A new record of *Lasiodiplodia pseudotheobromae* causing leaf spot of *Cynometra malaccensis* in Thailand

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Abstract

Lasiodiplodia species are well-known plant pathogens, causing fruit rot, stem-end rot and die-back on a wide range of hosts. They are characterized by hyaline or pigmented, aseptate or septate, thick-walled conidia, usually with longitudinal striations. In this study, *Lasiodiplodia pseudotheobromae* was found to cause leaf spots of *Cynometra malaccensis* in Thailand. Based on morphology and phylogenetic analyses of internal transcribed spacer regions (ITS) and translation elongation factor 1- α gene (TEF1- α), we identify the taxon as *L. pseudotheobromae*; it is the first confirmed record on *Cynometra malaccensis* in the world.

Key words – Botryosphaeriaceae – Fabaceae – Morphology – Multigene – Phylogeny – Taxonomy

Introduction

Lasiodiplodia Ellis & Everh., typified by *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Phillips et al. 2013, Jayasiri et al. 2019), is a genus in Botryosphaeriaceae (Alves et al. 2008, Phillips et al. 2019, Wijayawardene et al. 2020). *Lasiodiplodia* species have been recorded from many hosts, manifesting as pathogens (Abdollahzadeh et al. 2010), endophytes (Slippers & Wingfield 2007, Chen et al. 2015b) and saprobes (Abdollahzadeh et al. 2010, Liu et al. 2012, Dissanayake et al. 2016, Hyde et al. 2019). *Cynometra* (Fabaceae) contains approximately 85 species distributed in tropical regions (Mabberley 2008). Similar to other *Cynometra* species, *C. malaccensis* forms part of a large, pantropical group of woody plants ranging from 5-50 m in height (Radosavljevic et al. 2017).

Lasiodiplodia pseudotheobromae was first described from *Gmelina arborea* in Costa Rica (Alves et al. 2008). It was later isolated from different parts of many host plants including dead leaves of *Plukenetia volubilis* (Tennakoon et al. 2016), necrotic shoots and branches of *Mangifera indica* (Kwon et al. 2017), necrotic calyx of persimmon fruit (Nogueira et al. 2017), dead leaves of *Pandanus* sp. (Tibpromma et al. 2018), fruit rot of *Dimocarpus longan* (Pipattanapuckdee et al. 2019) and stems of *Ormosia pinnata* (Li et al. 2020). In this study, we provide a new record of *L. pseudotheobromae* causing leaf spots of *Cynometra malaccensis* from northern Thailand, based on identification using two-locus phylogeny and morphology.

Materials & Methods

Sample collection, fungal isolation and microscopic characterization

Leaves with brown spots were collected in plastic bags from a living *Cynometra malaccensis* tree at Mae Fah Luang Botanical Garden, Chiang Rai, Thailand on 9 October 2018. Each spot from the infected leaf was cut into four sections (5 mm²) consisting of both symptomatic and healthy tissue. All sections were surface sterilized in 70% ethanol for one minute, 5% sodium hypochlorite for one minute, rinsed thrice with sterilized water, dried on sterilized tissue paper, placed on potato dextrose agar (PDA) plates, and incubated at 25 ± 1°C for three days in the dark. Hyphal tips from the margin of developing colonies were transferred to fresh PDA plates and pure isolates were incubated for seven to 10 days at 25°C. To induce the formation of pycnidia, four to six autoclaved wooden toothpicks were placed on top of the culture medium, and the plates were kept at 25°C for two weeks. Subsequently, morphological characters such as conidia (size, shape, and colour) and mycelium were examined and digital images were captured using an Axio Cam ERc5s attached to a stereomicroscope (SteREO Discovery v8) and a Canon EOS 600D camera (Canon, Tokyo, Japan) attached to a Nikon ECLIPSE Ni-U compound microscope (Nikon, Tokyo, Japan). The features observed were measured by using Tarosoft® Image Frame Work software (version 0.97). The photomicrograph plate was prepared using Adobe Photoshop CS6 version (Adobe Systems). Ex-type living cultures are deposited in Mae Fah Luang University Culture Collection (MFLUCC) and the fungarium specimen is deposited in the Mae Fah Luang University Herbarium (MFLU), Thailand. Faces of Fungi (FoF) number was obtained following Jayasiri et al. (2015).

