673029	MK673000	MK672948	MK672975
<i>C. eucalypti</i>	LSEQ	AY347340	N/A	N/A	N/A	N/A	N/A
<i>C. eucalypti</i>	CBS 144241	MG971907	N/A	MG972056	N/A	MG971617	N/A
<i>C. eucalypti</i>	7G-62	MG971910	N/A	MG972060	N/A	MG971620	N/A
<i>C. eucalypti</i>	CBS 116814	KY051789	KX965253	KX964699	N/A	KX965062	KX964899
<i>C. eucalyptina</i>	CMW 5882	AY347375	N/A	N/A	N/A	N/A	N/A
<i>C. eucalyptina</i>	CBS 118085	KY051848	KX965315	N/A	N/A	N/A	KX964935
<i>C. eucalyptina</i>	CBS 116853	KY051822	KX965288	KX964727	N/A	N/A	KX964918
<i>C. fraxinigena</i>	MFLU 17-0880	MF190134	MF190079	N/A	N/A	N/A	N/A
<i>C. fraxinigena</i>	MFLUCC 14-0868	MF190133	MF190078	N/A	N/A	N/A	N/A
<i>C. gigaspora</i>	CFCC 50014	KR045630	KR045710	KU710999	KU710959	KU710922	KR045671
<i>C. gigaspora</i>	CFCC 89634	KF765671	KF765687	KU711000	KU710960	KU710923	KR045672
<i>C. italica</i>	MFLUCC 14-0440	KU900329	KU900301	N/A	N/A	N/A	N/A
<i>C. junipericola</i>	BBH 42444	MF190126	MF190071	N/A	N/A	MF377579	N/A
<i>C. junipericola</i>	MFLU 17-0882	MF190125	MF190072	N/A	N/A	MF377580	N/A
<i>C. kunzei</i>	CBS 118556	DQ243791	N/A	N/A	N/A	N/A	N/A
<i>C. kunzei</i>	CBS 118093	KY051855	KX965322	KX964754	N/A	KX965116	KX964941
<i>C. kunzei</i>	CBS 114651	KY051780	KX965243	KX964691	N/A	KX965055	KX964890
<i>C. leucostoma</i>	CFCC 50023	KR045635	KR045715	KU711003	KU710964	KU710926	KR045676
<i>C. leucostoma</i>	CFCC 53156	MN854447	MN854658	MN850762	MN850748	MN850755	MN861117
<i>C. leucostoma</i>	MFLUCC 16-0574	KY417731	KY417764	KY417696	KY417798	N/A	N/A

Table 2. Cont.

