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Research Article

A Novel Molecular Identification Technique for *Lasiodiplodia* species Associated with Avocado Diseases in Taiwan

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Abstract

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Lasiodiplodia theobromae and *L. pseudotheobromae* are widely distributed plant pathogens in tropical and subtropical regions, causing numerous diseases, but they are not easily identified and distinguished by traditional morphological techniques. In this study, we developed two species-specific primer pairs (Lt-F2/Lt-R1 and Lp-F/Lp-R2) based on sequence differences in translational elongation factor 1-α (*TEF1-α*) among *Lasiodiplodia* species. Polymerase chain reaction (PCR) amplification from genomic DNA verified that Lt-F2/Lt-R1 and Lp-F/Lp-R2 were specific for *L. theobromae* and *L. pseudotheobromae*, respectively; no amplification was observed from nontarget species. Using these primers to amplify DNA from 54 *Lasiodiplodia* isolates from avocado (*Persea americana*) in Taiwan, we successfully identified isolates of *L. theobromae* and *L. pseudotheobromae*. Meanwhile, we also detected the presence of other unknown *Lasiodiplodia* spp. Phylogenetic analyses using the DNA sequences of *TEF1-α*, β-tubulin 2 (*TUB2*), and RNA polymerase subunit II (*RPB2*) from these unknown species showed that *L. thailandica*, *L. hormozganensis*, and other unidentified *Lasiodiplodia* species also occurred on diseased avocado fruits, branches, or pedicels in Taiwan; their pathogenicity to avocado was confirmed on fruits. *L. thailandica* was reported as a pathogen of avocado for the first time, and the specific primers designed in this study provide a valuable tool for identifying the two *Lasiodiplodia* species.

Keywords: *Lasiodiplodia hormozganensis*, *Lasiodiplodia thailandica*, *Lasiodiplodia theobromae*, *Lasiodiplodia pseudotheobromae*, Species-specific primers.

INTRODUCTION

Lasiodiplodia spp. are widely distributed pathogens of woody plants in tropical and subtropical regions, causing numerous diseases (Phillips *et al*. 2013; Castro-Medina *et al*. 2014; Netto *et al*. 2014; Pipattanapuckdee *et al*. 2019). Among several *Lasiodiplodia* spp. associated with avocado (*Persea americana*), *L. theobromae* was reported to cause avocado branch canker, dieback, and stem end rot in Chile and Thailand (Trakunyingcharoen *et al*. 2015a; Valencia *et al*. 2019), dieback in Peru and Spain (Arjona-Girona *et al*. 2019; Rodríguez-Gálvez *et al*. 2021), branch canker in California (Avenot *et al*. 2023), postharvest fruit rot in Italy (Garibaldi *et al*. 2012), and branch blight in China (Qiu *et al*. 2020). In addition, *L. pseudotheobromae* has been demonstrated to cause avocado branch canker and stem-end

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rot in Thailand and dieback in Peru (Trakunyingcharoen *et al*. 2015a; Rodríguez-Gálvez *et al*. 2021). Moreover, *L. laeliocattleyae* and *L. citricola* were reported as pathogens causing avocado dieback in Peru and Italy, respectively (Rodríguez-Gálvez *et al*. 2021; Fiorenza *et al*. 2023), and *L. euphorbiaceicola* and *L. mahajangana* in Hainan, China (Chen *et al*. 2024), and *L. hormozganensis* in Sri Lanka (Nilmini *et al*. 2020) were reported as pathogens causing avocado stem-end rot. As mentioned above, *L. theobromae* and *L. pseudotheobromae* were the most frequently reported, and also were proved to be important pathogens causing branch canker and stem-end rot of avocado in Taiwan (Liang *et al*. 2021).

Due to their similar morphologies, *L. pseudotheobromae* was often misidentified as *L. theobromae* prior to the work of Alves *et al*. (2008), who distinguished them using a combination of morphological and phylogenetic analyses. Although some morphological and culture characteristics were suggested for distinguishing *L. pseudotheobromae* from *L. theobromae* (Alves *et al*. 2008), as more isolates from different geographic areas have been examined, has been called into question whether these characteristics are particular to *L. pseudotheobromae* (Liang *et al*. 2021). Because morphological and culture characteristics are not sufficient to distinguish between these species and also other closely related species of *Lasiodiplodia*, it is essential to use sequence-based identification based on multiple loci (Cruywagen *et al*. 2017; Rathnayaka *et al*. 2023). Sequences of the internal transcribed spacer (ITS) of genomic ribosomal DNA (rDNA) and the nuclear small subunit (SSU) rRNA, nuclear large subunit (LSU) rRNA, translational elongation factor 1-α (*TEF1-α*), β-tubulin 2 (*TUB2*), RNA polymerase subunit II (*RPB2*), and calmodulin (*cmdA*) genes have been used for phylogenetic analyses and identification of *Lasiodiplodia* spp. (Alves *et al*. 2008; Endes *et al*. 2016; Cruywagen *et al*. 2017; Li *et al*. 2019; Wang *et al*. 2021; Ko *et al*. 2023; Rathnayaka *et al*. 2023). However, identification using multi-locus phylogenetic analyses can be time-consuming and expensive.

Additionally, different *Lasiodiplodia* species can co-occur on the same plant and cause similar symptoms, further complicating identification (Ko *et al*. 2023). For example, both *L. theobromae* and *L. pseudotheobromae* have been demonstrated to cause branch canker and stem-end rot of avocado (Trakunyingcharoen *et al*. 2015a; Liang *et al*. 2021), dieback and stem-end rot of mango (*Mangifera indica*) (Ismail *et al*. 2012; Marques *et al*. 2013), and stem-end rot of papaya (*Carica papaya*) (Netto *et al*. 2014). A study by Rathnayaka *et al*. (2023) suggested that *L. theobromae* is the predominant species in tropical and subtropical regions and that *L. pseudotheobromae* is the second most prevalent species. Given their prevalence and economic importance, developing a cost-effective method to identify these species from non-purified cultures could facilitate epidemiological studies.