DNA extraction, PCR amplification and sequencing

Fresh mycelium was scraped from the margin of the colony grown on PDA plates (incubated at 25°C for 4 weeks) and transferred into 1.5 ml microcentrifuge tubes for genomic DNA extraction. Genomic DNA was extracted with an amended cetyltrimethyl ammonium bromide (CTAB) method (Guo et al. 2000). DNA amplification was performed by polymerase chain reaction (PCR) using the following primers: ITS4 and ITS5 (White et al. 1990) to amplify the partial rRNA of the internal transcribed spacer regions (ITS); and EF1-728F and EF2 (O'Donnell et al. 1998) to amplify the protein coding region of the translation elongation factor 1- α gene (TEF1- α). PCR was carried out using BIORAD C1000 Touch™ Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The PCR mixtures contained 16.2 μ l of ddH₂O, 1 μ l of each primer, 3 μ l of dNTPs (TaKaRa, China), 2.5 μ l of 10x Ex-Taq buffer (TaKaRa, China), 1 μ l of genomic DNA, and 0.3 μ l of TaKaRa Ex-Taq DNA polymerase (TaKaRa, China). The thermal cycling process was completed by an initial denaturation for 3 min at 95°C, followed by 34 cycles of denaturation for 30 s at 95°C, 30 s of annealing, elongation for 1 min at 72°C, and a final extension for 10 min at 72°C. The annealing temperatures for ITS and TEF1- α were 58°C and 52°C, respectively. Ethidium bromide (EtBr) was used for staining PCR products on 1% agarose electrophoresis gel under UV light. PCR products were sequenced with the same primers mentioned above at Beijing Biomed Gene Technology Co., China.

Phylogenetic analyses

Sequences obtained were subjected to BLAST search in the GenBank database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine the most probable closely related taxa. Based on the latest publications, ITS and TEF1- α sequences from type strains were retrieved from GenBank (Table 1). Single gene sequence datasets were aligned using the MAFFT website (v.7.036) (Kato et al. 2019) and manually adjusted in BioEdit (v.7.0) (Hall 2004) wherever necessary. BioEdit (v.7.0) was used to combine ITS and TEF1- α sequences. The combined alignment in FASTA format was converted to PHYLIP and NEXUS formats using the Alignment Transformation Environment (ALTER) website (Posada 2010). Phylogenetic analyses were performed for the combined ITS and TEF1- α sequence data. Maximum likelihood (ML) analysis was performed in the CIPRES Science Gateway (v.3.3) (Miller et al. 2010). Randomized Accelerated Maximum Likelihood (RAxML) rapid

bootstrapping and subsequent ML search were performed using distinct model/data partitions with joint branch length optimization. The number of replicates was inferred using the stopping criterion (Pattengale et al. 2010). Phylogenetic Analysis Using Parsimony (PAUP) (v.4.0b10) (Swofford 2001) was used to conduct the maximum parsimony (MP) analysis using the heuristic search option with 1,000 random taxa additions to building the phylogenetic tree. Bayesian inference (BI) analysis was conducted with MrBayes (v.3.1.2) (Huelsenbeck et al. 2001). Simultaneous Markov chains were run for 2,000,000 generations and trees were sampled every 200th generation. The first 2,000 trees, representing the burn-in phase of the analyses, were discarded, while the remaining trees were used for calculating posterior probabilities in the majority rule consensus tree. Adobe Illustrator CS3 (Adobe Systems, USA) software was used to present the tree. The new sequences generated from this study were submitted to GenBank (Table 1).

Pathogenicity test

Pathogenicity was confirmed by comparing morphology and colony characteristics with the original isolate. Since our isolate did not sporulate in culture, the experiment was carried out using the mycelium plug method on both attached and detached leaves of *Cynometra malaccensis*. Two-month old plants of *C. malaccensis* were used to conduct the pathogenicity test. For the attached method, each leaf selected was surface sterilized by spraying with 70% ethanol, followed by 5% sodium hypochlorite and sterilized water. For the detached method, each healthy leaf selected was surface sterilized in 70% ethanol for one minute, 5% sodium hypochlorite for one minute, rinsed thrice with sterilized water, and dried on sterilized tissue paper. Mycelium plugs of the growing culture were cut using sterilized straws. Leaves were wounded with syringe needles. The mycelium plugs were then placed on five wounded and five non-wounded attached leaves, and three wounded and three non-wounded detached leaves. The attached leaves with the inoculum were covered with a thin layer of Parafilm to prevent the plugs from drying. Sterile PDA plugs were used as controls.