Species Name	Culture Accession No.	Genbank Accession No.					
		ITS	LSU	ACT	RPB2	TEF1- α	TUB2
<i>C. lumnitzericola</i>	MFLUCC 17-0508	MG975778	MH253453	MH253457	MH253461	N/A	N/A
<i>C. magnoliae</i>	IMI259790	JX438623	N/A	N/A	N/A	JX438565	N/A
<i>C. mali-spectabilis</i>	CFCC 53181	MK673066	MK673096	MK673036	MK673006	MK672953	MK672982
<i>C. multicolis</i>	CBS 105.89	DQ243803	N/A	N/A	N/A	N/A	N/A
<i>C. nitschkii</i>	CMW 10180	AY347356	N/A	N/A	N/A	N/A	N/A
<i>C. nitschkii</i>	CMW 10184	AY347355	N/A	N/A	N/A	N/A	N/A
<i>C. nitschkii</i>	CBS 130.95	KY051879	KX965344	N/A	N/A	N/A	N/A
<i>C. nitschkii</i>	CBS 116854	MH863003	MH874559	KX964728	KX965487	KX965090	N/A
<i>C. nivea</i>	MFLUCC 15-0860	KY417737	KY417771	KY417703	KY417805	N/A	N/A
<i>C. nivea</i>	CFCC 89641	KF765683	KF765699	KU711006	KU710967	KU710929	KR045679
<i>C. notastroma</i>	K3	JX438631	N/A	N/A	N/A	JX438539	N/A
<i>C. notastroma</i>	K20	JX438629	N/A	N/A	N/A	JX438541	N/A
<i>C. olivacea</i>	CFCC 53175	MK673062	MK673092	MK673032	MK673003	N/A	MK672978
<i>C. olivacea</i>	CFCC 53176	MK673068	MK673098	MK673038	MK673008	MK672955	MK672984
<i>C. palm</i>	CXY 1276	JN402990	N/A	N/A	N/A	KJ781296	N/A
<i>C. palm</i>	CXY 1280	JN411939	N/A	N/A	N/A	KJ781297	N/A
<i>C. parapersoonii</i>	T28.1	AF191181	N/A	N/A	N/A	JX438545	N/A
<i>C. paratranslucens</i>	MFLUCC 15-0506	KY417741	KY417775	KY417707	KY417809	N/A	N/A
<i>C. paratranslucens</i>	MFLUCC 16-0627	KY417742	KY417776	KY417708	KY417810	N/A	N/A
<i>C. pavettae</i>	CBS 145562	MK876386	MK876427	MK876457	MK876483	MK876497	MK876503
<i>C. phitsanulokensis</i>	MFLUCC 21-0046	MZ356517	MZ356521	MZ451160	MZ451168	MZ451164	MZ451172
<i>C. pingbianensis</i>	MFLUCC 18-1204	MK912135	MK571763	MN685817	MN685826	N/A	N/A
<i>C. pini</i>	CBS 197.42	AY347332	N/A	N/A	N/A	N/A	N/A
<i>C. pini</i>	CBS 224.52	AY347316	N/A	N/A	N/A	N/A	N/A
<i>C. pini</i>	CPC 28408	KY051994	KX965452	KX964872	KX965591	N/A	KX965041
<i>C. platycladi</i>	CFCC 50504	MH933645	MH933679	MH933552	MH933610	MH933516	MH933581
<i>C. platycladi</i>	CFCC 50506	MH933647	MH933681	MH933554	MH933612	MH933518	MH933583
<i>C. plurivora</i>	CBS 144239	MG971861	N/A	MG972010	N/A	MG971572	N/A
<i>C. predappioensis</i>	MFLU 17-0327	MH253451	MH253452	MH253449	MH253450	N/A	N/A
<i>C. predappioensis</i>	MFLUCC 17-2458	MG873484	MG873480	N/A	N/A	N/A	N/A
<i>C. pruinosa</i>	CBS 201.42	DQ243801	N/A	N/A	N/A	N/A	N/A
<i>C. pruinosa</i>	CBS 199.42	KY051911	KX965369	KX964795	N/A	KX965159	KX964968
<i>C. pubescentis</i>	MFLUCC 18-1201	MK912130	MK571758	MN685812	MN685821	N/A	N/A
<i>C. punicae</i>	CBS 144244	MG971943	N/A	MG972091	N/A	MG971654	N/A
<i>C. punicae</i>	7C-11	MG971942	N/A	N/A	N/A	MG971653	N/A
<i>C. quercicola</i>	MFLU 17-0881	MF190128	MF190074	N/A	N/A	N/A	N/A
<i>C. quercicola</i>	MFLUCC 14-0867	MF190129	MF190073	N/A	N/A	N/A	N/A
<i>C. rhizophorae</i>	MUCC302	EU301057	N/A	N/A	N/A	N/A	N/A
<i>C. rhizophorae</i>	M225	KR056292	N/A	N/A	N/A	N/A	N/A
<i>C. rhizophorae</i>	ATCC 38475	DQ996040	N/A	N/A	N/A	N/A	N/A
<i>C. rhizophorae</i>	CBS 116861	N/A	KX965296	KX964735	N/A	N/A	N/A
<i>C. rosae</i>	MFLU 17-0885	MF190131	MF190076	N/A	N/A	N/A	N/A
<i>C. rusanovii</i>	MFLUCC 15-0853	KY417743	KY417777	KY417709	KY417811	N/A	N/A
<i>C. rusanovii</i>	MFLUCC 15-0854	KY417744	KY417778	KY417710	KY417812	N/A	N/A

Table 2. Cont.

Species Name	Culture Accession No.	Genbank Accession No.					
		ITS	LSU	ACT	RPB2	TEF1- α	TUB2
<i>C. shoreae</i>	MFLUCC 21-0047	MZ356515	MZ356519	MZ451158	MZ451166	MZ451162	MZ451170
<i>C. shoreae</i>	MFLUCC 21-0048	MZ356516	MZ356520	MZ451159	MZ451167	MZ451163	MZ451171
<i>C. sorbi</i>	MFLUCC 16-0631	KY417752	KY417786	KY417718	KY417820	N/A	N/A
<i>C. sorbicola</i>	MFLUCC 16-0584	KY417755	KY417789	KY417721	KY417823	N/A	N/A
<i>C. sorbicola</i>	MFLUCC 16-0633	KY417758	KY417792	KY417724	KY417826	N/A	N/A
<i>C. tamaricicola</i>	CFCC 50507	MH933651	MH933686	MH933559	MH933616	MH933525	MH933587
<i>C. tamaricicola</i>	CFCC 50508	MH933652	MH933687	MH933560	MH933617	MH933523	MH933588
<i>C. thailandica</i>	MFLUCC 17-0262	MG975776	MH253455	MH253459	MH253463	N/A	N/A
<i>C. thailandica</i>	MFLUCC 17-0263	MG975777	MH253456	MH253460	MH253464	N/A	N/A
<i>C. translucens</i>	CXY 1351	KM034874	N/A	N/A	N/A	N/A	KM034895
<i>C. valsoidea</i>	CBS 117003	KY051832	KX965298	N/A	KX965494	KX965097	N/A
<i>C. variostromatica</i>	CMW 6766	AY347366	N/A	N/A	N/A	N/A	N/A
<i>C. variostromatica</i>	PPRI5297	AF260264	N/A	N/A	N/A	N/A	N/A
<i>C. variostromatica</i>	CBS 116858	KY051828	KX965293	KX964732	N/A	N/A	KX964921
<i>C. vinacea</i>	CBS 141585	KX256256	N/A	N/A	N/A	KX256277	KX256235
<i>C. viridistroma</i>	CBS 202.36	MN172408	MN172388	N/A	N/A	MN271853	N/A
<i>C. viridistroma</i>	CRY 1534	AF452120	N/A	N/A	N/A	N/A	N/A
<i>C. xinglongensis</i>	CFCC 52458	MK432622	MK429892	MK442946	MK578082	N/A	N/A
<i>C. xinglongensis</i>	CFCC 52459	MK432623	MK429893	MK442947	MK578083	N/A	N/A
<i>C. xylocarpi</i>	MFLUCC 17-0251	MG975775	MH253454	MH253458	MH253462	N/A	N/A
<i>C. xylocarpi</i>	111_03_01	MT507847	N/A	N/A	N/A	N/A	N/A
<i>Diaporthe eres</i>	CBS 138594	KJ210529	N/A	KJ420760	N/A	KJ210550	KJ420799
<i>D. vaccinii</i>	CBS 160.32	KC343228	N/A	JQ807297	N/A	KC343954	KC344196