One cost-effective method that has been widely used for the accurate identification of fungal pathogens is polymerase chain reaction (PCR) with species-specific primers (Hariharan & Prasannath 2021). Although a species-specific primer pair for *L. theobromae* was developed in a previous study (Ni *et al*. 2012), it failed to distinguish *L. pseudotheobromae* from *L. theobromae*, as it amplified a PCR product of a similar size from both species. Therefore, we aimed to develop species-specific primers for cost-effective identification of these two species using PCR. Subsequently, these primers were used to identify *Lasiodiplodia* isolates from avocado in Taiwan. For some isolates, no product could be amplified by either pair of primers. Therefore, we next identified these isolates by performing multi-locus phylogenetic analyses and assessing their pathogenicity to avocado.

MATERIALS AND METHODS

DNA extraction

Mycelia were collected from colonies cultivated on potato dextrose agar (PDA, Difco Inc., Detroit, MI, USA). All DNA was extract-

ed using QuickExtract™ Plant DNA Extraction Solution (Epicenter, Madison, WI, USA), except for the DNA used in the primer sensitivity test, which was extracted using MasterPure yeast DNA purification kit (Lucigen, Middleton, WI, USA) and quantified using a microplate spectrophotometer (Epoch, Bio Tek Instruments, Inc., Winooski, VT, USA). Purified genomic DNA was stored at -20℃ until use.

Design of *L. theobromae*- and *L. pseudotheobromae*-specific primers

Eight isolates of *L. theobromae* and five isolates of *L. pseudotheobromae* were selected for designing species-specific primers (Appendix 1). The *TEF1-α* sequences of these isolates retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) (Appendix 1) were analyzed by multiple sequence alignment using Vector NTI Advance 11.5 (Invitrogen, Carlsbad, CA, USA). Two primer pairs were first designed based on the sequence variability between *L. theobromae* and *L. pseudotheobromae*: Lt-F2 (5′-AGTTCGAGAAGGTCCGTGCAC-3′), Lt-R1 (5′-TTCCTGTAGTGGGGCGC-3′), Lp-F (5′-AACCCCGCTTGGCTCTGT-3′), and Lp-R2 (5′-GTCCATCTTGTTGATGGCGAC-3′). The predicted sizes of the products amplified by primer pairs Lt-F2/Lt-R1 and Lp-F/Lp-R2 were 272 bp and 567 bp for *L. theobromae* and *L. pseudotheobromae*, respectively. To further evaluate the specificity of the primers, the sequences of *TEF1-α* at the primer annealing sites in *L. theobromae* and *L. pseudotheobromae* were compared with those of 31 other *Lasiodiplodia* spp., each represented by a single isolate (Appendix 1).

Assessment of primer specificity and sensitivity

Because only *L. theobromae* and *L. pseudotheobromae* have been reported on avocado in Taiwan previously, the isolates used for the specificity test included 6 *L. theobromae* isolates, 4 *L. pseudotheobromae* isolates, and other Botryosphaeriaceae spp. isolated from avocado, including *Neofusicoccum parvum* B1118, *N. parvum* B1174, *Neofusicoccum mangiferae* B1183, *N. mangiferae* B1640, *Botryosphaeria dothidea* B1134, and *B. dothidea* B1147 (Ni *et al*. 2011). PCR was performed in a 25-μL reaction mixture containing 5 μL of Fast-Run™ Taq Master Mix 5× (Protech Technology Enterprise Co., Ltd., Taipei, Taiwan), 18 μL of ddH_2O, 0.5 μL of each primer (10 pmole μL^{-1}), and 1 μL of DNA template. The PCR conditions were 4 min of initial denaturation at 94℃, followed by 31–33 cycles of 30 s of denaturation at 94℃, 30 s of annealing at 51–68℃, and 30 s of extension at 72℃, and a final extension of 10 min at 72℃. The optimum annealing temperature was empirically investigated by testing temperatures ranging from 51–68℃. The sensitivity of each primer pair was analyzed by using a serial dilution of purified fungal DNA as the template for PCR. Given the difference in expected amplicon sizes, the two primer pairs were also combined in a single PCR reaction to assess their suitability for multiplex PCR applications. The amplified products were stained with EZ-Vision® Three DNA Dye, and analyzed by 1.5% agarose gel electrophoresis in Tris-borate-EDTA buffer.

Identification of *Lasiodiplodia* isolates from avocado using species-specific primers

Lasiodiplodia isolates were isolated from avocado fruits with stem-end rot and branches with cankers between 2018–2020. The symptomatic tissues were disinfected by immersion in 0.5% NaClO for 30 s, rinsed with sterile water, and then placed in Petri dishes containing acidified potato dextrose agar [APDA, 300 mL PDA with 750 μL 50% (v/v) lactic acid]. The plates were incubated at 25℃ for 1 to 4 d. Colonies were transferred to 2% water agar, and single hyphal tips were transferred to PDA to obtain a pure culture. The DNA of each isolate was extracted and subjected to PCR using the species-specific primer pairs developed in this study as aforementioned.

Identification of *Lasiodiplodia* isolates from avocado by phylogenetic analyses

To identify the *Lasiodiplodia* isolates used in this study, phylogenetic analyses were conducted using partial sequences of the *TEF1-α*, *TUB2*, and *RPB2* genes because they were suggested as reliable molecular markers for the identification of *Lasiodiplodia* (Rathnayaka *et al*. 2023). The *TEF1-α*, *TUB2*, and *RPB2* nuclear gene regions were amplified using primers EF688F and EF1251R (Alves *et al*. 2008) or EF1F and EF2R (for B2885 and B2900) (Jacobs *et al*. 2004), Bt2a and Bt2b (Glass & Donaldson 1995), and rpb2-LasF and rpb2-LasR (Cruywagen *et al*. 2017), respectively. The PCR reaction mix was prepared as aforementioned. The PCR product was purified and sequenced by Tri-I Biotech, Inc. (Taipei, Taiwan).

