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Morphological and Phylogenetic Evidences Reveal *Lasiodiplodia chonburiensis* and *L. theobromae* Associated with Leaf Blight in *Hevea brasiliensis* in Southern Thailand

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Abstract: The rubber tree is an important economic tree in Thailand. Recently, the cultivation of rubber trees in Thailand has suffered from a novel leaf fall disease with diverse symptoms, including leaf spot and leaf blight, resulting in severe leaf defoliation. Fungi from the *Lasiodiplodia* genus, which causes leaf disease in rubber trees, have not been reported in Thailand. Our research aimed to identify *Lasiodiplodia* associated with leaf blight disease in Thailand by examining morphological characteristics and completing a multi-gene sequence analysis and pathogenicity test to fulfill Koch's postulates. The internal transcribed spacer regions, translation elongation factor 1-α, and β tubulin 2 were sequenced for the multi-gene sequence analysis. In total, we recovered 14 isolates with 6 of those isolates. Of the six pathogenetic isolates, LST001, LST002, LYT003, LSrt001, and LSrt002 were determined to be *Lasiodiplodia chonburiensis*, and isolate LYL005 was determined to be *L. theobromae*. To the best of our knowledge, this is the first report of *L. chonburiensis* and *L. theobromae* being associated with leaf blight disease in rubber trees in Thailand or elsewhere.

Keywords: rubber tree; morphology; molecular techniques; pathogenicity test

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1. Introduction

Fungal diseases of economically important crops occur worldwide and have been implicated as a significant problem for rubber trees (*Hevea brasiliensis*) [1,2]. In particular, fungal species causing abnormal rubber tree defoliation appear more frequently than in the past and are now considered the most destructive diseases to affect rubber plantations [3]. To this day, the phenomenon of leaf fall has extended beyond the normal 'wintering' of the annual dry season. One case report described a diagnosis of leaf fall disease in Papua New Guinea and Vietnam in 2021, which resulted in a reduction of thousands of hectares in rubber plantations worldwide, with an average reduction of 380,000 ha in Indonesia, 5000 ha in Malaysia, and 52,000 ha in Thailand [3]. Of interest is a novel leaf disease first observed in Thailand rubber trees in 2019 [4]; however, rubber trees demonstrating similar symptoms were previously reported in other regions including North Sumatra in 2016, South Sumatra and Malaysia in late 2017 [5], and Cameroon in 2018 [1].

The rubber tree or rubber plant, belonging to the family Euphobiaceae, is an angiosperm plant and is a fast growing, medium to tall tree. Rubber trees are commonly grown in tropical areas as the climate is suitable for their growth and development [6,7]. The cultivation of rubber trees in Thailand suffers from a variety of fungal diseases. Fungal disease outbreaks in rubber plantations occur due to a variety of reasons including temperature, humidity, and rainfall [7]. Increased fluctuations in extreme climate change have a major potential to lead to a higher pest risk and disease severity in rubber plantations [8]. Several reports showed that during 2019–2022 there was a prolonged monsoon season from September to January in southern Thailand [4,9]. During this period, there was an increase

in overall fungal outbreaks on rubber plants and a novel leaf disease was also reported to appear during this time [4,9]. Other factors favorable to a fungal disease outbreak were also reported, including a lower-than-average temperature (24–26 °C), higher rainfall averages (>100 mm), and a higher average relative humidity (>90% RH).

In addition to the unidentified novel leaf disease, incidences of Phytophthora leaf fall disease caused by *Phyotophthora citrophthora* [10] and leaf fall caused by *Neopestalotiopsis cubana* and *N. formicarium* were also detected during this time frame in Thailand [4]. Lastly, another fungus that causes leaf fall in rubber trees, *Calonectria foliicola*, was also documented in the southern part of Thailand [9]. In addition, both fungal genera *Neopestalotiopsis* and *Lasiodiplodia* are species that can cause leaf disease in a wide variety of commercial plant species. *Lasiodiplodia* spp. have been known to infect almond (Prunus amygdalus), blueberry (*Vaccinium corymbosum*), castor (*Ricinus communis*), Chinese hackberry (*Celtis sinensis*), citrus (*Citrus* spp.), cocoa (*Theobroma cacao*), coconut (*Cocos nucifera*), grapevine (*Vitis* spp.), groundnut (*Arachis hypogaea*), jackfruit (*Artocarpus heterophyllus*), mango (*Mangifera indica*), mulberry (*Morus* spp.), melon (*Cucumis* spp.), olive (*Olea europaea*), pine (*Pinus* spp.), strawberry (*Fragaria* spp.), rice (*Oryza* spp.) [5,11–18], and durian (*Durio* spp.) crops [19]. It is generally accepted that *Lasiodiplodia* spp. have an endophytic lifestyle in addition to causing dieback disease. Host symptoms include trunk canker, yellow leaves, and wilting due to stem and root infection [5,17,18]. The severity of host symptoms has been shown to be influenced by climatic factors [20]. Dianda et al. [20] reported isolating six different *Lasiodiplodia* species from mango seedlings, including *L. euphorbicola* that accounted for 36 of the 47 isolates. The occurrence and incidence of this species were widely found in the drier and warmer regions in Burkina Faso, Africa. It is difficult to distinguish between *Lasiodiplodia* spp. based on morphological features alone; therefore, a DNA-sequence-based approach has been recommended [14] since there is a clear phylogenetic boundary [21] between currently known species. For example, Meng et al. [22] confirmed a new species, *Lasiodiplodia syzygii*, from post-harvest water-soaked brown lesions on *Syzygium samarangense* using the DNA sequencing method. Although *Lasiodiplodia* spp. have been studied in numerous plant species, there are limited studies of *Lasiodiplodia*-associated leaf blight disease in rubber trees in Thailand.