Maximum likelihood (ML) analysis was performed by RAxML-HPC2 (v.8.2.12) on XSEDE implemented in the CIPRES Science Gateway web server (<http://www.phylo.org> accessed on 1 August 2021; [37] using 1000 rapid bootstrap replicates and the GTR + GAMMA + I substitution model. Maximum parsimony (MP) analysis was generated by PAUP (Phylogenetic Analysis Using Parsimony) v.4.0b10 [38] using the heuristic search option and 1000 random sequence additions. The branch-swapping was analysed using tree-bisection reconnection (TBR) algorithm. Maxtrees was set up at 1000 and all characters were unordered and of equal weight. The branches of zero length were collapsed and gaps were treated as missing data. All multiple and equally parsimonious trees were saved. The stability of the most parsimonious tree was evaluated by a bootstrap analysis with 1000 replicates, each with 100 replicates of random stepwise addition of taxa. The descriptive parsimonious tree, Tree Length [TL], Consistency Index [CI], Retention Index [RI], Relative Consistency Index [RC] and Homoplasy Index [HI] were calculated.

The evolutionary models for Bayesian inference (BI) analysis were determined for each locus using MrModeltest v.2.3 [39]. GTR + I + G was selected as the best-fitting model for LSU, RPB2, TEF- α and TUB2 datasets, SYM + I + G for the ITS dataset and HKY + I + G for the ACT dataset. BI analysis was conducted by Markov chain Monte Carlo sampling (BMCMC) to assess posterior probabilities (PP) [40,41] using MrBayes v3.1.2 [42]. Six simultaneous Markov chains were run for random trees for 10,000,000 generations, and trees were sampled every 1000th generation. The effective sampling sites (ESS) of initial trees were checked using the Tracer v. 1.6 [43]. The first 10% of generated trees were discarded, and the remaining trees were used to calculate posterior probabilities (PP).

in the majority rule consensus tree (the standard deviation of split frequency lower than 0.01). Bootstrap support values for ML and MP equal to or greater than 60% and Bayesian posterior probabilities (PP) equal to or greater than 0.95 were given above the nodes in the phylogenetic tree (Figure 1).

Figure 1. Cont.

Figure 1. Phylogenetic tree obtained from RAxML analyses of a combined ITS, LSU, ACT, RPB2, TEF1- α and TUB2 sequence dataset. Bootstrap support values for ML, MP equal to or greater than 60% and PP equal to greater than 0.95 are indicated at the nodes as ML/MP/PP. The ex-type strains are in bold and the new isolates of this study are in red. The tree is rooted to *Diaporthe eres* (CBS138594) and *D. vaccinii* (CBS16032).

Phylograms were visualized by FigTree v1.4.0 program [44] and reorganized by Microsoft PowerPoint 2013 (Microsoft Inc., Redmond, WA, USA) and Adobe Photoshop CS6 software (Adobe Systems, San Jose, CA, USA). The generated sequences of the new taxa were registered for the GenBank accession numbers. The final alignment and phylogram were submitted in TreeBASE (<http://purl.org/phylo/treebase/phyloids/study/TB2:S28804> accessed on 1 August 2021).

2.5. Genealogical Concordance Phylogenetic Species Recognition Analysis

New species and phylogenetically related species were analyzed using the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) model by conducting a pairwise homoplasy index (PHI) test as described by Bruen et al. [45] and Quaadvlieg et al. [46]. The PHI test was conducted in SplitsTree4 [47,48] to examine the recombination level within phylogenetically closely related species using a six-locus concatenated dataset (ITS, LSU, ACT, RPB2, TEF1- α and TUB2). The significant recombination in the dataset was indicated by PHI value below 0.05 ($\Phi_w < 0.05$). The results were visualized by generating a split graph, using both the LogDet transformation and split decomposition options.