The trays containing the plants and the detached leaves were covered with plastic for 48 h to maintain the humidity near saturation, at $25\pm1^{\circ}\text{C}$, and kept under fluorescent light for 12 h per day. The leaves were observed daily for any symptoms and lesion lengths were measured after two and five days. Fungal isolation was carried out on the fifth day. Tissue culture method was used for fungal isolation on the attached leaves, since no conidiomata were formed, while single spore method was used for the detached leaves.

Table 1 GenBank accession numbers, isolate numbers, host information and origin of the taxa used in the phylogenetic analyses. Sequences generated in this study are in bold. Ex-type strains are denoted with *.

Taxa	Isolate No.	Host	Country	GenBank accession numbers		References
				ITS	TEF1- α	
<i>Diplodia mutila</i>	CMW 7060	<i>Fraxinus excelsior</i>	Netherlands	AY236955	AY236904	Slippers et al. (2004)
<i>D. seriata</i>	CBS 112555*	<i>Vitis vinifera</i>	Montemor-o-Novo, Portugal	AY259094	AY573220	Alves et al. (2004)
<i>Lasiodiplodia americana</i>	CERC 1961*	Twigs of <i>Pistacia vera</i>	Arizona, USA	KP217059	KP217067	Chen et al. (2015a)
<i>L. aquilariae</i>	CGMCC 3.18471*	<i>Aquilaria crassna</i>	Vientiane, Laos	KY783442	KY848600	Wang et al. (2019)
<i>L. avicenniae</i>	CBS 139670*	Asymptomatic branches of <i>Avicennia marina</i>	Kwazulu-natal, South Africa	KP860835	KP860680	Osorio et al. (2017)

Table 1 Continued.

Taxa	Isolate No.	Host	Country	GenBank accession numbers		References
				ITS	TEF1- α	
<i>L. avicenniarum</i>	MFLUCC 17-2591*	Decaying fruit pericarp of <i>Avicennia marina</i>	Krabi, Thailand	MK347777	MK340867	Jayasiri et al. (2019)
<i>L. brasiliense</i>	CMM 4015*	<i>Mangifera indica</i>	Brazil	JX464063	JX464049	Marques et al. (2013)
<i>L. bruguiera</i>	CBS 139669*	Asymptomatic branches of <i>Bruguiera gymnorrhiza</i>	Kwazulu-natal, South Africa	KP860832	KP860677	Osorio et al. (2017)
<i>L. caatinguensis</i>	CMM 1325*	Trunk canker of <i>Citrus sinensis</i>	Itarema, Brazil	KT154760	KT008006	Coutinho et al. (2017)
<i>L. chinensis</i>	CGMCC 3.18061*	Stem blight or dieback of blueberries	Hainan, China	KX499889	KX499927	Dou et al. (2017a)
<i>L. chonburiensis</i>	MFLUCC 16-0376*	Dead leaf of <i>Pandanus</i> sp.	Chonburi, Thailand	MH275066	MH412773	Tibpromma et al. (2018)
<i>L. cinnamomi</i>	CFCC 51997*	Twigs and branches of <i>Cinnamomum camphora</i>	Jiangsu, China	MG866028	MH236799	Jiang et al. (2018)
<i>L. citricola</i>	CBS 124707*	Twigs of <i>Citrus</i> sp.	Gilan, Iran	GU945354	GU945340	Abdollahzadeh et al. (2010)
<i>L. crassispora</i>	CBS 118741*	Canker of <i>Santalum album</i>	Western Australia	DQ103550	EU673303	Burgess et al. (2006)
<i>L. curvata</i>	CGMCC 3.18456	<i>Aquilaria crassna</i>	Vientiane, Laos	KY783437	KY848596	Wang et al. (2019)
<i>L. egyptiaca</i>	BOT10*	Leaf of <i>Mangifera indica</i>	Sharkia, Egypt	JN814397	JN814424	Ismail et al. (2012)
<i>L. endophytica</i>	MFLUCC 18-1121	Fresh leaves of <i>Magnolia candolii</i>	Yunnan, China	MK501838	MK584572	de Silva et al. (2019)
<i>L. euphorbiicola</i>	CMM 3609*	Collar and root rot of <i>Jatropha curcas</i>	Colatina, Brazil	KF234543	KF226689	Machado et al. (2014)
<i>L. exigua</i>	CBS 137785*	Branch canker of <i>Retama raetam</i>	Nabeul, Tunisia	KJ638317	KJ638336	Linaldeddu et al. (2015)
<i>L. gilanensis</i>	CBS 124704*	Twigs of unknown woody plant	Gilan, Iran	GU945351	GU945342	Abdollahzadeh et al. (2010)
<i>L. gonubiensis</i>	CBS 115812*	<i>Syzygium cordatum</i>	Eastern Cape, South Africa	AY639595	DQ103566	Pavlic et al. (2004)
<i>L. gravistriata</i>	CMM 4564*	Stems of <i>Anacardium humile</i>	Minas Gerais, Brazil	KT250949	KT250950	Netto et al. (2017)
<i>L. hormozganensis</i>	CBS 124709*	Twigs of <i>Olea</i> sp.	Hormozgan, Iran	GU945355	GU945343	Abdollahzadeh et al. (2010)
<i>L. hyalina</i>	CGMCC 3.17975*	<i>Acacia confusa</i>	Hainan, China	KX499879	KX499917	Dou et al. (2017b)
<i>L. indica</i>	PAN 30202*	Fallen twig of angiospermous tree	Chandigarh, India	NR_155317	-	Prasher et al. (2014)
<i>L. iraniensis</i>	CBS 124710*	Twigs of <i>Salvadora persica</i>	Hormozgan, Iran	GU945346	GU945334	Abdollahzadeh et al. (2010)