Bayesian inference was used to construct phylogenetic trees. Sequences of 96 *Lasiodiplodia* isolates from the GenBank database and this study were included in the trees (Appendix 1). For each gene, a multiple sequence alignment was conducted using ClustalX v.2.1 (Larkin *et al*. 2007), and these alignments were concatenated using SequenceMatrix v.2.1.10 (Vaidya *et al*. 2011). jModelTest (Darriba *et al*. 2012) was used to select the best-fit DNA substitution model under the Bayesian information criterion. The DNA substitution models used for *TEF1-α*, *TUB2*, and *RPB2* were HKY + I + G, HKY + I, and K80 + I, respectively. For confirming the identification of *L. theobromae* and *L. pseudotheobromae* using species-specific primers, only the partial sequences of *TEF1-α* and *TUB2* were used to construct a phylogenetic tree. The DNA substitution models used for *TEF1-α* and *TUB2* were HKY + I + G and HKY + G, respectively. Phylogenetic tree construction was conducted with MrBayes v.3.2.6 (Ronquist *et al*. 2012). The analyses were run for 1.5×10^7 generations, and samples were taken from the posterior every 15,000 generations. The first 25% of generations were discarded as a burn-in period. Both trees were rooted with *Diplodia seriata* CBS112555.

Morphological characterization

Four isolates of *L. hormozganensis* (B2828, B2831, B2847, and B2858), 5 isolates of *L. thailandica* (ASS65, B2844, B2848, B2870, and B2913), and 8 isolates of unknown *Lasiodiplodia* spp. (AZP74, B2850, B2881, B2903, B2915, B2917, B2928, and B2930) were selected for morphological characterization. Colony morphology was characterized by growing isolates on 9-mm Petri dishes containing PDA for 3 d at 25℃ and 14 d at 35℃ in darkness. Pycnidia and conidia were induced by placing mycelial plugs on 2% (w/v) water agar (Merck, Darmstadt, Germany) supplemented with autoclaved horsetail stem (*Casuarina equisetifolia* L.) (Ni *et al*. 2011; Kee *et al*. 2017) and incubating for over 21 d at 25℃ under black light (TL-D 18W BLB, Philips, Amsterdam, The Netherlands). Morphological characteristics of conidia (e.g., color, shape, and septation) were observed with a Nikon 80i microscope (Tokyo, Japan), and pictures were captured with a Progres Gryphax camera (Jenoptik, Jena, Germany). The length and width of 50 conidia were measured.

Pathogenicity test on fruits

L. hormozganensis B2828, *L. pseudotheobromae* B2854, *L. thailandica* B2848, *L. theobromae* B2891, and *Lasiodiplodia* sp. B2903 were used in the pathogenicity test. ‘Choquette’ fruits were harvested at maturity and surface disinfected with 75% ethanol. The fruits were wounded with a flame-sterilized needle (5 mm in depth), and 2-day-old mycelial plugs (5 mm in diameter) of the isolates were placed on the wounds with the mycelial side facing the fruits. Control fruits were treated with sterile PDA discs (5 mm in diameter). There were 5 replicates for each isolate. The fruits were kept at 25℃ in 100% relative humidity for the first 2 d after inoculation, and then the relative humidity was reduced to 65% for the remainder of the trial. The diameters of the necrotic lesions on fruits were measured at 7 d after inoculation. Statistical analysis was conducted using SAS Enterprise Guide version 7.15 (SAS

Institute Inc., Cary, NC, USA). Before conducting a one-way analysis of variance (ANOVA), a Levene's test was performed to assess the assumption of homogeneity of variance. The results were non-significant ($P = 0.31$), indicating that the assumption of equal variances was met. Subsequently, the data were subjected to one-way ANOVA followed by Fisher's least significant difference test. Pathogens were re-isolated from lesions, and *L. pseudotheobromae* B2854 and *L. theobromae* B2891 were re-identified by PCR with species-specific primers, while *L. hormozganensis* B2828, *L. thailandica* B2848, and *Lasiodiplodia* sp. B2903 were re-identified by analysis of *TEF1-α* sequences. The experiment was replicated twice.

RESULTS

Design and assessment of *L. theobromae*- and *L. pseudotheobromae*-specific primers

PCR was conducted with 31 cycles, and the optimal annealing temperatures for the Lt-F2/Lt-R1 and Lp-F/Lp-R2 primer pairs were 59℃ and 60℃, respectively. The primer pairs Lt-F2/Lt-R1 and Lp-F/Lp-R2 amplified a single PCR band of the expected size from *L. theobromae* and *L. pseudotheobromae* isolates, respectively, but not from other Botryosphaeriaceae spp. (Fig. 1). The expected amplicon sizes were 272 bp and 567 bp, respectively. Primer sensitivity was analyzed by using a serial dilution of purified fungal DNA as the template for PCR. Analysis of the amplified products by agarose gel electrophoresis indicated that the detection limit of PCR was approximately 1 ng for each of the primer pairs (Fig. 2).

To further evaluate the specificity of the primers, the sequences of *TEF1-α* at the primer annealing sites of *L. theobromae* and *L. pseudotheobromae* were compared with those of other *Lasiodiplodia* spp. (Fig. 3). The alignment of *TEF1-α* sequences showed that the Lt-F2 sequence was unique to *L. theobromae*. Because there was insufficient sequence information for *L. viticola* CBS 128313, the *TEF1-α* sequences of two other *L. viticola* isolates with more sequence information were analyzed. The results showed that the sequence of *L. viticola* CMW40944 (GenBank accession number KP872376) differed from that of *L. theobromae* at this site, while that of *L. viticola* CMM1472 (GenBank accession number JX464040) was the same