Hence, our study aimed to identify the fungal pathogen (*Lasiodiplodia* sp.) that causes the novel leaf blight disease in Thailand using both morphological and molecular approaches and verify its pathogenicity using healthy rubber tree leaves. This is important because this pathogen could represent a new *Lasiodiplodia* sp. and identifying the etiological agent is an important step in treating this disease in rubber trees.

2. Materials and Methods

2.1. Sample Collection and Fungal Isolation

A total of 25 symptomatic leaf samples were collected from infected areas in southern Thailand. The codes LST, LSrt, and LYL indicate that the samples were from the Satun, Surattani, and Yala provinces, respectively. Symptomatic leaf samples were kept in plastic bags and in a cool box and brought to the Plant Pathology Laboratory, Faculty of Natural Resources, Prince of Songkla University, Thailand, where the isolation was subsequently conducted. Fungal isolation was conducted using the tissue transplantation method. Pieces of symptom-containing healthy parts of 3 × 3 mm were cut and surface disinfected using 70% ethanol and 0.1% sodium hypochlorite (NaOCl). Excess NaOCl was removed by sterile distilled water (DW) 3 times, and the samples were hung to dry on sterile filter paper. The samples were directly placed on water agar (WA) and incubated at ambient temperature (28 ± 2 °C) for 3 days. Hyphal tips recovered from the infected tissues were cut and directly placed on potato dextrose agar (PDA; HiMedia, Mumbai, India) for purification. The fungal samples were transferred to a PDA slant and stored at 10 °C for further analyses.

2.2. Pathogenicity Test

A pathogenicity test was conducted to fulfill Koch's postulates. Healthy rubber leaves were surface disinfected using 70% ethanol. Inoculation was then performed using the agar

plug method, as previously described by Thaochan et al. [9]. Each fungal isolate was cultured on PDA and incubated at an ambient temperature for 3 days. A mycelial plug (5 mm) was cut from the edge of the 3-day-old incubated colony. We compared inoculations on rubber leaves with and without wounding. For the wounded samples, the rubber leaves were wounded using fine needles. Mycelial plugs were directly placed onto the rubber leaves. Only plugs that consisted of the PDA medium were used as the control. Each treatment was composed of 5 leaves (replicates) and the experiment was repeated twice. The inoculated samples were incubated in a humid chamber to maintain humidity (approximately 80–90%) for 7 days. The symptoms that developed on the inoculated leaves were observed and photographed daily. Symptomatic leaf samples were re-isolated using the tissue transplantation method indicated in Section 2.1. The morphology was observed using a compound microscope.

2.3. Morphology Study

Each fungal isolate was cultured on PDA and incubated at a temperature of 28.0 ± 2.0 °C. The colony diameter was measured daily to observe the growth rate. The macroscopic characteristics of pycnidia formation were observed using a stereo microscope (Leica Microsystems, Wetzlar, Germany). The microscopic features of the conidia were observed using a compound microscope (Leica Microsystems). The dimensions of the conidia (n = 20) were measured and photographed. The fungal cultures were deposited in the Culture Collection of the Pest Management Division, Faculty of Natural Resources, Prince of Songkla University.

2.4. Molecular Study

Each fungal isolate was cultured on PDA for 2 days to obtain young mycelia, which were subjected to DNA extraction using the mini-preparation method [23]. Portions of internal-transcribed spacer (ITS), translation elongation factor 1-α (*tef1-α*), and β-tubulin 2 (*tub2*) regions were amplified using the primer pairs ITS1/ITS4 [24], EF1-728F/EF1-986R [25], and Bt2a/Bt2b [26], respectively. A PCR mixture containing a DNA template, 10 *p*mol of each primer, 2× Green PCR Master Mix (Thermo Scientific, Waltham, MA, USA), and DNase/RNase free distilled water was placed in a 50 µL microtube. PCR amplification was performed using a BIO-RAD T100™ Thermal Cycler (Hercules, CA, USA). The PCR profile was denatured at 95 °C for 3 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at 50 °C for 30 s, elongation at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were observed via 1% agarose gel electrophoresis using novel juice DNA staining.