Table 1 Continued.

Taxa	Isolate No.	Host	Country	GenBank accession numbers		References
				ITS	TEF1- α	
<i>L. irregularis</i>	CGMCC 3.18468*	<i>Aquilaria crassna</i>	Vientiane, Laos	KY783472	KY848610	Wang et al. (2019)
<i>L. jatrophiicola</i>	CMM 3610*	Collar and root rot of <i>Jatropha curcas</i>	Colatina, Brazil	KF234544	KF226690	Machado et al. (2014)
<i>L. krabiensis</i>	MFLU 17-2617*	Decaying submerged wood of <i>Bruguiera</i> sp.	Krabi, Thailand	MN047093	MN077070	Dayarathne et al. (2020)
<i>L. laeliocattleyae</i>	CBS 167.28*	Necrotic branch of <i>Mangifera indica</i>	Piura, Peru	KU507487	KU507454	Rodríguez-Gálvez et al. (2017)
<i>L. laosensis</i>	CGMCC 3.18464*	<i>Aquilaria crassna</i>	Vientiane, Laos	KY783471	KY848609	Wang et al. (2019)
<i>L. lignicola</i>	CBS 134112*	Dead wood of unknown plant	Chiang Rai, Thailand	JX646797	KU887003	Liu et al. (2012)
<i>L. macroconidia</i>	CGMCC 3.18479*	<i>Aquilaria crassna</i>	Vientiane, Laos	KY783438	KY848597	Wang et al. (2019)
<i>L. macrospora</i>	CMM 3833*	Collar and root rot of <i>Jatropha curcas</i>	Colatina, Brazil	KF234557	KF226718	Machado et al. (2014)
<i>L. magnolia</i>	MFLUCC 18-0948*	Dead leaves attached to <i>Magnolia candolii</i>	Yunnan, China	MK499387	MK568537	de Silva et al. (2019)
<i>L. mahajangana</i>	CBS 124925*	Healthy branches of <i>Terminalia catappa</i>	Mahajanga, Madagascar	FJ900595	FJ900641	Begoude et al. (2010)
<i>L. margaritacea</i>	CBS 122519*	<i>Adansonia gibbosa</i>	Western Australia	EU144050	EU144065	Pavlic et al. (2008)
<i>L. marypalmiae</i>	CMM 2275*	<i>Carica papaya</i> fruit	Pernambuc, Brazil	NR_147341	-	Netto et al. (2014)
<i>L. mediterranea</i>	CBS 137783*	Branch canker of <i>Quercus ilex</i>	Bortigiadas, Italy	KJ638312	KJ638331	Linaldeddu et al. (2015)
<i>L. microcondia</i>	CGMCC 3.18485	<i>Aquilaria crassna</i>	Vientiane, Laos	KY783441	KY848614	Wang et al. (2019)
<i>L. missouriana</i>	CBS 128311*	Interspecific hybrid grape	Saint James, USA	HQ288225	HQ288267	Urbez-Torres et al. (2012)
<i>L. pandanicola</i>	MFLUCC 16-0265*	Dead leaves of <i>Pandanus</i> sp.	Phatthalung, Thailand	MH275068	MH412774	Tibpromma et al. (2018)
<i>L. parva</i>	CBS 456.78*	Cassava field soil	Colombia	EF622083	EF622063	Alves et al. (2008)
<i>L. plurivora</i>	CBS 120832*	V-shaped necrotic lesion of <i>Prunus salicina</i>	Western Cape, South Africa	EF445362	EF445395	Damm et al. (2007)
