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Lasiodiplodia pseudotheobromae causes postharvest fruit rot of longan in Thailand

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Abstract

Fruit rot disease of postharvest longan is a major limiting factor for the longan market in Thailand. The causal fungus was identified as *Lasiodiplodia pseudotheobromae* based on morphology and analysis of the internal transcribed spacer (ITS) and translation elongation factor 1-alpha (EF1-α) partial genes. This is the first record of *L. pseudotheobromae* causing fruit rot disease of postharvest longan in Thailand.

Keywords *Dimocarpus longan* · Fruit decay · Identification · Phylogenetic tree · Botryosphaeriaceae

Longan (*Dimocarpus longan*) is commercially grown in many countries including China, India, Taiwan, Thailand and Vietnam (Jiang et al. 2002). In Thailand, longan growing areas cover about 188,574 ha with total production of 1,027,298 t per year. The major production areas in Thailand are located in the northern region consisting of Chiang Mai, Chiang Rai, Lamphun and Phayao Provinces (Office of Agricultural Economics 2017). Longan fruit is non-climacteric fruit and will not continue to ripen once removed from the tree (Drinnan 2004). Consequently, fruit must be harvested when their skin become yellow-brown and their flesh reaches optimal eating quality. Browning of longan fruits are associated with desiccation, heat stress, senescence, chilling injury and pest or pathogen attack (Pan 1994). The most important fruit rot disease of longan caused by fungi including *Lasiodiplodia* sp., *Pestalotiopsis* sp. and *Xylaria* sp. (Chang-ngern et al. 2010). *In this study,* we conducted morphological and phylogenetic analyses using ITS and *EF1-α* genes to identify the potential causal agent of longan fruit rot disease.

Longan fruit rot symptoms were observed and collected from a longan orchard during August 2018 until October 2018 at Lamphun Province, Thailand. Longan fruits were incubated in a plastic box for observation of symptom development. The fungal pathogen was isolated from the peel of longan fruit by tissue transplanting technique on potato dextrose agar (PDA) (Barnes 1968) and incubated at room temperature for three days. The fungi were re-isolated by hyphal tip isolation and stored on a PDA slant at 4 °C for further experimentation.

Samples of longan fruit rot were obtained and characterised by observation of the colour of the pericarp. The infected pericarp became brown and developed to a dark-brown and black colour (Fig. 1a). After incubation of longan fruit in a moist chamber, fungal mycelia grew on fruit and formed conidiomata (Fig. 1b). Conidia were released from conidiomata when stored in a moist chamber for three days (Fig. 1c). There were nine fungal pathogens (FRLP1-FRLP9) isolated from infected fruit.

The pathogenicity test of nine isolates were conducted on postharvest longan fruits according to the method of Than et al. (2008) by placing mycelial discs on both wounded (wounded with sterilised needle pricks) and unwounded longan fruits and kept at room temperature. Longan fruits with PDA discs were used as controls. The spore suspension technique was also used to compare. The symptom development was observed at 3, 5 and 7 days after inoculation.

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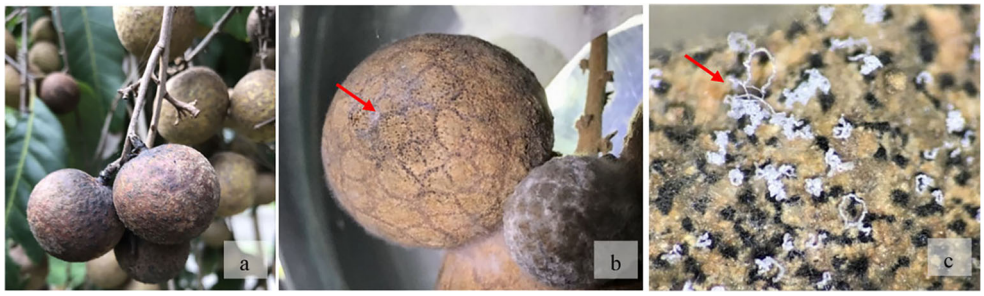