A portion of the PCR product was sequenced at the Macrogen Sequencing Service (Seoul, Republic of Korea) using the same primers as the PCR reaction. The DNA sequences were aligned with the known sequences in the NCBI (National Center for Biotechnology Information) database using the MAFFT v. 7 online servers (http://mafft.cbrc.jp/alignment/server/index.html) (accessed on 21 March 2022); these were then manually adjusted using MEGA X [27]. The phylogenetic tree estimation for each alignment was performed using maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI) methods. The ML tree was constructed using MEGA X, based on the T92 + G + I evolutionary model. The MP tree was obtained using the heuristic search option, with 1000 random additions of sequences and using Tree Bisection and Reconnection (TBR) as the branch-swapping algorithm of MEGA X. The Bayesian tree was generated using MrBayes ver. 3.2.7 [28]. Two parallel Markov chain Monte Carlo (MCMC) runs were performed for 2,000,000 generations; these were sampled every 100 generations. The initial 1000 generations were discarded as burn-in. The remaining trees were used to calculate the Bayesian inference posterior probability (BIPP) values. The phylogenetic trees were visualized using FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) (accessed on 29 March 2022).

3. Results

3.1. Symptom Recognition and Fungal Isolates

Disease incidence was about 10–15% in the observed field. The disease appeared as small orange circular or irregular spots on the rubber leaves. Subsequently, the spots

enlarged and coalesced into regular or irregular brown necrotic lesions with a dark-brown margin (Figure 1). Dark grey to black pycnidia were observed within the brown spots (Figure 1C,D). In total, we isolated 14 isolates from necrotic lesions.

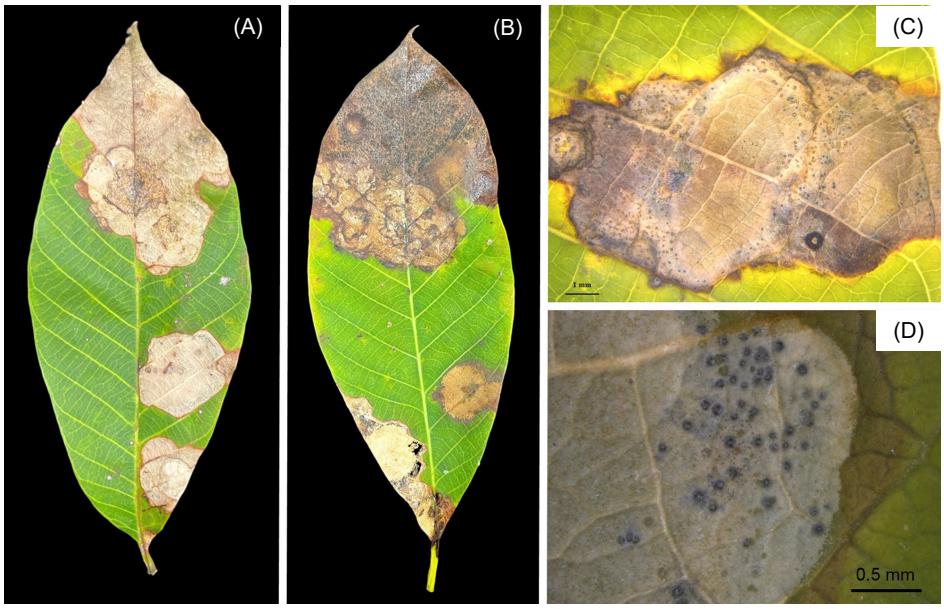

Figure 1. Symptomatic leaf samples observed in the rubber field: brown lesions expanded with dark-brown margins (**A**); dark-brown to green lesions (**B**); zoomed in view of lesions with black pycnidia (**C**,**D**).

3.2. Pathogenicity of Lasiodiplodia

During the pathogenicity analysis of the 14 fungal isolates on healthy rubber leaves, we observed that only 6 isolates resulted in the production of the necrotic lesions observed in the field. The fungal isolates LST001, LST002, LST003, LSrt001, LSrt002, and LYL005 showed dark-brown necrotic lesions on both wounded and unwounded samples (Figure 2). The wounded samples displayed more rapid and severe lesion development than the unwounded samples. There were no visible symptoms produced on the control leaves with and without wounding (Figure 2). The same fungal isolates were re-isolated from symptomatic inoculated leaves to confirm Koch's postulates.