2.6. Preliminary Screening of Antagonistic Activity against Fungal Pathogens

The fungal pathogens, *Colletotrichum artocarpicola* (MFLUCC 18-1167), *Co. fruticicola* (MFLUCC 18-1160), *Co. siamense* (MFLUCC 18-1162), *Co. viniferum* (MFLUCC 18-1179) and *Fusarium sambucinum* (MFLUCC 17-1056) were obtained from MFLUCC and used for the antagonistic activity test. Fungal isolates were screened using in vitro dual culture assays for their ability to suppress the mycelial growth of fungal pathogens. An antagonism test was performed with 10-day-old cultures of pathogens and new strains. Fresh cultures (pathogens and our strains) 10 days after incubation were used for the antagonism test. A fungal pathogen disc (5 mm) was placed 3 cm from the margin of the PDA plate (9 cm in diam.). An antagonist fungus disc (5 mm) was also placed in a similar manner but on the direct opposite of the pathogen disc. The plate was incubated at room temperature (28 °C) for 10 days. Plates inoculated with a fungal pathogen in the absence of an antagonistic fungus were used as negative controls. The assay was replicated three times. Observations were carried out for the 3rd, 5th, 7th and 10th days. Clear inhibition zone was recorded and the percentage inhibition in mycelial growth was calculated using the following formula [49]: $I\% = [(R1 - R2) / R1] \times 100$, where $I\%$ = the percentage inhibition, $R1$ = the radial growth of test pathogen in a control plate and $R2$ = the radial growth of test pathogen in the direction of antagonistic fungus. Data were statistically analyzed with ANOVA using SPSS version 22 (SPSS, Inc., Chicago, IL, USA). Tukey's HSD test was used to determine the significant differences between treatments at $p \leq 0.05$.

3. Results

3.1. Phylogenetic Analyses

The concatenated sequence dataset of ITS, LSU, ACT, RPB2, TEF1- α and TUB2 gene regions comprised 120 strains of *Cytospora* and two outgroup taxa, *Diaporthe eres* (CBS 138594) and *D. vaccinia* (CBS 160.32). The dataset contained 3339 characters according to the order of ITS: 1–416, LSU: 417–1199, ACT: 1200–1429, RPB2: 1430–2027, TEF- α : 2028–2624, β -TUB: 2625–3339. The tree topologies of combined sequence data obtained from ML, MP and BI analyses were not significantly different.

The RAxML analysis of the combined dataset yielded a best scoring tree with a final ML optimization likelihood value of -24983.102448 (Figure 1). The aligned sequence matrix comprises 1399 distinct alignment patterns, with 55.00% of undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.239305, C = 0.269679, G = 0.260287, T = 0.230728; substitution rates AC = 1.822160, AG = 4.370331, AT = 1.771054, CG = 1.304544, CT = 8.574513, GT = 1.000000; gamma distribution shape parameter α = 0.219288. The maximum parsimonious dataset consisted of 3354 characters, of which 2127 were constant, 1052 were parsimony-informative and 175 were parsimony-uninformative. The descriptive

statistics of the most parsimony tree was TL = 4294, CI = 0.468, RI = 0.780, RC = 0.365, HI = 0.532.

In the phylogenetic analyses (Figure 1), two new species, *Cytospora chiangmaiensis* (MFLUCC 21-0049) and *Cytospora shoreae* (MFLUCC 21-0047, MFLUCC 21-0048), clustered in a monophyletic lineage but well separated branch with strong bootstrap support (94% ML/99% MP/1.00 PP). Another new species, *Cytospora phitsanulokensis* (MFLUCC 21-0046), formed an independent branch adjacent to *C. acaciae*, *C. magnoliae* and *C. italica* with high bootstrap support (100% ML, 100% MP, 1.00 PP; Figure 1).

A pairwise homoplasy index (PHI) test revealed no significant recombination event between *Cytospora phitsanulokensis* and the closely related taxa, *C. acaciae*, *C. italica* and *C. magnoliae* (Figure 2). There was also no significant recombination among *Cytospora chiangmaiensis*, *C. shoreae*, *C. thailandica*, *C. diopuiensis* and *C. xinglongensis* (Figure 2). This evidence supports that they are different species. The significant recombination between two strains of *Cytospora shoreae* (MFLUCC 21-0047 and MFLUCC 21-0048) indicate that they are conspecific (Figure 3).

Figure 2. Results of the pairwise homoplasy index (PHI) test of *Cytospora phitsanulokensis* and closely related species using both LogDet transformation and splits decomposition. PHI test results (Φ_w) < 0.05 indicate significant recombination within the dataset. New species described in this study are indicated in red.

Figure 3. Results of the pairwise homoplasy index (PHI) test of *Cytospora chiangmaiensis* and *C. shoreae* and closely related species using both LogDet transformation and splits decomposition. PHI test results (Φ_w) < 0.05 indicate significant recombination within the dataset. New species described in this study are indicated in red.

3.2. Taxonomy

3.2.1. *Cytospora chiangmaiensis* Monkai and K.D. Hyde, sp. nov.

Index Fungorum number: IF558524; Facesoffungi number: FoF 09935; Figure 4.

Figure 4. *Cytospora chiangmaiensis* (MFLU 21-0048, holotype). (A) Conidiomata on host substrate. (B–D) Longitudinal sections through conidioma. (E) Conidiomata with ostiole and the arrangement of locules. (F,G) Peridium. (H) Conidiophore and conidiogenous cells with attached conidia. (I) Germinating conidia. (J) Conidia. (K,L) Colony on PDA plate (9 cm diam.) ((K) from above, (L) from reverse). Scale bars: (A–C) = 500 μ m, (D,E) = 100 μ m, (G) = 50 μ m, (F,H–J) = 10 μ m.