Fig. 1 Fruit rot symptoms on longan; **a** Longan fruit pericarp browning; **b** Conidiomata formed on pericarp (arrow); **c** Conidia released from conidiomata (arrow)

The results showed that at three days after inoculation by mycelial disc and spore suspension, the fungus isolate FRLP1 caused the most severe fruit rot symptoms using both methods. Symptoms on wounded longan fruits, showed the exocarp and endocarp turning dark brown in colour with greyish mycelial growth around the inoculation site, progressing to cover the whole fruit in five days. During the development of the disease, conidia and conidiomata were observed on inoculated fruits while the control showed no symptoms. The isolate FRLP1 was re-isolated from infected longan fruits, fulfilling Koch's postulates.

The fungus was cultured on PDA and incubated at room temperature for observation of colony characteristics. Fungal sporulation was induced by transferring fungal discs onto 2% water agar (WA) overlaid with sterilised grass leaf (*Imperata cylindrica*) as a substrate and incubated at room temperature (Suwanakood et al. 2005). Observation and measurement of conidial characteristics were conducted under a compound microscope with a digital camera (Carl Zeiss, Germany).

The colony of the fungus isolate FRLP1 was initially white with woolly aerial mycelia on PDA (Fig. 2a), then became pale grey in colour after 2 weeks of incubation. Conidiomata were produced on 2% WA overlaid with grass within 10 days (Fig. 2b). Conidiomata were uniloculated, dark brown to black in colour and appeared on the surface of the grass leaf (Fig. 2c). Fungal paraphyses were hyaline, cylindrical shape, aseptate, ends rounded and arising between conidiogenous cells (Fig. 2d). Conidiogenous cells were hyaline, cylindrical, base swollen, holoblastic, proliferating percurrently to form one or two closely spaced annellations. The conidia size was 23.7–28.2 × 12.4–14.9 μm and the shape was ellipsoidal with rounded shape at both apex and base. The immature conidia were hyaline and aseptate when released from conidiomata (Fig. 2e). Mature conidia became dark brown with middle one septate and longitudinal striations formed by melanin deposit on inner surface wall of conidia (Fig. 2f). The morphological features of the fungus isolate FRLP1 were similar to other *Lasiodiplodia* species. However, *Lasiodiplodia* species could be distinguished by size and shape of conidia and paraphyses (Abdollahzadeh et al. 2010; Alves et al. 2008; Burgess et al. 2006; Coutinho et al. 2017; Dou et al. 2017; Kwon et al. 2017; Linaldeddu et al. 2015;