<i>L. pontae</i>	CMM 1277*	Necrotic canker on <i>Spondias purpurea</i>	Brazil	KT151794	KT151791	Coutinho et al. (2017)
<i>L. pseudotheobromae</i>	CBS 116459*	<i>Gmelina arborea</i>	San Carlos, Costa Rica	EF622077	EF622057	Alves et al. (2008)
<i>L. pseudotheobromae</i>	CGMCC 3.18047	<i>Pteridium aquilinum</i>	China	KX499876	KX499914	Dou et al. (2017a)
<i>L. pseudotheobromae</i>	Gr26	<i>Grevillea robusta</i>	Kenya	FJ904834	JF682854	Njuguna et al. (2011)
<i>L. pseudotheobromae</i>	MFLUCC 20-0137	leaf spot of <i>Cynometra malaccensis</i>	Chiang Rai, Thailand	MT947087	MT951067	This study

Table 1 Continued.

Taxa	Isolate No.	Host	Country	GenBank accession numbers		References
				ITS	TEF1- α	
<i>L. pyriformis</i>	CBS 121770*	<i>Acacia mellifera</i>	Dordabis, Namibia	EU101307	EU101352	Van der Walt (2009)
<i>L. rubropurpurea</i>	CBS 118740*	Canker of <i>Eucalyptus grandis</i>	Queensland, Australia	DQ103553	EU673304	Burgess et al. (2006)
<i>L. sterculiae</i>	CBS 342.78*	<i>Sterculia oblonga</i>	Braunschweig, Germany	KX464140	KX464634	Yang et al. (2017)
<i>L. subglobosa</i>	CMM 3872*	Collar and root rot of <i>Jatropha curcas</i>	Colatina, Brazil	KF234558	KF226721	Machado et al. (2014)
<i>L. swieteniae</i>	MFLUCC 18-0244*	Decaying fruit pericarp of <i>Swietenia</i> sp.	Chiang Rai, Thailand	MK347789	MK340870	Jayasiri et al. (2019)
<i>L. tenuiconidia</i>	CGMCC 3.18449*	<i>Aquilaria crassna</i>	Vientiane, Laos	KY783466	KY848619	Wang et al. (2019)
<i>L. thailandica</i>	CBS 138760*	<i>Mangifera indica</i>	Chiang Mai, Thailand	KJ193637	KJ193681	Trakunyingcharoen et al. (2014)
<i>L. theobromae</i>	CBS 164.96*	Fruit	Papua New Guinea	AY640255	AY640258	Phillips et al. (2005)
<i>L. tropica</i>	CGMCC 3.18477*	<i>Aquilaria crassna</i>	Vientiane, Laos	KY783454	KY848616	Wang et al. (2019)
<i>L. vaccinii</i>	CGMCC 3.19022*	Blighted branches of <i>Vaccinium corymbosum</i>	Beijing, China	MH330320	MH330328	Zhao et al. (2019)
<i>L. venezuelensis</i>	CBS 118739*	Wood of living <i>Acacia mangium</i>	Portuguesa, Venezuela	DQ103547	EU673305	Burgess et al. (2006)
<i>L. viticola</i>	CBS 128313*	Interspecific hybrid grape	Altus, USA	HQ288227	HQ288269	Urbez-Torres et al. (2012)
<i>L. vitis</i>	CBS 124060*	Canker of <i>Vitis vinifera</i>	Sicily, Italy	KX464148	KX464642	Yang et al. (2017)