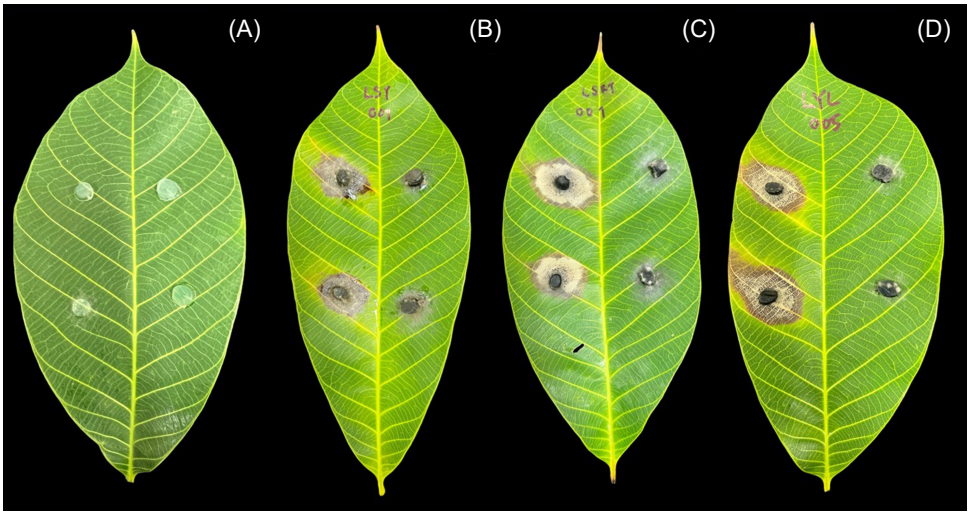

Figure 2. Pathogenicity test of fungal isolates on healthy rubber leaves: control uninoculated (**A**) and leaves inoculated with LST (**B**), LSRT (**C**), and LYL (**D**). The isolates shown were treated with wounded (left part of leaf) and unwounded (right part of leaf) methods.

3.3. Morphological Characteristics of Lasiodiplodia

All six fungal isolates were fast growing in PDA medium and covered the 8.5 cm Petri dishes within 7 days. This indicated a growth rate of 1.17 ± 0.1 cm/day. Colonies were white to pale greenish-gray and gradually became dark-grayish with age (Figures 3 and 4). The pycnidia developed on the substrate were solitary, globose to subglobose, black, and L × W (avr ± SD; *n* = 20). Immature and mature conidia were observed in this study. Both immature and mature conidia were subovoid to ellipsoid in shape, with a rounded apex and tapering to a truncated base. The conidiogenous cells were hyaline and cylindrical with a thin wall. The paraphyses were aseptate and hyaline. The immature conidia were aseptate, double-layered, hyaline, unicellular, and 21.10–24.69 × 10.73–13.79 µm (avr ± SD; *n* = 20). The mature conidia were dark-brown, septate, and 21.41–25.06 × 10.79–13.26 (avr ± SD; *n* = 20). Based on the morphological characteristics, we tentatively identified the six fungal isolates as *Lasiodiplodia* sp. [29]. The morphologies of the fungal isolates were compared with known species, as demonstrated in Table 1.

Table 1. Morphology dimension of representative *Lasiodiplodia* spp.

Species	Conidia		Paraphyses	Sources
	Size	Septation		
Lasiodiplodia chonburiensis	15.0–30.0 × 10.0–15.0	-	-	[30]
	19.4–25.2 × 10.3–13.4	1	Septate	This study (LSrt001)
	20.7–25.4 × 10.5–13.6	1	Septate	This study (LSrt002)
	17.7–24.9 × 11.2–12.4	1	Septate	This study (LST001)
	18.2–24.6 × 10.0–11.4	1	Septate	This study (LST002)
	19.3–26.9 × 11.1–14.6	1	Septate	This study (LST003)
L. pseudotheobromae	25.5–30.5 × 14.8–17.2	1	Septate	[31]
	25.5–27.3 × 12.7–14.6	1	Septate	[32]
	23.7–28.2 × 12.4–14.9	1	Septate	[33]
L. theobromae	26.2–27.0 × 14.0–14.4	1	Septate	[31]
	19.7–26.7 × 10.9–15.3	1	Septate	[34]
	23.0–27.4 × 12.2–14.7	1	Septate	This study (LYL005)