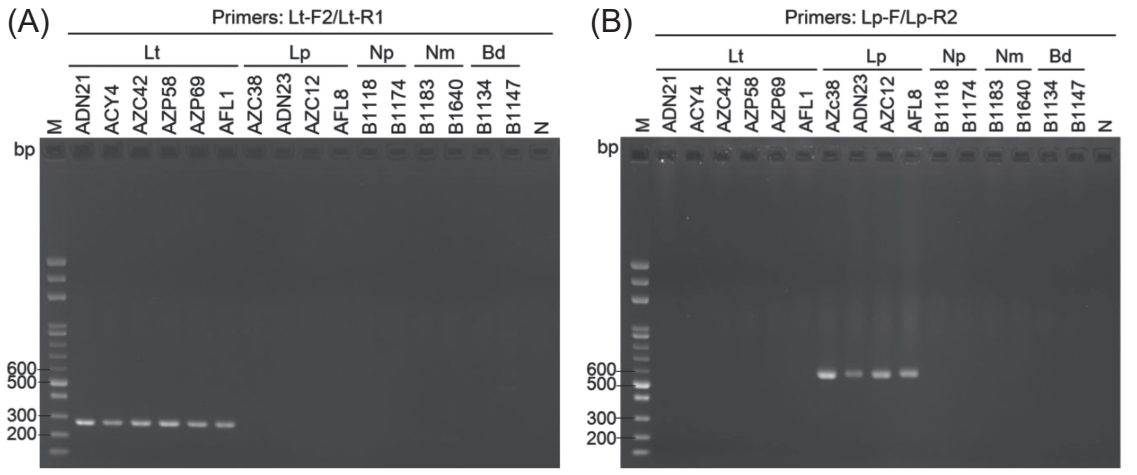

Fig. 1. Polymerase chain reaction (PCR) amplified products with species-specific primer pairs (A) Lt-F2/Lt-R1 and (B) Lp-F/Lp-R2. M: 100-bp DNA ladder (Protech Technology Enterprise, Taipei, Taiwan). N: negative control. Abbreviations: Lt: *Lasiodiplodia theobromae*; Lp: *Lasiodiplodia pseudotheobromae*; Np: *Neofusicoccum parvum*; Nm: *Neofusicoccum mangiferae*; Bd: *Botryosphaeria dothidea*.

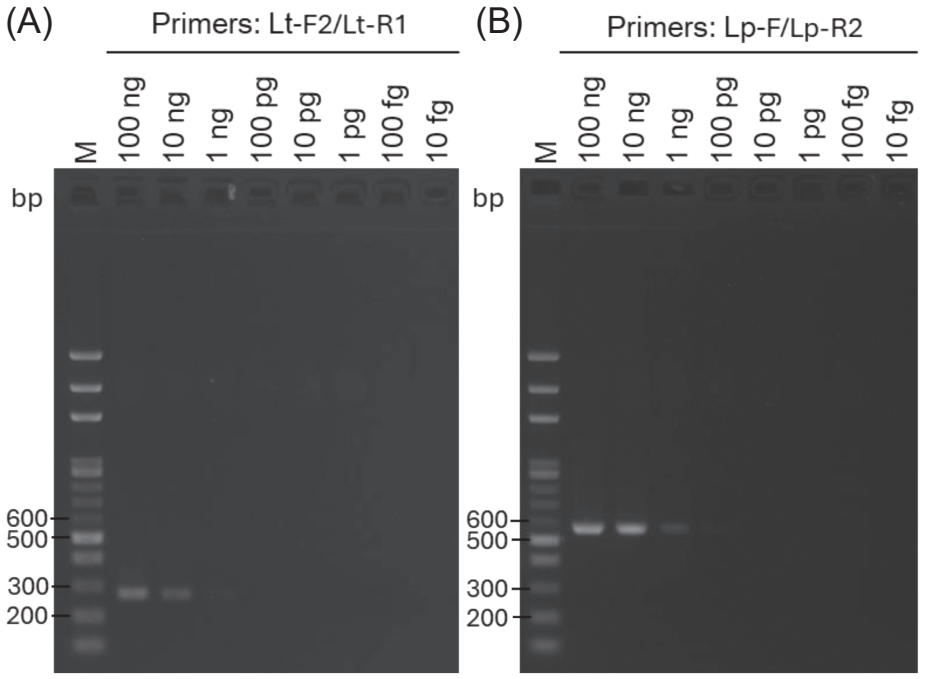

Fig. 2. Sensitivities of (A) Lt-F2/Lt-R1 and (B) Lp-F/Lp-R2 determined using 10-fold serial dilutions of *L. theobromae* ADN21 and *L. pseudotheobromae* AZC38 genomic DNA, respectively, ranging from 100 ng to 10 fg, as templates. M, 100-bp DNA ladder (Protech Technology Enterprise, Taipei, Taiwan).

(data not shown). In the region corresponding to Lt-R1, *L. mahajangana* CMW27801, *L. viticola* CBS128313, *L. bruguierae* CMW 41470, *L. chonburiensis* MFLUCC 16-0376, and *L. gilanensis* IRAN1523C shared the identicalsequences with *L. theobromae*. The Lp-F sequence was unique to *L. pseudotheobromae*. Most of the *TEF1-α* sequences of *Lasiodiplodia* spp. in NCBI lack information about the sequence from which Lp-R2 was designed. Among the species with such details, *L. theobromae* AZP58, *L. mahajangana* CMW27801, and *L. euphorbiacieco-la* CMM3609 shared the same sequence as *L. pseudotheobromae* (Fig. 3).

Although the sizes of the amplified products differed, the two primer pairs amplified two PCR products (200–300 bp and 500–600 bp) from *L. theobromae* DNA when combined (Appendix 3). Therefore, these primer pairs are not suitable for multiplex PCR. It is probably because there was at most one-nucleotide difference between *L. theobromae* and *L. pseudotheobromae* at the annealing site of Lp-R2, so Lt-F2/Lp-R2 could also amplify an amplicon from *L. theobromae* DNA (Appendix 3).

Identification of *Lasiodiplodia* isolates from avocado using species-specific primers

A total of 54 *Lasiodiplodia* isolates were collected from avocado fruits, pedicels, and branches in 2018–2020 (Appendix 2). Among them, 16 isolates (B2852, B2854, B2855, B2857, B2864, B2866, B2871, B2876, B2880, B2885, B2888, B2894, B2895, B2942, B2944, and B2946) were amplified by Lp-F/Lp-R2 with a 500–600 bp product, indicating that they were *L. pseudotheobromae*, while 19 isolates (B2825, B2827, B2843, B2849, B2862, B2863, B2865, B2873, B2875, B2877, B2886, B2887, B2891, B2900, B2926, B2937, B2940, B2943, B2953) were amplified by Lt-F2/Lt-R1 with a 200–300 bp product, indicating that they were *L. theobromae* (Fig. 4).