*CBS – Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, CERC – Culture collection of China Eucalypt Research Centre, Zhanjiang, Guangdong Province, China, CFCC – China Forestry Culture Collection Center, CGMCC – China General Microbiological Culture Collection Center, CMM – Culture Collection of Phytopathogenic fungi “Prof. Maria Menezes”, CMW – Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, MFLUCC – Mae Fah Luang University Culture Collection, Chiang Rai, Thailand.

Results

Phylogenetic analyses

The combined ITS and TEF1- α sequences for the fungus obtained in this study were aligned with 61 sequences retrieved from GenBank, representing Botryosphaeriaceae. All three phylogenetic trees (ML, MP and BI) showed similar topologies. The RAxML analysis yielded a best scoring tree with the final ML optimization likelihood value of -3256.889118. The matrix had 247 distinct alignment patterns, with 7.77% of undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.216125, C = 0.289643, G = 0.255338, T = 0.238894; substitution rates AC = 1.353360, AG = 5.161491, AT = 1.777014, CG = 1.550618, CT = 6.747365, GT = 1.000000; gamma distribution shape parameter alpha = 0.682453 and invar = 0.467834. The maximum parsimonious dataset consisted of 770 characters, of which 576 were conserved and 99 were variable. The parsimony analysis resulted in 5,000 equally parsimonious trees (length = 363) with CI = 0.691, RI = 0.776, RC = 0.537 and HI = 0.309. Our strain of *L. pseudotheobromae* (MFLUCC 20-0137) clustered with other strains of *L. pseudotheobromae* (CBS 116459, CGMCC 3.18047, Gr26) with

bootstrap support as follows: 68% ML, 64% MP and 0.94 BI, confirming its phylogenetic position (Fig. 1).

Fig. 1 – Maximum parsimony tree resulting from analysis of combined ITS and TEF1- α sequence data alignment. Bootstrap support values for maximum likelihood and maximum parsimony greater than 50 and Bayesian posterior probabilities (BYPP, third set) greater than 0.90 are indicated at the

nodes as ML/MP/BYPP. The tree is rooted with *Diplodia mutila* and *D. seriata*. Ex-type strains are denoted with * and our strain is indicated in **red bold** (MFLUCC 20-0137).

Taxonomy

Lasiodiplodia pseudotheobromae A.J.L. Phillips, A. Alves & Crous

Fig. 2

Index Fungorum number: IF510941; Facesoffungi number: FoF00166

Causing brown leaf spots on *Cynometra malaccensis* van Meeuwen. Sexual morph: not observed. Asexual morph: *Pycnidia* 1.0–1.5 × 0.6–0.9 mm ($\bar{x}$ = 1.2 × 0.8 mm, n = 10), on toothpick surfaces solitary, globose to subglobose, uniloculate, black, irregularly surrounded by grey-white mycelium. *Pycnidial wall* 75.0–103 µm ($\bar{x}$ = 92.0 µm, n = 10) comprising cells of *textura angularis*, consisting of 2 layers of thick-walled, brown to hyaline cells. *Mycelium* hyaline to brown, septate. *Conidiophores* 6.0–33.0 × 2.6–8.0 µm ($\bar{x}$ = 16.7 × 5.7 mm, n = 20), hyaline, pyriform and cylindrical. *Conidiogenous cells* 6.4–27.0 × 5.1–10.0 µm ($\bar{x}$ = 16.2 × 8.7 µm, n = 20), hyaline, smooth, cylindrical, holoblastic. *Conidia* 23.0–33.5 × 14.7–16.9 µm ($\bar{x}$ = 29.7 × 15.8 µm, n = 30), oblong, sub-obovoid to ellipsoidal with rounded apex and base, thick-walled, hyaline and aseptate when immature, becoming dark brown, 1-septate and striated longitudinally.