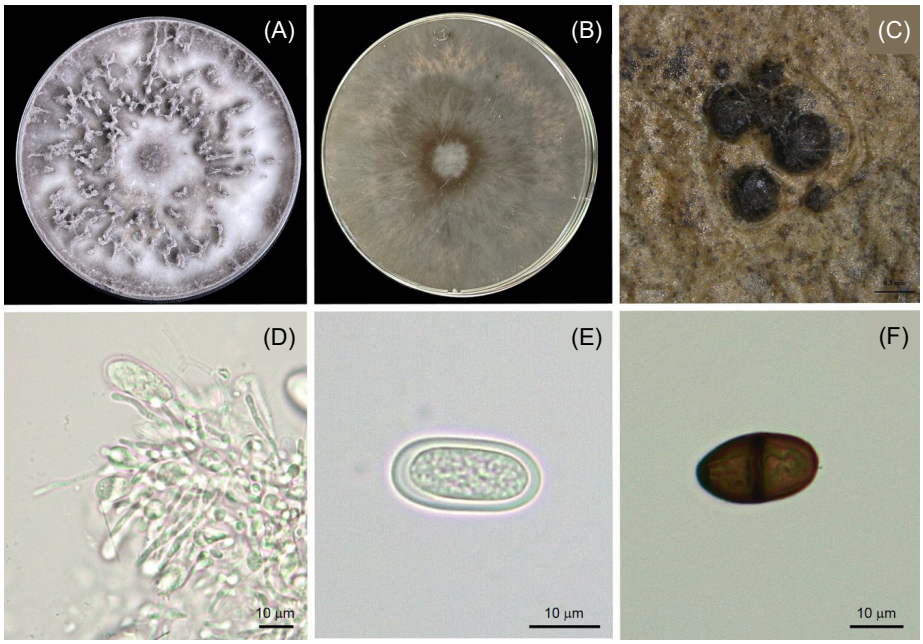

Figure 3. Morphological characteristics of *Lasiodiplodia* sp. Isolate LST001: top view of colony on PDA (**A**); bottom view of colony on PDA (**B**); pycnidia (**C**); conidiogenous cells and paraphyses (**D**); immature conidium (**E**); mature conidium (**F**).

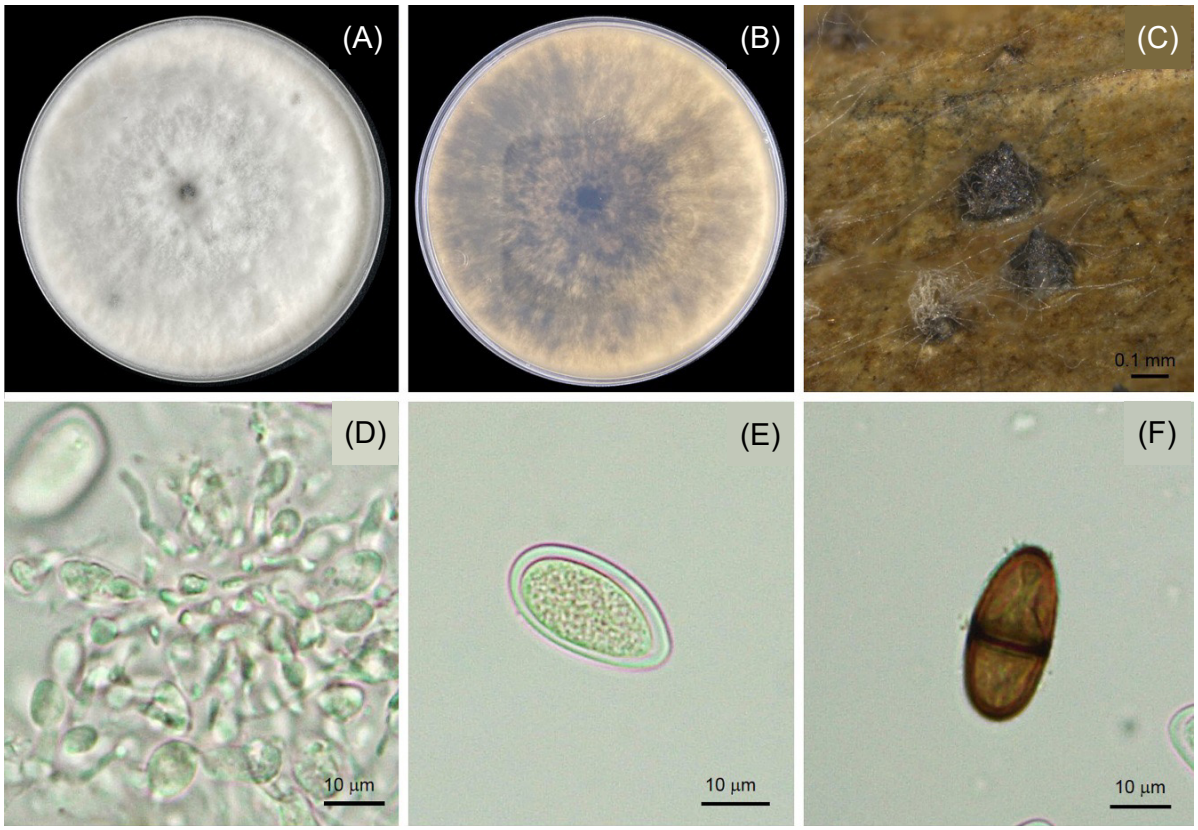

Figure 4. Morphological characteristics of *Lasiodiplodia* sp. isolate LYL005: top view of colony on PDA (**A**); bottom view of colony on PDA (**B**); pycnidia (**C**); conidiogenous cells and paraphyses (**D**); immature conidium (**E**); mature conidium (**F**).