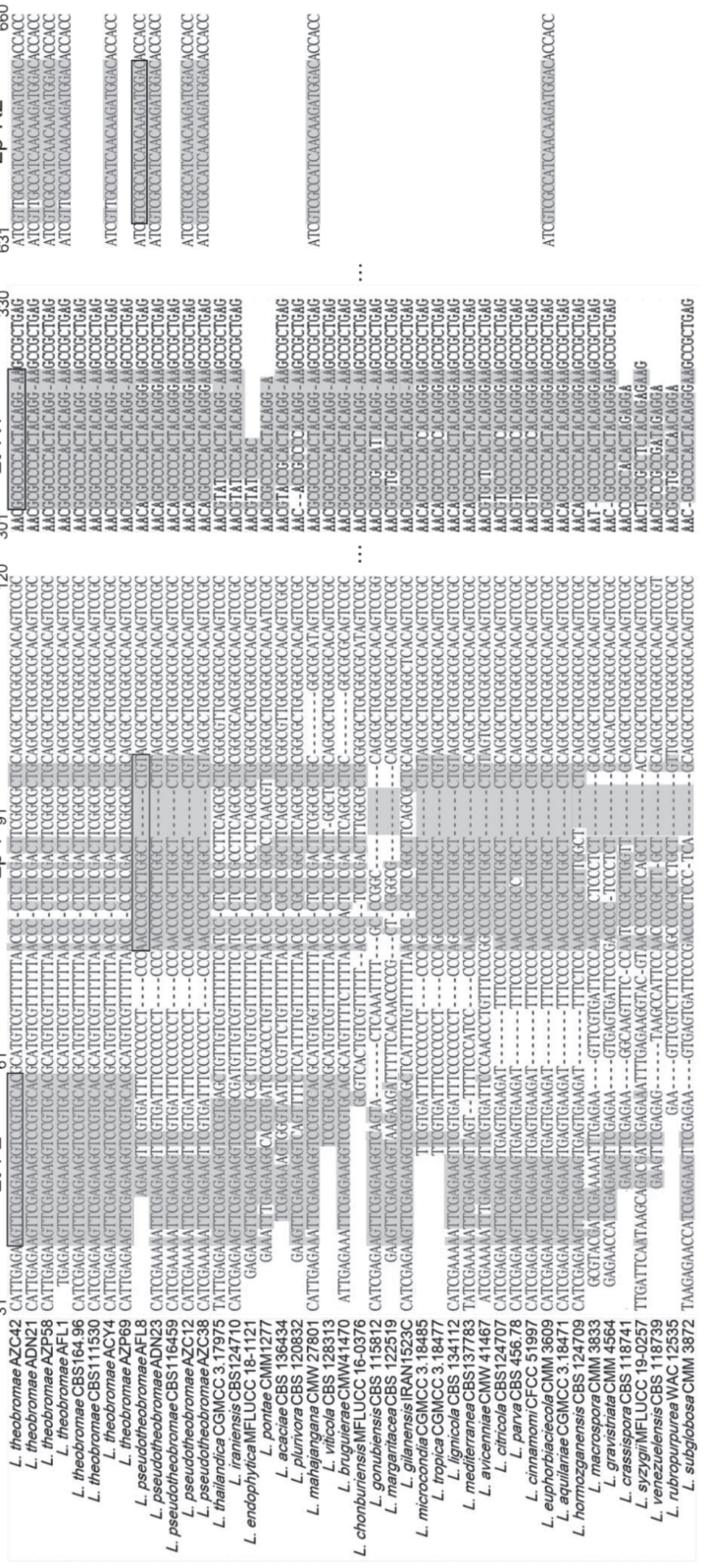

Fig. 3. Nucleotide sequence alignment of the translation elongation factor 1-α genes from different *Lasiodiplodia* species. Positions of the primers for species-specific detection of *L. theobromae* and *L. pseudotheobromae* are boxed with rectangles. Sequences that are identical to the primers are shaded in gray.