Fig. 2 – *Lasiodiplodia pseudotheobromae* (MFLUCC 20-0137). a–b Herbarium specimen. c–d Colonies on PDA after 3 days of incubation at 25°C. e–f Colonies on PDA after 15 days of

incubation at 25°C. g Pycnidia on toothpick surfaces. h Close up of pycnidium covered with grey-white mycelium. i Close up of pycnidium. j Mycelium. k Section through pycnidium. l Pycnidial wall. m Conidiogenesis and developing conidia. n–r Conidia. Scale bars: h–i = 1.00 mm, j = 10 µm, k = 100 µm, l = 50 µm, m = 20 µm, n–r = 10 µm.

Culture characteristics – Colonies growing on PDA, reaching 8 cm diam. in three days at 25°C, initially grey-white, turning grey-black after 15 days, mycelium dense.

Material examined – THAILAND, Chiang Rai Province, Mae Fah Luang Botanical Garden, on attached leaves of *Cynometra malaccensis* (Fabaceae), 9 Oct 2018, N. Huanraluek, culture collection no: MFLUCC 20–0137, Fungarium no: MFLU 20–0654.

The morphology of our specimen is compared with other isolates of *L. pseudotheobromae* in Table 2.

Pathogenicity test

Lesions were formed after two days on both the attached and detached leaves, but only on those that were wounded (Fig. 3b, c, h). Measurements of the lesions are shown in Table 3. Conidiomata were formed only on the detached leaves. No symptoms were produced on non-wounded leaves (Fig. 3d, i) and controls (Fig. 3e–f, j–k). After re-isolation, the isolate was identified as *Lasiodiplodia pseudotheobromae*, based on morphology and colony characteristics, which is similar to those shown in Fig. 2.

Table 2 Comparison of conidial dimensions of *Lasiodiplodia pseudotheobromae* isolates.

Isolate No.	Conidial dimensions (µm)	Disease & country	References
CBS 116459*	25.5–30.5 × 14.8–17.2	From <i>Gmelina arborea</i> , Costa Rica	Alves et al. (2008)
CMM4875–CMM4877	21.4–30.7 × 11.4–15.1	Coffee dieback, Brazil	Freitas-Lopes et al. (2020)
FRLP1	23.7–28.2 × 12.4–14.9	Postharvest fruit rot of longan, Thailand	Pipattanapuckdee et al. (2019)
GXJG4.5	25.0–30.5 × 12.5–16.5	Husk rot of macadamia, China	Chang et al. (2019)
HNSY003	21.5–31.85 × 12.06–14.49	Leaf spots of <i>Hevea brasiliensis</i> , China	Wu et al. (2019)
IRAN 1518C	21.7–26.3 × 13.4–14.8	From <i>Citrus</i> sp., Iran	Abdollahzadeh et al. (2010)
MFLUCC 14-1192	25.0–30.0 × 10.0–15.0	Grapevine fruit peduncle and pedicel, China	Dissanayake et al. (2015)
MFLUCC 18-1120, MFLUCC 18-0950	20.0–26.0 × 10.0–14.0 (hyaline conidia) 19.0–25.0 × 12.0–15.0 (brown conidia)	Dead twigs of <i>Magnolia candolii</i> , China	de Silva et al. (2019)
MFLUCC 20-0137	23.0–33.5 × 14.7–16.9	Leaf spot of <i>Cynometra malaccensis</i>, Thailand	Current study
MHGNU F120	24.0–27.0 × 13.0–16.0	Mango dieback, South Korea	Kwon et al. (2017)
R1757	25.5–27.3 × 12.7–14.6	Pre-harvest fruit rot of mango, Malaysia	Munirah et al. (2017)

Table 3 Size of lesions (mm) formed in wounded attached and detached leaves

Leaf	Attached leaves		Detached leaves	
	After 2 days (mm)	After 5 days (mm)	After 2 days (mm)	After 5 days (mm)
1	7.0 × 9.0	10.0 × 12.0	1.0 × 3.0	5.0 × 6.0
	7.0 × 8.0	8.0 × 9.0	1.0 × 2.0	6.0 × 9.0
2	7.0 × 10.0	11.0 × 14.0	2.0 × 2.0	5.0 × 6.0
	6.0 × 11.0	12.0 × 17.0	2.0 × 2.0	5.0 × 7.0

Table 3 Continued.