Etymology: Name reflects the locality, Chiang Mai Province, Thailand, where the holotype was collected.

Holotype: MFLU 21-0048

Saprobic on decaying leaves (vein and petioles) of *Shorea* sp. **Sexual morph:** Undetermined. **Asexual morph:** *Conidiomata* 650–800 μ m diam., pycnidial, solitary, semi-immersed to erumpent, circular to ovoid, multi-loculate. *Conceptacle* black. *Ectostromatic disc* 170–230 μ m diam., brown to dark brown, circular to ovoid, one ostiole per disc. *Ostioles* 55–65 μ m diam., conspicuous, circular, dark brown, at the same level as the disc surface. *Peridium* comprising few layers of cells, *textura globosa*, dark brown to black. *Locules*

numerous, regularly arranged with a column, independent wall. *Conidiophores* hyaline, unbranched or occasionally branched at the bases, formed from the innermost layer of pycnidial wall, embedded in a gelatinous layer. *Conidiogenous cells* $8.5\text{--}12.2 \times 1\text{--}1.7 \mu\text{m}$ ($\bar{x} = 10.4 \times 1.3 \mu\text{m}$, $n = 20$), enteroblastic, phialidic, sub-cylindrical to cylindrical, tapering towards apices. *Conidia* $5\text{--}7 \times 0.9\text{--}1.7 \mu\text{m}$ ($\bar{x} = 6 \times 1.2 \mu\text{m}$, $n = 30$), hyaline, unicellular, elongate-allantoid, guttulate, smooth, thin-walled.

Culture characteristics: Conidia germinating on PDA within 24 h germ tubes produced from one pole. Colonies on PDA reached at 5 cm diam. after 7 days at 28 °C, irregular in shape, surface slightly rough, effuse, slightly raised, with undulate margin, medium dense, white to cream, in reverse pale yellowish to white.

Material examined: THAILAND, Chiang Mai, Doi Lor district, Yang Kram, on decaying leaves of *Shorea* sp. (Dipterocarpaceae), 15 October 2019, J. Monkai, CH1-2 (MFLU 21-0048, holotype), ex-type living culture, MFLUCC 21-0049.

Notes: *Cytospora chiangmaiensis* formed an independent lineage sister to *Cytospora shoreae* with 94% ML/99% MP/1.00 PP bootstrap support (Figure 1). Based on phylogenetic analyses, *Cytospora chiangmaiensis* grouped in the same clade with *C. diopuiensis*, *C. lumnitzericola*, *C. pingbianensis*, *C. platycladi*, *C. shoreae*, *C. thailandica* and *C. xinglongensis* (Figure 1). *Cytospora diopuiensis* and *C. pingbianensis* are only known from their sexual morph [2], and our strain was found as an asexual morph. Thus, we are unable to compare the morphological characters of those species. However, *Cytospora lumnitzericola* can be distinguished from *C. chiangmaiensis* by its shorter conidia [5] (Table 4). *Cytospora platycladi* is similar to *C. chiangmaiensis* in having multi-loculate conidiomata with central ostiole but it has smaller conidiomata and shorter conidia [1] (Table 4). *Cytospora thailandica* differs from *C. chiangmaiensis* in having larger conidiomata with an ostiolar neck, shorter conidiogenous cell and shorter conidia [5] (Table 4). *Cytospora xinglongensis* differs from *C. chiangmaiensis* in having undivided locules, inconspicuous ostioles and shorter conidiogenous cells and longer conidia [16] (Table 4).

3.2.2. *Cytospora shoreae* Monkai and K.D. Hyde, sp. nov.

Index Fungorum number: IF558525; Facesoffungi number: FoF 09936; Figure 5.

Etymology: The specific epithet *shoreae* refers to host plant genus *Shorea*, on which the fungus was first collected.

Holotype: MFLU 21-0049

Saprobic on decaying leaves (vein and petioles) of *Shorea* sp. Sexual morph: Undetermined. Asexual morph: *Conidiomata* 400–1000 μm diam., pycnidial, semi-immersed in host tissue, solitary, erumpent, flask shaped to ovoid, multi-loculate. *Conceptacle* black. *Ectostromatic disc* 120–580 μm diam., brown to dark brown, circular to ovoid, one ostiole per disc. *Ostioles* 19–52 μm diam., conspicuous, circular, dark brown, at the same level as the disc surface. *Peridium* comprising few layers of cells, *textura globosa*, dark brown to black. *Locules* numerous, regularly arranged with a column, independent wall. *Conidiophores* hyaline, unbranched or occasionally branched at the bases, formed from the innermost layer of pycnidial wall, embedded in a gelatinous layer. *Conidiogenous cells* $6.7\text{--}11.8 \times 1\text{--}1.9 \mu\text{m}$ ($\bar{x} = 10 \times 1.4 \mu\text{m}$, $n = 10$), enteroblastic, phialidic, subcylindrical to cylindrical, tapering towards apices. *Conidia* $5\text{--}7 \times 1\text{--}1.6 \mu\text{m}$ ($\bar{x} = 6 \times 1.3 \mu\text{m}$, $n = 30$), hyaline, unicellular, elongate-allantoid, guttulate, smooth, thin-walled.