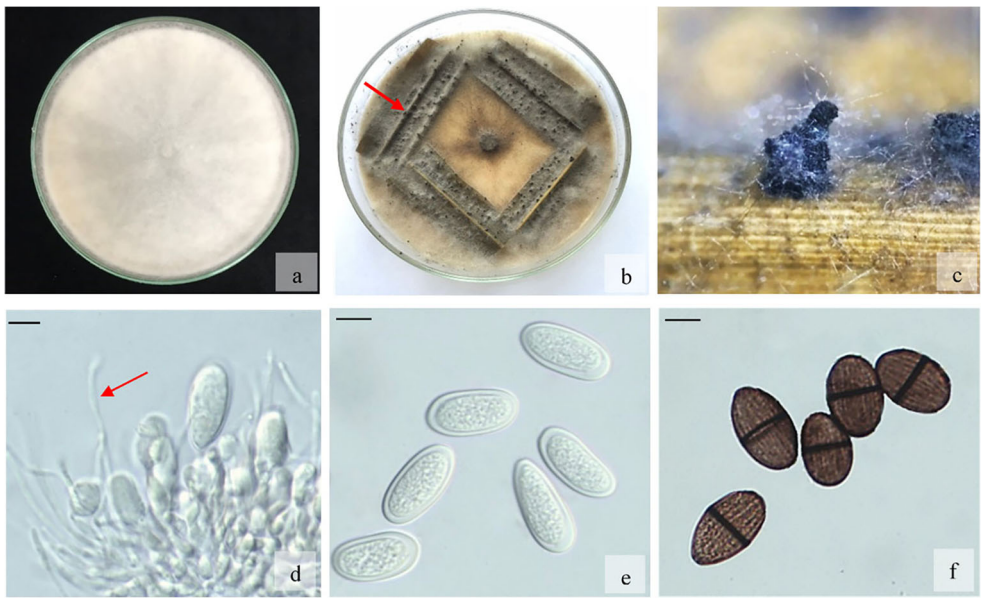

Fig. 2 Morphological characteristic of the fungus isolate FRLP1; **a** White and woolly colony and aerial mycelium on PDA; **b** Conidiomata on grass (arrow); **c** Conidiomata formed; **d** paraphyses hyaline, cylindrical and aseptate (arrow); **e** immature conidia hyaline and aseptate; **f** mature conidia dark brown colour with one-septate. Bar = 10 μm

Machado et al. 2014; Munirah et al. 2017; Pavlic et al. 2004; Phillips et al. 2013; Trakunyingcharoen et al. 2015; Yang et al. 2017). For example the conidial size of *Lasiodiplodia theobromae* was 19.5–27 × 9.1–15.3 μm with ovoid shape and septate paraphyses compared to *L. pseudotheobromae* which conidia size were 23.5–32 × 12.7–18 μm, which were larger than *L. theobromae*, had more ellipsoid and did not taper as strongly towards the base (Table 1).

Moreover, the fungus isolate FRLP1 was confirmed by molecular characterisation using PCR amplification of ITS and EF1-α genes. Genomic DNA was extracted from fungal mycelia according to the method of Myoung et al. (2009). The PCR amplification was performed by EconoTag PLUS GREEN 2X Master Mixed (Lucigen, USA) using ITS gene primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990) and the EF1-α gene was amplified by using primers EF1-688F (5'-CGGTCACTTGATCTACAAGTGC-3′) and EF1-1251R (5'-CCTCGAAC TCACCAGTACCG-3′) (Cruywagen et al. 2017). The PCR products were analysed by 1.7% agarose gel electrophoresis stained by RedSafe (iNtRON, South Korea).

Nucleotide sequences were directly analysed using fluorescent dye-terminator sequencing on ABI Prism™ 3730xl DNA sequencers (Applied Biosystems, Foster City, CA). All obtained sequences were analysed and aligned using BLAST and MEGA 7 software (Kumar et al. 2016), and then deposited in GenBank. The phylogenetic tree was inferred by using the Maximum Likelihood (ML) method based on the Tamura-Nei model with 1,000 replicates of bootstrap compared to previously reported *L. pseudotheobromae*, *L. theobromae* and other members in the family Botryosphaeriaceae (Table 2) which was performed in MEGA 7.

The combined ITS and EF1-α dataset of *Lasiodiplodia* sp. isolate FRLP1 was deposited in Thailand Bioresource Research Center (TBRC) and deposition number was TBRC10378. The combined dataset containing 1,098 characters (563 from ITS accession MK368390 and 535 from EF1-α accession MK376951) was grouped into *L. pseudotheobromae* clade and separated from *L. theobromae* clade and other members of the family Botryosphaeriaceae (Fig. 3). The nucleotides of *Lasiodiplodia* sp. isolate FRLP1 shared 99% identity to the