Leaf	Attached leaves		Detached leaves	
	After 2 days (mm)	After 5 days (mm)	After 2 days (mm)	After 5 days (mm)
3	9.0 × 10.0	9.0 × 12.0	1.0 × 2.0	8.0 × 9.0
	7.0 × 11.0	8.0 × 12.0	2.0 × 3.0	6.0 × 7.0
4	7.0 × 10.0	8.0 × 10.0	-	-
	7.0 × 9.0	8.0 × 10.0	-	-
5	7.0 × 8.0	10.0 × 12.0	-	-
	8.0 × 9.0	12.0 × 14.0	-	-
$\bar{x}$ (mm)	6.5 × 9.5	9.6 × 12.2	1.5 × 2.3	7.2 × 7.3

Fig. 3 – Pathogenicity test results on attached and detached leaves of *Cynometra malaccensis*. a Experimental plant (*Cynometra malaccensis*). b Leaf spots formed after 5 days in wounded leaf. c Close up of leaf spot on wounded leaf. d Mycelium plugs on non-wounded leaf. e Control for wounded leaf. f Control for non-wounded leaf. g Pathogenicity test results on detached leaves of *Cynometra malaccensis*. h Wounded leaf. i Non-wounded leaf. j Control for wounded leaf. k Control for non-wounded leaf.

Discussion

Lasiodiplodia pseudotheobromae was recorded for the first time from *Cynometra malaccensis*. *Lasiodiplodia* consists of 66 species (Species Fungorum 2020), exhibiting pathogenic, saprobic and endophytic lifestyles, occurring mainly on woody hosts (Phillips et al. 2013, 2019, Hyde et al. 2019, Phookamsak et al. 2019). From our collection and isolation, we found that *L. pseudotheobromae* causes brown leaf spots of *Cynometra malaccensis* (Fig. 3).

Morphological examination of our isolate confirmed that it is characterized by thick-walled conidia, initially hyaline and aseptate and becoming pigmented and one-septate, bearing longitudinal striations on maturation (Alves et al. 2008, Phillips et al. 2013). However, only hyaline conidia were observed in an endophytic strain of *L. pseudotheobromae* (MFLUCC 18-0951) (de Silva et al. 2019). Morphologically, *L. pseudotheobromae* resembles *L. theobromae*, the type species of the genus. Nonetheless, these two species can be delineated based on size and shape of their conidia. Conidia of *L. pseudotheobromae* are larger and more ellipsoid than those of *L. theobromae* (Alves et al. 2008). Conidial sizes of our collection are similar to those described by Alves et al. (2008) and for other strains of *L. pseudotheobromae* (Table 2).

Botryosphaeriaceae consists of 24 genera and more than 100 species (Slippers et al. 2017, Yang et al. 2017, Index Fungorum 2020), with a cosmopolitan distribution. Most members of Botryosphaeriaceae cause infection to plants via wounds or natural openings (van Niekerk et al. 2010, Úrbez-Torres & Gubler 2011, Chethana et al. 2016, Massonnet et al. 2017). They can infect a wide range of unrelated hosts, although some appear to be host-specific (De Wet et al. 2008). *Lasiodiplodia pseudotheobromae* is a common plant pathogen (Kwon et al. 2017, Munirah et al. 2017, Chang et al. 2019, Wu et al. 2019), but not host-specific (Phillips et al. 2013, Dissanayake et al. 2015, 2016, Li et al. 2019). These fungi can enter the hosts and manifest as endophytes but becoming pathogens when the host is stressed (Chethana et al. 2016, Paolinelli-Alfonso et al. 2016). Thus, they are considered as opportunistic pathogens (Yan et al. 2018). Our pathogenicity test results validate that *L. pseudotheobromae* is an opportunistic pathogen when lesions were formed only in wounded leaves.

Research on new records of fungal species in hosts are crucial as it provides useful information in understanding the interactions between hosts and fungi as well as determining their geographical distribution (Li et al. 2019).

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