3.4. Molecular Properties of Lasiodiplodia

A BLAST search in the GenBank database revealed that five isolates (LST001, LST002, LST003, LSrt001, and LSrt002) had a 99–100% sequences similarity to *L. caatingensis*, *L. citricola*, *L. exigua*, *L. mahajangana*, *L. pandanicola*, and *L. theobromae*. Another isolate (LYL005) showed 100% sequence similarity to *L. theobromae*. To construct the phylogenetic tree of the combined DNA sequences of ITS, *tef1-α*, and *tub2*, the sequences of the six fungal isolates from the present study were aligned with the sequences of 76 reference isolates of *Lasiodiplodia* and two outgroup taxa (*Diplodia mutila* and *D. seriata*). The gene boundaries in the alignment were ITS 1–486, *tef1-α* 487–702, and *tub2* 704–1019. The maximum likelihood (ML) tree revealed that the phylogenetic position of the five isolates from the rubber leaves clustered with the ex-type isolate of *L. chonburiensis* (-/56/-; ML/MP/BPP), whereas another isolate clustered with the ex-type isolate of *L. theobromae* (73/97/-; ML/MP/BPP) (Figure 5). Based on the results from this study, fungal isolates LST001, LST0 02, LST003, LSrt001, and LSrt002 were identified as *L. chonburiensis* and isolate LYL005 was verified as *L. theobromae*.