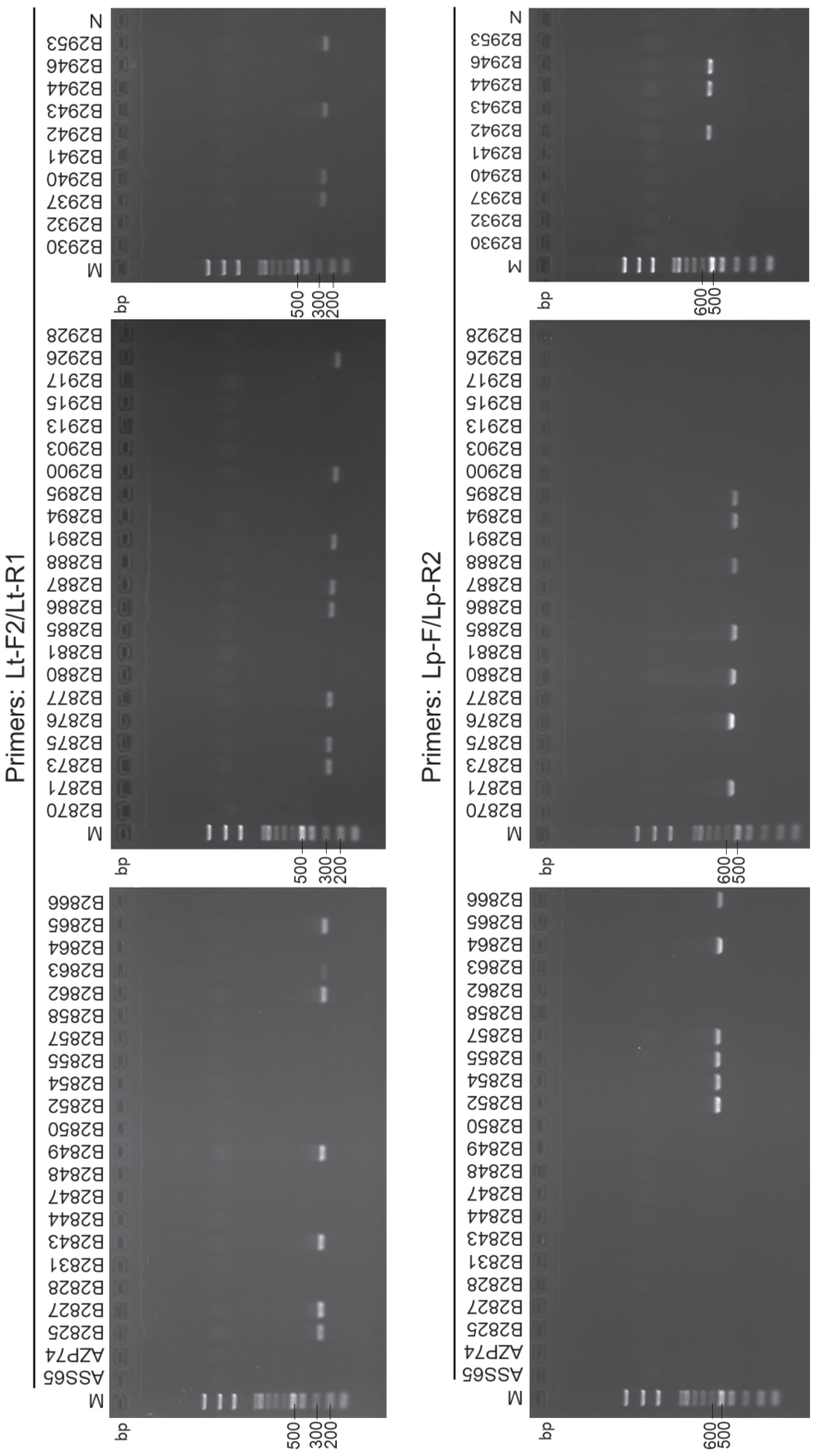

Fig. 4. Identification of *Lasiodiplodia* isolates from avocado using species-specific primers pairs Lt-F2/Lt-R1 (top) and Lp-F/Lp-R2 (bottom). M: 100-bp DNA ladder (Protech Technology Enterprise, Taipei, Taiwan). N: negative control.

To confirm the PCR-based identification of these isolates, the partial *TEF1-α* and *TUB2* sequences of these isolates and 73 isolates from the GenBank database were concatenated to form a supermatrix of 1,267 bp and used for phylogenetic analysis (Appendix 1). In this tree, B2852, B2854, B2888, B2866, B2855, B2857, B2864, B2871, B2876, B2880, B2885, B2894, B2895, B2942, B2944, and B2946 were clustered with *L. pseudotheobromae*, while B2825, B2827, B2843, B2849, B2862, B2863, B2865, B2873, B2875, B2877, B2886, B2887, B2891, B2900, B2926, B2953, B2937, B2940, and B2943 were clustered with *L. theobromae*, with Bayesian posterior probability (BPP) values of 69% and 100%, respectively, confirming the results obtained by PCR with Lt-F2/Lt-R1 and Lp-F/Lp-R2 (Appendix 4).

Discovery of novel *Lasiodiplodia* pathogens on avocado

No PCR product was amplified from 19 isolates (ASS65, AZP74, B2828, B2831, B2844, B2847, B2848, B2850, B2858, B2870, B2881, B2903, B2913, B2915, B2917, B2928, B2930, B2932, and B2941) by the 2 primer pairs (Fig. 4). Therefore, these isolates were subjected to phylogenetic analysis.

To identify these isolates, DNA sequences of the three gene regions (*TEF1-α*, *TUB2*, and *RPB2*) from these isolates, *L. pseudotheobromae* B2854 and B2885, *L. theobromae* B2891 and B2900, and 73 isolates from the GenBank database were concatenated to form a supermatrix of 1,918 bp and used for phylogenetic analysis (Appendix 1). The results showed that 4 isolates (B2828, B2831, B2847, and B2858) were clustered with *L. hormozganensis*, while 7 isolates (ASS65, B2844, B2848, B2870, B2913, B2932, and B2941) were in the *L. thailandica* clade; in both cases the BPP value was 100%. Eight isolates (AZP74, B2850, B2858, B2903, B2915, B2917, B2928, and B2930) clustered with *L. chiangraiensis*, *L. endophytica*, *L. ireniensis*, and *L. thailandica*, but their taxonomical positions could not be determined (Fig. 5).

To confirm the phylogenetic analysis-based identification, the morphological characteristics of the 19 isolates that did not yield a PCR product with the species-specific primers were examined. All isolates grew rapidly on PDA, covering the entire surface of the Petri dishes within 3 d. Colonies of all isolates were gray-white on the upper surface with fluffy aerial mycelia, and for some of the isolates, the middle of the colonies turned grayish-gray (Appendix 5). When cultured in PDA at 35℃, none of the 4 *L. hormozganensis* isolates produced red pigments, while all 7 of the *L. thailandica* isolates produced a red pigment, ranging in color from light pink to deep crimson (Appendix 6). Among the 8 isolates of unidentified *Lasiodiplodia* spp., AZP74, B2903, B2928, and B2930 showed the production of red pigments, while B2850, B2881, B2915, and B2917 did not. All isolates formed pycnidia and conidia on horsetail stems on water agar within 2–4 wk. Conidia of all isolates were subovoid to ellipsoid, smooth, initially hyaline and aseptate, becoming dark brown, 1-septate, and striated when mature. Conidia of *L. thailandica* isolates were 17–29 × 10–16 µm, L/W = 1.7–1.9 (n = 50) (Table 1, Appendix 7), while those of *L. hormozganensis* isolates were 15–24 × 10–14 µm, L/W = 1.4–1.9 (n = 50) (Table 1, Appendix 7). The conidia of unidentified *Lasiodiplodia* spp. isolates were 19–29 × 10–16 µm, L/W = 1.7–2.0 (n = 50) (Table 1, Appendix 7). No sexual (teleomorph) structures were observed during this study.

Pathogenicity test on fruits

To confirm that the *Lasiodiplodia* spp. isolated in this study were causal agents of avocado stem-end rot, pathogenicity tests were conducted on avocado fruits. The tests showed that *L. hormozganensis* B2828, *L. pseudotheobromae* B2854, *L. thailandica* B2848, *L. theobromae* B2891, and *Lasiodiplodia* sp. B2903 caused black lesions on avocado fruits. The external lesions were visible at 2 d after inoculation and continued to grow larger. Underneath the lesions, the pulp turned soft, water-soaked,