Table 1 Morphological characteristics of *Lasiodiplodia* species

Species	Conidia		paraphyses	Source of data
	Conidial size (μm)	septation		
L. theobromae	26.2–27.0 × 14.0–14.4	1	septate	Alves et al. 2008
	19.7–26.7 × 10.9–15.3	1	septate	Coutinho et al. 2017
	20.0–21.8 × 9.1–10.9	1	septate	Munirah et al. 2017
L. pseudotheobromae	23.5–32.0 × 14.0–18.0	1	aseptate	Alves et al. 2008
	25.5–27.3 × 12.7–14.6	1	aseptate	Munirah et al. 2017
	24.0–27.0 × 13.0–16.0	1	aseptate	Kwon et al. 2017
	23.7–28.2 × 12.4–14.9	1	aseptate	This study
L. crassispora	27.0–33.0 × 14.0–17.0	1	septate	Burgess et al. 2006
L. citricola	20.0–31.0 × 11.0–19.0	1	septate	Abdollahzadeh et al. 2010
L. venezuelensis	26.0–33.0 × 12.0–15.0	1	septate	Burgess et al. 2006
L. lignicola	15.0–17.5 × 8.0–11.0	UN[1]	aseptate	Phillips et al. 2013
L. sterculiae	14.0–16.0 × 10.0–11.0	UN	UN	Yang et al. 2017
L. chinensis	19.0–25.0 × 12.0–14.0	1	septate	Dou et al. 2017
L. mediterranea	26.3–37.0 × 13.5–18.0	1–2	septate	Linaldeddu et al. 2015
L. vitis	26.0–28.0 × 15.0–16.0	1	aseptate	Yang et al. 2017
L. subglobosa	16.0–23.0 × 11.0–17.0	1	aseptate	Machado et al. 2014
L. macrospora	28.0–35.0 × 15.0–17.0	1–3	septate	Machado et al. 2014
L. thailandica	20.0–26.0 × 12.0–16.0	1	septate	Trakunyingcharoen et al. 2015
L. parva	15.5–24.5 × 10.0–14.5	1	septate	Alves et al. 2008
L. gonubiensis	28.0–39.0 × 14.0–21.0	1–3	aseptate	Pavlic et al. 2004
L. parva	15.5–24.5 × 10.0–14.5	1	septate	Alves et al. 2008

Note: [1] UN = unknown

Table 2 Accession code of the sequences used in the phylogenetic analysis

Isolate Number[a]	Species	Location	Host	GenBank[b]	
				ITS	EF1-α
CGMCC3.18066	*L. chinensis*	China	*Hevea brasiliensis*	KX499899	KX499937
CGMCC3.18067	*L. chinensis*	China	*Sterculia lychnophora*	KX499901	KX499939
CBS 124707	*L. citricola*	Iran	*Citrus* sp.	GU945354	GU945340
IRAN 1521C	*L. citricola*	Iran	*Citrus* sp.	GU945353	GU945339
CBS 118741	*L. crassispora*	Australia	*Santalum album*	DQ103550	DQ103557
CMW 13488	*L. crassispora*	Venezuela	*Eucalyptus urophylla*	DQ103552	DQ103559