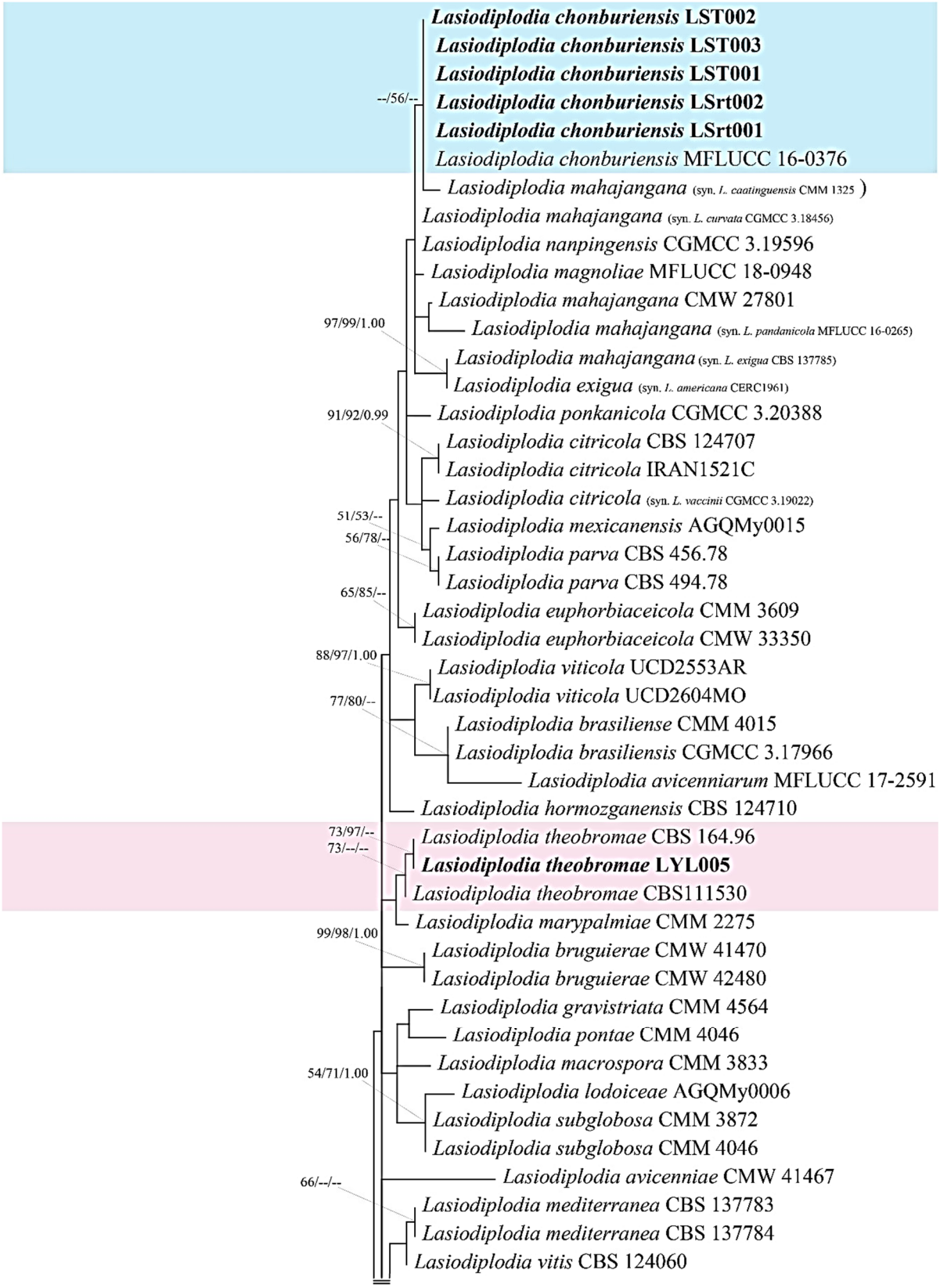

Figure 5. *Cont.*

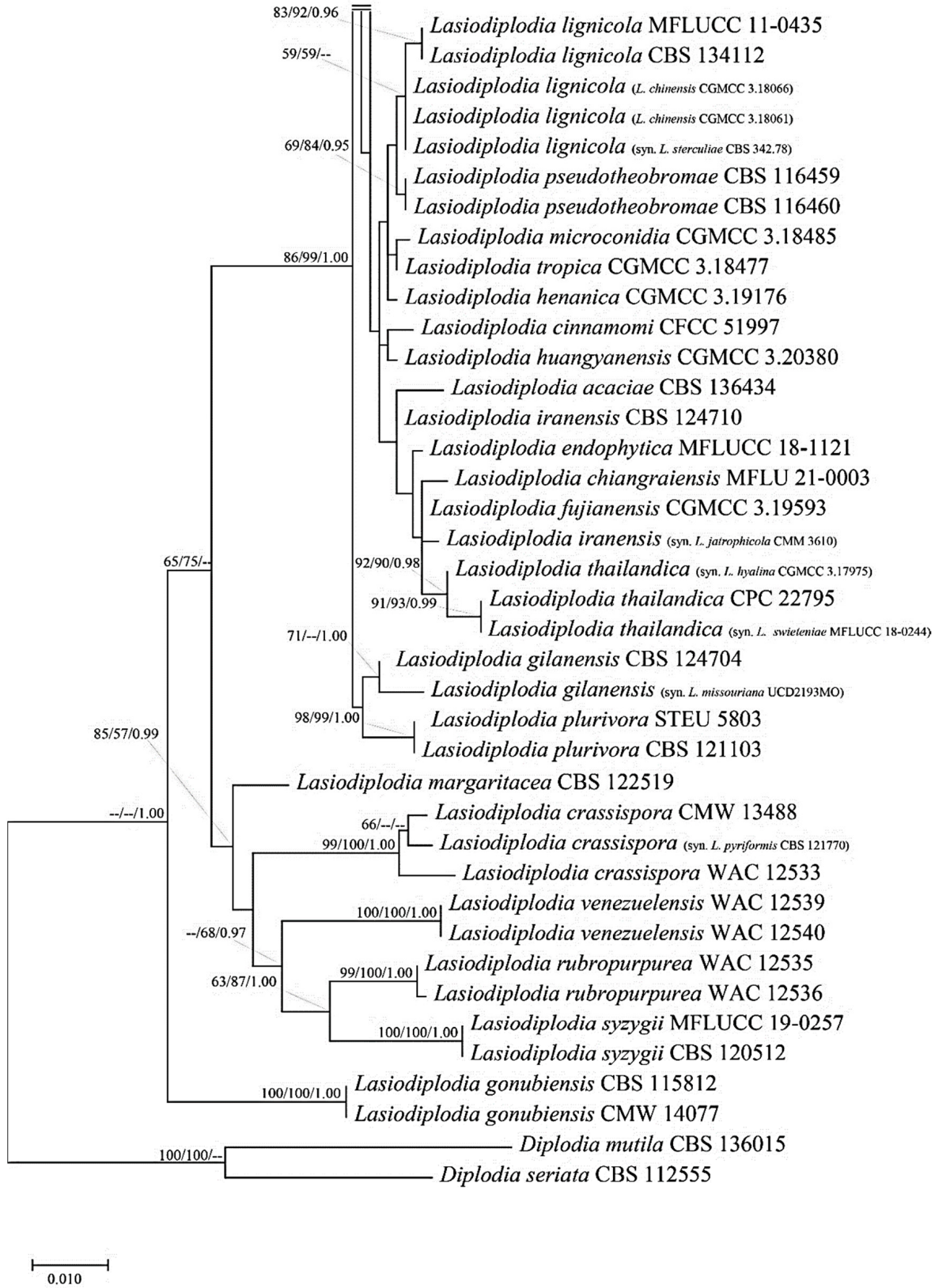

Figure 5. The maximum likelihood (ML) tree of combined DNA sequences of ITS, *tef1-α*, and *tub2*. Isolates in bold represent isolates in the present study. Bootstrap support values for MP and ML equal to or greater than 50% and Bayesian inference (BI) equal to or greater than 0.90 are defined as ML/MP/BPP above or below the nodes. *Lasiodiplodia chonburiensis* and *L. theobromae* are highlighted in blue and pink color, respectively. *Diplodia mutila* and *D. seriata* were used as outgroups.

4. Discussion

In the present study, we identified the pathogens causing leaf blight on rubber leaves as the fungi *L. chonburiensis* and *L. theobromae*. The identification was based on the morphological characteristics and molecular similarity was based on multiple DNA loci (ITS, *tef1-α*, and *tub2*). Fungi of the genus *Lasiodiplodia* belong to the family Botryosphaeriaceae and are commonly found in tropical and subtropical areas [29], causing several diseases in many plant species [35–37]. In Thailand, *Lasiodiplodia* species have been associated with various diseases, for instance, canker in rubber trees [38], spadix rot of *Anthurium andraeanum* [39], and fruit rot in longan [33].