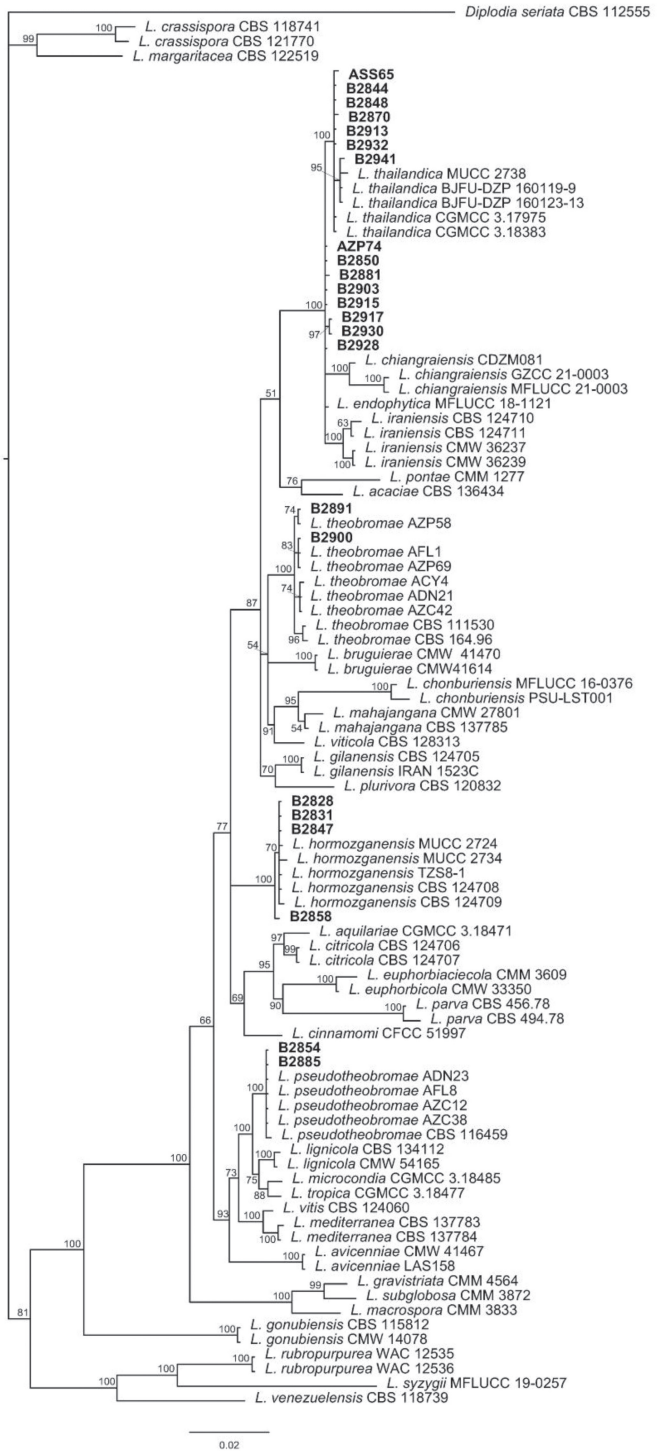

Fig. 5. Bayesian phylogenetic tree of *Lasiodiplodia* isolates from avocado in Taiwan and from the GenBank database. The phylogenetic tree was built using concatenated sequences of partial *TEF1-α*, *TUB2*, and *RPB2* gene regions. The Bayesian posterior probabilities are indicated next to the nodes. The tree is rooted with *Diplodia seriata* CBS 112555. Isolates from this study are emphasized in bold.

Table 1. Morphological characteristics of the *Lasiodiplodia* isolates examined in this study.

Species	Isolate	Conidial size (μm)			Colony pigmentation at 35℃
		Length	Width	L/W[z]	
L. hormozganensis	B2828	15.2–18.3–21.7	10.4–12.4–13.9	1.48	None
L. hormozganensis	B2831	19.1–21.4–23.5	9.3–12.1–13.7	1.78	None
L. hormozganensis	B2847	19.0–21.1–23.9	11.1–12.2–13.4	1.74	None
L. hormozganensis	B2858	17.6–20.8–23.6	10.0–11.3–12.7	1.83	None
L. thailandica	ASS65	20.9–23.8–27.6	10.9–12.9–15.4	1.86	Red
L. thailandica	B2844	19.7–23.5–27.2	10.7–13.7–15.3	1.73	Slightly pink
L. thailandica	B2848	18.9–23.2–26.4	10.8–13.2–14.5	1.77	Slightly pink
L. thailandica	B2870	21.0–24.0–28.8	11.4–13.9–15.6	1.73	Pink
L. thailandica	B2913	19.9–23.2–27.3	10.7–12.3–15.0	1.89	Slightly pink
L. thailandica	B2941	18.4–23.3–26.8	11.2–13.3–15.2	1.75	Pink
L. thailandica	B2932	17.2–23.2–27.4	10.3–12.6–14.3	1.85	Pink
Lasiodiplodia sp.	AZP74	22.1–23.8–25.7	10.3–12.4–15.6	1.93	Pink
Lasiodiplodia sp.	B2850	19.6–24.9–28.0	12.1–13.3–15.1	1.87	None
Lasiodiplodia sp.	B2881	21.5–24.9–27.7	11.0–12.6–14.4	1.98	None
Lasiodiplodia sp.	B2903	19.9–22.6–26.0	10.5–12.9–14.5	1.76	Pink
Lasiodiplodia sp.	B2915	18.8–23.1–26.8	11.5–12.6–14.6	1.84	None
Lasiodiplodia sp.	B2917	18.8–22.6–26.9	10.5–12.1–13.3	1.87	None
Lasiodiplodia sp.	B2928	19.1–24.6–28.2	11.9–13.8–16.1	1.78	Pink
Lasiodiplodia sp.	B2930	20.5–23.8–27.0	10.7–12.3–14.1	1.93	Slightly pink

[z] L/W: average length/average width.

and black (Appendix 8). The average diameters of the lesions caused by *L. hormozganensis* B2828, *L. thailandica* B2848, and *L. theobromae* B2891 were all more than 100 mm, which is significantly larger than the lesions caused by *L. pseudotheobromae* B2885 and *Lasiodiplodia* sp. B2903 (*P*-value < 0.0001) (Table 2). All control fruits remained symptomless at the treatment sites, and no fungi were re-isolated. All of the isolates were successfully re-isolated from necrotic pulps and re-identified based on species-specific primers or *TEF1-α* sequences, and thus, Koch's postulates were fulfilled.

Table 2. Pathogenicity tests on 'Choquette' fruits 7 d post inoculation.