Culture characteristics: Conidia germinating on PDA within 24 h germ tubes produced from both poles. Colonies on PDA reached at 6 cm diam. after 7 days at 28 °C, irregular in shape, surface slightly rough, effuse, slightly raised, with undulate margin, medium dense, pale brown to white, in reverse pale yellowish to white.

Figure 5. *Cytospora shoreae* (MFLU 21-0049, holotype (C–E,G,H,J,L,N,O) and MFLU 21-0050, paratype (A,B,F,I,K,M,P,G)). (A,D) Conidiomata on host substrate. (B,C,E,F) Longitudinal sections through conidioma. (G) Peridium. (H,I) Conidiophores and conidiogenous cells with attached conidia. (J,K) Conidia. (L,M) Germinating conidia. (N–Q) Colonies on PDA plate (9 cm diam.) ((N,P) from above, (O,Q) from reverse). Scale bars: (A–E) = 500 µm, (F) = 50 µm, (G–M) = 10 µm.

Material examined: THAILAND, Chiang Mai, Omkoi district, Yang Piang, on decaying leaves of *Shorea* sp. (Dipterocarpaceae), 16 October 2019, J. Monkai, CH2-2 (MFLU 21-0049, holotype), ex-type living culture, MFLUCC 21-0047; THAILAND, Chiang Mai, Omkoi district, Yang Piang, on unidentified decaying leaves, 16 October 2019, J. Monkai, CH2-3 (MFLU 21-0050, paratype), ex-paratype living culture MFLUCC 21-0048.

Notes: *Cytospora shoreae* formed a sister clade to *C. chiangmaiensis* with 94% ML, 99% MP and 1.00 PP statistical support (Figure 1). *Cytospora chiangmaiensis* and *C. shoreae* share similar morphology in the size and characteristics of conidiogenous cells and conidia. However, *C. chiangmaiensis* has circular shaped conidiomata. *Cytospora shoreae* has larger, flask-shaped conidiomata. The single gene comparison of ACT, RPB2, TEF1- α and TUB2 showed that there are significant nucleotide differences (more than 1.5%) between *Cytospora chiangmaiensis* and two strains of *C. shoreae* (MFLUCC 21-0047 and MFLUCC 21-0048; Table 3) and this provides evidence that they are different species [50]. *Cytospora shoreae* differs from *C. lumnitzericola* and *C. platycladi* by its longer conidia [1,5] (Table 4). *Cytospora shoreae* is distinguished from *C. thailandica* by having longer conidiogenous cell and conidia [5] (Table 4). *Cytospora shoreae* is distinct from *C. xinglongensis* in having longer conidiogenous cells and shorter conidia [16] (Table 4). *Cytospora shoreae*, strains MFLUCC 21-0047 and MFLUCC 21-0048 showed similar characteristics of conidiomata, conidiogenous cells, conidia and culture characteristics, with no significant differences. They were also collected from the same site with the notes of different hosts, where the holotype was isolated from *Shorea* sp. and the paratype was isolated from unidentified host. The single gene comparison of ITS, LSU, ACT, RPB2, TEF1- α and TUB2 showed that there is no significant difference between two strains of *Cytospora shoreae* (MFLUCC 21-0047 and MFLUCC 21-0048; Table 3), and this confirms that these two strains are the same species [50].

Table 3. Nucleotide differences of *Cytospora* species discussed in this study.

New Taxa/Strains	New Taxa/Strains Compared with	Number of Different Nucleotides/Number of All Nucleotides (% Base Pairs Difference)					
		ITS	LSU	ACT	RPB2	TEF1- α	TUB2
<i>Cytospora shoreae</i> (MFLUCC 21-0047)	<i>C. shoreae</i> (MFLUCC 21-0048)	0/494 (0%)	0/510 (0%)	0/180 (0%)	4/726 (0.6%)	0/291 (0%)	3/375 (0.8%)
<i>C. chiangmaiensis</i> (MFLUCC 21-0049)	<i>C. shoreae</i> (MFLUCC 21-0047)	0/494 (0%)	0/510 (0%)	5/180 (2.8%)	13/726 (1.8%)	16/291 (5.5%)	13/375 (3.5%)
<i>C. chiangmaiensis</i> (MFLUCC 21-0049)	<i>C. shoreae</i> (MFLUCC 21-0048)	0/494 (0%)	0/510 (0%)	5/180 (2.8%)	11/726 (1.5%)	16/291 (5.5%)	12/375 (3.2%)

Table 4. Synopsis of asexual morph of *Cytospora* species discussed in this study.