CBS 115812	*L. gonubiensis*	South Africa	*Syzygium cordatum*	DQ458892	DQ458877
CPC 22781	*L. gonubiensis*	Thailand	*Phyllanthus emblica*	KM006443	KM006474
CBS 134112	*L. lignicola*	Thailand	Dead wood	JX646797	KU887003
CMM 3833	*L. macrospora*	Brazil	*Jatropha curcas*	KF234557	KF226718
CBS 137783	*L. mediterranea*	Italy	*Quercus ilex*	KJ638312	KJ638331
CBS 137784	*L. mediterranea*	Italy	*Vitis vinifera*	KJ638311	KJ638330
CBS 456.78	*L. parva*	Columbia	Cassava field-soil	EF622083	EF622063
CBS 356.59	*L. parva*	Sri Lanka	*Theobroma cacao*	EF622082	EF622062
MHGNUF120	*L. pseudotheobromae*	Korea	*Mangifera indica*	KY404091	KY404090
CBS 116460	*L. pseudotheobromae*	Costa Rica	*Gmelina arborea*	EF622078	EF622058
CPC 22756	*L. pseudotheobromae*	Thailand	*Osmanthus fragrans*	KM006434	KM006465
CPC 22758	*L. pseudotheobromae*	Thailand	*Hevea brasiliensis*	KJ607141	KJ607151
CPC 22770	*L. pseudotheobromae*	Thailand	*Persea Americana*	KJ607146	KJ607156
CPC 22777	*L. pseudotheobromae*	Thailand	*Coffea arabica*	KM006439	KM006470
TBRC10378	*L. pseudotheobromae*	Thailand	*Dimocapus longan*	MK368390	MK376951
CBS 342.78	*L. sterculiae*	Germany	*Sterculia oblonga*	KX464140	KX464634
CMM 3872	*L. subglobosa*	Brazil	*Jatropha curcas*	KF234558	KF226721
CPC 22795	*L. thailandica*	Thailand	*Mangifera indica*	KJ193637	KJ193681
CGMCC3.18382	*L. thailandica*	China	*Podocarpus macrophyllus*	KY767662	KY751303
CBS 164.93	*L. theobromae*	Papua New Guinea	Fruit along coral reef coast	AY640255	AY640258
CBS190.73	*L. theobromae*	Tanzania	*Persea americana*	EF622068	EF622048
CBS 287.47	*L. theobromae*	Unknown	*Musa sapientum*	EF622069	EF622049
PHLO9	*L. theobromae*	Puerto Rico	*Dimocapus longan*	KC964546	KC964552
CPC 22766	*L. theobromae*	Thailand	*Pinus kesiya*	KM006436	KM006467
CMW 13512	*L. venezuelensis*	Venezuela	*Acacia mangium*	DQ103548	DQ103569
CBS 118739	*L. venezuelensis*	Venezuela	*Acacia mangium*	DQ103547	DQ103568
CBS 128313	*L. viticola*	USA	*Vitis vinifera*	HQ288227	HQ288269
CBS 124060	*L. vitis*	Italy	*Vitis vinifera*	KX464148	KX464642
CBS 110299	*N. luteum*	Portugal	A.J.L. Phillips	AY259091	AY573217