Fungi of the family Botryosphaeriaceae display an abundant morphology that is different from other fungi; they are easily recognized and can be rapidly identified [32]. Certain fungi in the *Lasiodiplodia* species have shown overlapping morphological characteristics (such as growth rate, colony morphology, and conidia size and shape [40]) that could not be identified on a species level, as also observed in this study. The fungus *L. chonburiensis* could not be distinguished from *L. theobromae* based on morphology identification alone (Figures 3 and 4). The use of a combination of morphologic and molecular tools can be sufficient to identify *Lasiodiplodia* at a species level, as previously described by several researchers [5,39,40]. In the present study, the use of multiple DNA sequences (ITS, *tef1-α*, and *tub2*) successfully identified *Lasiodiplodia* obtained from rubber leaves to a species level as *L. chonburiensis* and *L. theobromae* with different clades (Figure 5). Our results are in agreement with de Silva et al. [41], who used DNA sequences of ITS, *tef1-α*, and *tub2* to study *Lasiodiplodia* species associated with *Magnolia*.

A pathogenicity test using the agar plug method fulfilled Koch's postulates in this study. We did not use conidia for inoculation on the rubber leaves for three reasons: (i) the formation of pycnidia required a significant amount of time (at least 4 months); (ii) pycnidia rarely developed on PDA; and (iii) we could not obtain conidia with a concentration of $\times 10^6$ conidia/mL. Therefore, we used the mycelial plug method for the pathogenicity test on rubber leaves, as previously described by Xia et al. [42]. The pathogenicity test revealed that both *L. chonburiensis* and *L. theobromae* could initiate infections and colonization on plant tissues with wounding. The wounding method appeared to be more effective at inoculating the inoculum (mycelia or conidia) into the plant tissues earlier than the unwounded method. This phenomenon may have been due to the wounded tissues helping the fungal mycelia to penetrate the plant, causing colonization and infection [43]. Several fungal pathogens use appressoria to form a penetration peg in host plants to cause infection [44,45]. *Lasiodiplodia* species do not form appressoria or other structures to penetrate plant tissues, but are pathogenic to host plants. Several publications have revealed that fungi in Botryophaeriaceae can invade host plants through endophytic ability, injuries, soil contamination, or insect infestation [5,35–46]. To date, the infection mechanism of *Lasiodiplodia* species and how they become pathogenic is still unclear.

Lasiodiplodia chonburiensis was first described from the dead leaves of *Pandanus* sp. in Thailand as a saprophyte [30]. There is no other report indicating that *L. chonburiensis* can cause disease in plants. In the present study, *L. chonburiensis* was confirmed as a causal pathogen of leaf blight in rubber leaves in Thailand. *Lasiodiplodia theobromae* was first described as causing charcoal rot of cocoa in Ecuador [29]. *Lasiodiplodia* was then recorded as causing diseases in several plant species in different regions, including dieback and gummosis in mangoes in Pakistan [47], bot canker and gummosis in nectarines in Turkey [48], and panicle blight in grapes in India [49]. Our result is the first report of *L. theobromae* causing leaf blight in rubber leaves. Trakunyingcharoen et al. [38] reported that only *L. pseudotheobromae* caused canker in rubber trees in northern Thailand. There has been no report prior to this study that *L. theobromae* also causes leaf blight in rubber leaves in Thailand. Our study revealed that both *L. chonburiensis* and *L. theobromae* act as plant pathogens associated with leaf-blight disease in rubber trees.

Although, the primary cause of leaf disease outbreaks remains unknown, the multiple fungal infections in plants belonging to the genus *Pestalotiopsis* [1], *Neopestalotiopsis* [8],

or *Calonectria foliicola* [4] cause serious damage to rubber production and other plants [2]. A long-established report showed that canopy reductions from rubber leaf fall disease have been increasingly impacted, already resulting in severely affected observations of tree health and latex yield [50]. The above-mentioned phenomenon can reduce the tree canopy density by up to 90% in severely affected areas [51]. Similarly, it should be noted that rubber harvesting and production in Thailand and other countries result in an estimated yield loss of between 15% and 50% [4]. In order to determine a fundamental method to reduce leaf fall disease and a reduced disease risk policy, additional research should be performed.

5. Conclusions

In the current study, pathogens causing leaf blight of rubber trees in Thailand were identified as *L. chonburiensis* and *L. theobromae* based on morphological and molecular studies of multiple DNA sequences. This study has expanded the knowledge of *Lasiodiplodia* species by providing a record of two species in a new host (*H. brasiliensis*), creating a new record of fungal distribution and host ranges. The precise identification of fungal pathogens is the first step in disease management. Studies of the management of *Lasiodiplodia* diseases need to be conducted in the near future.

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Data Availability Statement: The DNA sequence data obtained from this study were deposited in GenBank under accession numbers ITS (LC762191–LC762196), *tef1-α* (LC762197–LC762202), and *tub2* (LC762203–LC762208).

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Conflicts of Interest: The authors declare no conflict of interest.

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