Species	Conidiomata (μ m)	Conidiogenous Cell (μ m)	Conidia (μ m)	References
<i>Cytospora chiangmaiensis</i>	650–800	8.5–12.2 $\times$ 1–1.7	5–7 $\times$ 0.9–1.7	This study
<i>C. italica</i>	580–730	10–32 $\times$ 0.7–2.4	3.7–5.2 $\times$ 1–1.3	[51]
<i>C. lumnitzericola</i>	Undetermined	8–14 $\times$ 0.6–1.6	3.7–4.5 $\times$ 1–1.5	[5]
<i>C. platycladi</i>	210–330	5–12 $\times$ 1–1.5	4–5.5 $\times$ 1–1.5	[1]
<i>C. shoreae</i>	400–1000	6.7–11.8 $\times$ 1–1.9	5–7 $\times$ 1–1.6	This study
<i>C. thailandica</i>	400–1200	3.3–9.1 $\times$ 1–1.7	3.3–4 $\times$ 1–1.5	[5]
<i>C. xinglongensis</i>	Undetermined	4.5–12 $\times$ 1–1.5	7.5–10.5 $\times$ 1–1.5	[16]

3.2.3. *Cytospora phitsanulokensis* Monkai and K.D. Hyde, sp. nov.

Index Fungorum number: IF558526; Facesoffungi number: FoF 09937; Figure 6.

Figure 6. *Cytospora phitsanulokensis* (MFLU 21-0051, holotype). (A) Conidiomata on host substrate. (B,C) Longitudinal sections through conidioma. (D,E) Longitudinal sections of locules. (F,G) Conidiophores and conidiogenous cells with attached conidia. (H) Germinating conidia. (I) Conidia. (J,K) Colonies on PDA plate (9 cm diam.) ((J) from above, (K) from reverse). Scale bars: (A–C) = 500 µm, (D) = 30 µm, (E) = 50 µm, (F–I) = 10 µm.

Etymology: Name reflects the locality, Phitsanulok Province, Thailand, where the holotype was collected.

Holotype: MFLU 21-0051

Saprobic on unidentified decaying leaves (vein and petioles). Sexual morph: Undetermined. Asexual morph: *Conidiomata* 900–1100 µm diam., pycnidial, semi-immersed in host tissue, solitary, erumpent, nearly flat, multi-loculate. *Conceptacle* black. *Ectostromatic disc*

500–640 µm diam., brown to dark brown, circular to ovoid, one ostiole per disc. *Ostioles* inconspicuous, black. *Peridium* comprising few layers of cells of *textura angularis*, brown to dark brown. *Locules* numerous, irregular, subdivided frequently by invaginations with independent walls. *Conidiophores* hyaline, unbranched or occasionally branched at the bases, formed from the innermost layer of pycnidial wall, embedded in a gelatinous layer. *Conidiogenous cells* 6.7–14.1 × 1.1–2.5 µm ($\bar{x}$ = 9.8 × 1.9 µm, n = 30), enteroblastic, phialidic, sub-cylindrical to cylindrical. *Conidia* 5.2–6.9 × 1.2–1.7 µm ($\bar{x}$ = 6 × 1.5 µm, n = 30), hyaline, unicellular, allantoid, guttulate, smooth, thin-walled.

Culture characteristics: Conidia germinating on PDA within 24 h germ tubes produced from both poles. Colonies on PDA reached at 8 cm diam. after 7 days at 28 °C, circular in shape, effuse, slightly raised, with entire margin, medium dense, floccose, white, in reverse pale yellowish to white.

Material examined: THAILAND, Phitsanulok, Wang Thong, on unidentified decaying leaves, 25 July 2019, E. Yasanthika, E5-2 (MFLU 21-0051, holotype), ex-type living culture, MFLUCC 21-0046.

Notes: Phylogenetically, *Cytospora phitsanulokensis* forms a distinct lineage and is closely related to *C. acaciae*, *C. magnoliae* and *C. italica* with 100% ML, 100% MP and 1.00 PP statistical support (Figure 1). *Cytospora phitsanulokensis* differs from *C. italica* by its larger conidiomata, shorter conidiogenous cell and longer conidia [51] (Table 4). Complete descriptions of *C. acaciae* and *C. magnoliae* were not available for morphological comparison [11,52]. Adam et al. [11] reported that *Cytospora acaciae* produces phialides with a long narrow channel of apical pores with lipid globules at one end of conidium; however, these characteristics were not observed in *C. phitsanulokensis*.

3.3. Preliminary Screening of Antagonistic Activity against Fungal Pathogens

Cytospora phitsanulokensis (MFLUCC 21-0046) exhibited the highest inhibition against *Co. viniferum* and *F. sambucinum* at 75.1 ± 4.0% and 67.5 ± 1.3%, respectively (Table 5, Figure 7). *Cytospora shoreae* (MFLUCC 21-0047 and MFLUCC 21-0048) showed a high inhibition effect against *Co. viniferum* at 64.0 ± 1.5% and 61.8 ± 1.4%, respectively (Table 4, Figure 7). However, *Cytospora Chiangmaiensis* (MFLUCC 21-0049) had moderate inhibition activity against five tested plant pathogenic fungi (Table 5).

Table 5. In vitro antagonistic activity of *Cytospora* spp. against five plant pathogenic fungi after 10 days incubation at 28 °C.