[a] Abbreviation of isolate and culture collection: CBS - Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW - M.J. Wingfield, FABI, Universityy of Pretoria, South Africa; CPC - Culture Collection of P.W. Crous, housed at CBS; MHGNU - Department of Plant Medicine Gyeongsang National University, Republic of Korea

[b] ITS - internal transcribed spacer; EF1-α - encoding translation elongation factor 1-alpha gene

reference *L. pseudotheobromae* isolate MHGNUF120 (accessions KY404091 and KY404090) (Kwon et al. 2017).

From morphological and phylogenetic analysis, the result showed that the fungal isolate FRLP1 could be identified as *L. pseudotheobromae*, a member of *Botryosphaeriaceae,* which was commonly found as endophytes and pathogens of various plants in tropical and subtropical regions (Rosado et al. 2016). These fungi are generally regarded as opportunistic pathogens with a latent endophytic stage causing numerous diseases when the host plants are exposed to stress or favorable conditions for disease development (Slippers

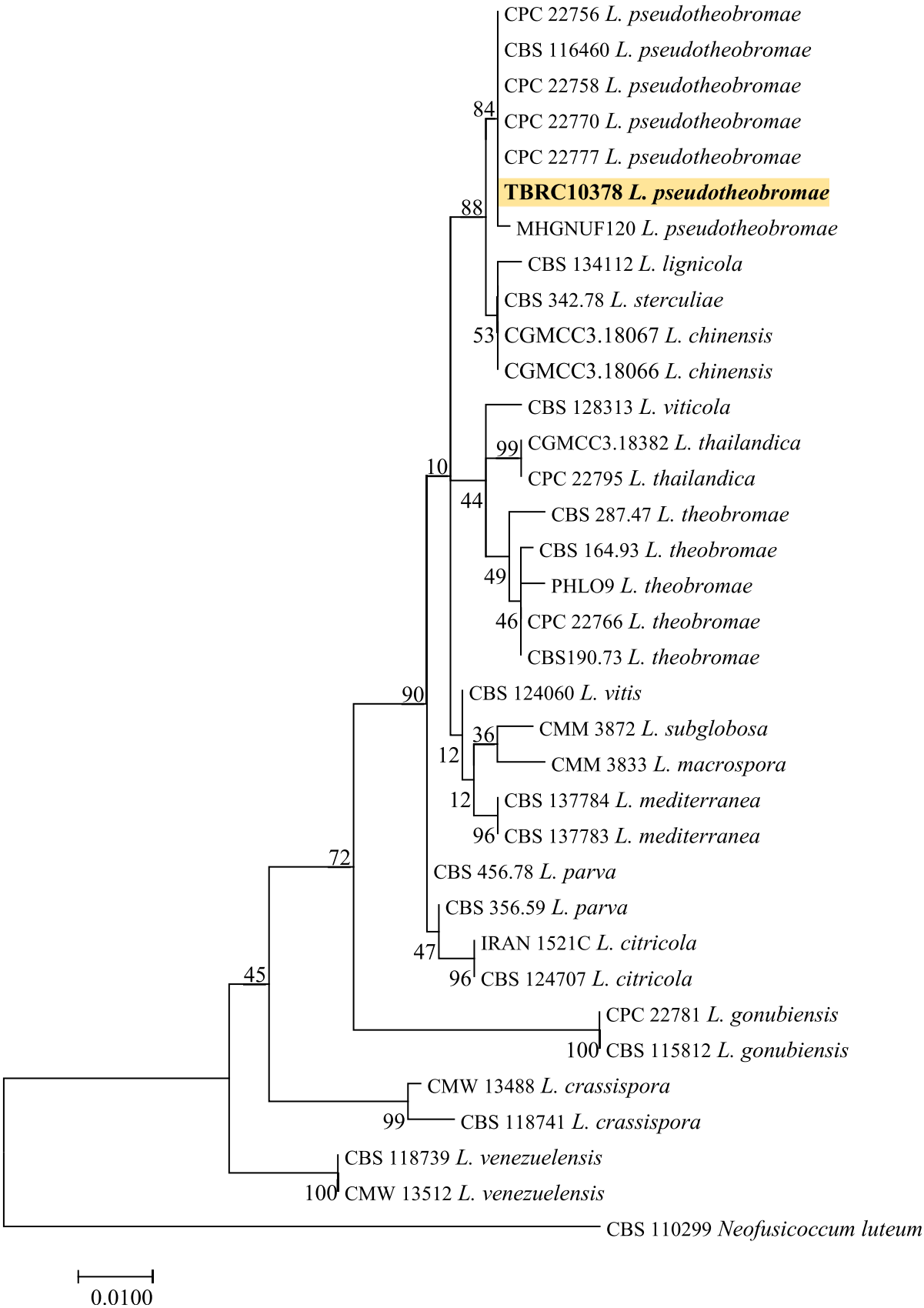

Fig. 3 The maximum likelihood tree generated from the combine analysis of ITS and EF1-α dataset. Designated out group taxon is *Neofusicoccum luteum*. Bootstrap support values from 1,000 replications are shown in the branches

and Wingfiled 2007). Botryosphaeriaceae were entering the fruit through scars at the stem-end of fruit and fungi colonised and latently remained in the pericarp (Ladanyia 2008). Symptoms of the fruit rot disease cause by *L. pseudotheobromae,* will develop after harvest especially under high temperature and high humidity conditions (Zhang 2014).

Lasiodiplodia pseudotheobromae was reported for the first time on grapevine in Brazil as a grapevine trunk pathogen (Correia et al. 2013) and mostly found in Africa, Europe and Latin America (Adetunji and Oloke 2013). In the case of Thailand, *L. pseudotheobromae* has been reported to cause canker, decline, dieback, stem end rot, and fruit rot on a wide range of plants (Farungsang et al. 1992; *Trakunyingcharoen* et al. 2013*). L. pseudotheobromae* has been identified as a causal agent of inflorescence blight of longan in Puerto Rico (Serrato-Diaz et al. 2014). To our knowledge, this is the first report of *L. pseudotheobromae* causing fruit rot disease of postharvest longan in Thailand.

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