Fungal Name	% Growth Inhibition			
	<i>Cytospora Chiangmaiensis</i> (MFLUCC 21-0049)	<i>C. shoreae</i> (MFLUCC 21-0047)	<i>C. shoreaeb</i> (MFLUCC 21-0048)	<i>C. phitsanulokensis</i> (MFLUCC 21-0046)
<i>Colletotrichum fructicola</i> (MFLUCC 18-1160)	44.2 ± 2.5 ^a	49.6 ± 0.0 ^a	50.5 ± 5.7 ^a	37.4 ± 1.5 ^a
<i>Co. siamense</i> (MFLUCC 18-1162)	18.1 ± 4.4 ^a	31.9 ± 0.4 ^b	53.5 ± 1.8 ^c	39.5 ± 2.2 ^b
<i>Co. artocarpicola</i> (MFLUCC 18-1167)	11.7 ± 3.9 ^a	14.9 ± 5.5 ^a	32.3 ± 3.6 ^b	58.0 ± 1.0 ^c
<i>Co. viniferum</i> (MFLUCC 18-1179)	30.0 ± 3.9 ^a	64.0 ± 1.5 ^b	61.8 ± 1.4 ^b	75.1 ± 4.0 ^b
<i>Fusarium sambucinum</i> (MFLUCC 17-1056)	17.4 ± 3.2 ^a	39.1 ± 4.2 ^b	33.1 ± 4.4 ^b	67.5 ± 1.3^c

The values represent I% ± S.E. Different letters indicate significant differences between tested pathogens ($p \leq 0.05$) according to Tukey's HSD test. The values greater than 60% with clear inhibition zone are bold.

Figure 7. The antagonistic activity of *Cytospora* species (left colonies) against fungal pathogens (right colonies). (A) *Cytospora shoreae* (MFLUCC 21-0047) vs. *Colletotrichum viniferum* (MFLUCC 18-1179). (B) *C. shoreae* (MFLUCC 21-0048) vs. *Co. viniferum* (MFLUCC 18-1179). (C) *C. phitsanulokensis* (MFLUCC 21-0046) vs. *Co. viniferum* (MFLUCC 18-1179). (D) *C. phitsanulokensis* (MFLUCC 21-0046) vs. *Fusarium sambucinum* (MFLUCC 17-1056).

4. Discussion

This study provides taxonomic novelties of *Cytospora* species discovered from Thailand and their antagonistic activities against fungal pathogens. We show that six-locus phylogeny (ITS, LSU, ACT, RPB2, TEF1- α and TUB2) facilitates species delineation in *Cytospora* which is consistent with previous studies [1,3,17,18]. In addition, *Cytospora chiangmaiensis* and *C. shoreae* are phylogenetically closely related to *C. diopuiensis*, which was also collected from Chiang Mai, Thailand [2], indicating the close geographical relationship of these taxa. Based on the Fungus-Host USDA database [53], two new species, *Cytospora chiangmaiensis* and *C. shoreae*, are recorded for the first time on *Shorea* sp. (Dipterocarpaceae). *Cytospora phitsanulokensis* collected from Phitsanulok, Thailand, is phylogenetically closely related to *C. acaciae*, *C. italica* and *C. magnoliae*. The GCPSR analyses provided evidence for *Cytospora phitsanulokensis* as a separate species. However, the presence of recombination among strains of *C. acaciae*, *C. italica* and *C. magnoliae* shown in the spilt graph (Figure 2) may be caused by the lack of some gene regions in the dataset. Thus, more strains and new sequence data of *C. italica* and *C. magnoliae* should be obtained to better confirm their taxonomic placements in the *C. acaciae* clade.

Cytospora species are discovered in woody substrates such as bark, branches and twigs [1]. It should be noted that the stromata of *Cytospora* were only detected on vein and petioles, but not on leaf lamina for all our samples. Adams et al. [11] mentioned that the formation of stroma of *Cytospora* could possibly decrease in leaves compared to bark. *Cytospora* species are known as saprobes on dead plants and are one of the important plant pathogens causing dieback and canker diseases on a wide range of hosts [1–3,5,9,16–18,23]. Considering that the species number of *Cytospora* is ever-increasing and related taxonomic knowledge continues to expand [1], more extensive sampling of both fresh and dead plant samples in selected hosts and locations should be implemented to improve and stabilize

the identification and classification of *Cytospora* species. In addition, *Cytospora* are interesting fungal taxa for screening potential biological activities [24,25,27]. Previous studies reported novel bioactive compounds produced by *Cytospora* spp. [24,25,27,54]; however, species identification of these potential strains was not established. The taxonomic classification and phylogenetic relationships can be used as important tools for the screening of biologically active strains and their biological activities for further applications [55,56].

In this study, we identified new *Cytospora* species and demonstrated that different strains and/or species can inhibit fungal pathogens. However, our study only conducted a preliminary screening of *Cytospora* that can inhibit fungal pathogens, using the dual culture method. Although our new species were identified as saprobes based on their occurrence on decaying leaf substrates, their pathogenicity should be clarified, as this genus is well known for pathogens. Therefore, to confirm whether our *Cytospora* strains are true saprobes, further research is needed. Further studies will be carried out in the near future to check the pathogenicity of the new *Cytospora* species, elucidate the biology of these fungi and screen secondary metabolites.

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