Isolates	Lesion diameter (mm, mean ± *SD*)[z]
L. hormozganensis B2828	133.6 ± 13.6 a
L. pseudotheobromae B2885	95.7 ± 15.7 b
L. thailandica B2848	123.7 ± 11.9 a
Lasiodiplodia sp. B2903	95.1 ± 14.6 b
L. theobromae B2891	137.6 ± 15.9 a
Control	0.0 ± 0.0 c
P-value	< 0.0001

[z] Mean and standard deviation (*SD*) are derived from 5 replicates. Means in the same column followed by the same letter are not significantly different according to Fisher's least significant difference test ($P = 0.05$). There were five replicates for each isolate.

DISCUSSION

Accurate and cost-effective identification of plant fungal pathogens is crucial for disease management, as their sensitivity to fungicides differ slightly (Chen *et al*. 2024). Because *L. theobromae* and *L. pseudotheobromae* are two of the most prevalent *Lasiodiplodia* species in tropical and subtropical regions (Rathnayaka *et al*. 2023) and are frequently isolated from

avocado and difficult to distinguish by morphological and culture characteristics (Alves *et al*. 2008; Liang *et al*. 2021), it is necessary to develop a molecular method for prompt identification. Toward this end, we developed 2 pairs of species-specific primers based on the differences in *TEF1-α* sequences between *L. theobromae* and *L. pseudotheobromae*, and these primers proved to be helpful in distinguishing and identifying these 2 species.

In addition, the primers we developed can detect *L. theobromae* and *L. pseudotheobromae* from a DNA mixture of these 2 species (unpublished data). Therefore, even when these species co-occur on the same plant tissue, diagnosis can be conducted by performing PCR with species-specific primers using DNA directly extracted from mycelia growing from the plant tissues without purifying the fungal isolates first.

Because no DNA from other *Lasiodiplodia* spp. was available when testing the specificity of the primer pairs, the *TEF1-α* sequences of 31 other *Lasiodiplodia* spp., each represented by 1 isolate, were compared at the primer annealing sites. The results showed that the *TEF1-α* sequence of *L. viticola* CMM1472 at both the Lt-F2 and Lt-R1 sites was the same as that of *L. theobromae*. Besides, *L. microconidia*, *L. lignicola*, and *L. tropica* only have one nucleotide difference at the Lp-F site from *L. pseudotheobromae*, and the phylogenetic analyses showed that they were also closely related to it. Although the possibility that the primer pairs might also amplify a product from these *Lasiodiplodia* species cannot be ruled out, the identities of the 19 isolates identified as *L. theobromae* by Lt-F2/Lt-R1 and 16 isolates identified as *L. pseudotheobromae* by Lp-F/Lp-R2 in this study were confirmed based on phylogenetic analysis of partial *TEF1-α* and *TUB2* sequences. Because of the similarity between available DNA sequences of all *Lasiodiplodia* species, it is difficult to design a single primer pair to distinguish *L. theobromae* from all other species. Further studies could select other gene sequences for designing a second primer pair to verify identification.

Using Lt-F2/Lt-R1 and Lp-F/Lp-R2 to screen 54 *Lasiodiplodia* isolates from avocado revealed that most *Lasiodiplodia* isolates from avocado in Taiwan are either *L. theobromae* or *L. pseudotheobromae*. Moreover, phylogenetic analyses revealed that the remaining isolates were *L. thailandica*, *L. hormozganensis*, and some unidentified *Lasiodiplodia* spp. These results suggest that primer pairs Lt-F2/Lt-R1 and Lp-F/Lp-R2 can be used not only to identify *L. theobromae* and *L. pseudotheobromae* but also to distinguish them from other *Lasiodiplodia* spp. associated with avocado.

Lasiodiplodia has a global distribution and occurs on a wide range of hosts (Ko *et al*. 2023; Rathnayaka *et al*. 2023). In recent years, multi-locus phylogenetic analyses combined with morphological characteristics have been used to re-examine previously identified *Lasiodiplodia* isolates, revealing cryptic species (Hattori *et al*. 2023; Ko *et al*. 2023). We found that the specific primers Lt-F2/Lt-R1 and Lp-F/Lp-R2 could identify *L. theobromae* and *L. pseudotheobromae* not only from avocado but also from other hosts (unpublished data), which means researchers could use these primers to screen through their collections of *Lasiodiplodia* spp. and identify isolates of *L. theobromae* and *L. pseudotheobromae* first, and then the remaining isolates could be subjected to further analysis. Therefore, these primers could be useful and cost-effective tools for investigating the diversity of *Lasiodiplodia* spp.

The pathogenicity tests in this study showed that *L. thailandica* B2848, *L. hormozganensis* B2828, and *Lasiodiplodia* sp. B2903 were all pathogenic to avocado fruits. *L. thailandica* was first reported on *M. indica* and *Phyllanthus acidus* in Thailand (Trakunyingcharoen *et al*. 2015b). Since then, this species has been reported to be associated with *Podocarpus macrophyllus* and *Albizia chinensis* in China, *M. indica* and *Kalanchoe pinnata* in Japan, and a *Cerasus* sp. in

Taiwan (Dou *et al*. 2017; de Silva *et al*. 2019; Hattori *et al*. 2023; Rathnayaka *et al*. 2023), but it has not previously been reported as a pathogen of avocado. The reported conidial dimensions of *L. thailandica* were (20–)22–25(–26) × (12–)13–15(–16) µm, and those of the *L. thailandica* isolates identified in this study were all within this range, confirming the identification from phylogenetic analysis. To our knowledge, this is the first study describing the pigmentation of *L. thailandica* at 35℃, and demonstrating that *L. thailandica* is also a pathogen causing avocado stem-end rot disease.

L. hormozganensis has been reported to be associated with avocado in Sri Lanka (Nilmini *et al*. 2020), *M. indica* and an *Olea* sp. in Iran (Abdollahzadeh *et al*. 2010), *C. papaya*, *M. indica*, and *Ricinus communis* in Brazil (Abdollahzadeh *et al*. 2010; Marques *et al*. 2013; Netto *et al*. 2014), several herbaceous and woody plants in Japan (Hattori *et al*. 2023), and *Syzygium samarangense*, *M. indica*, *Psidium guajava*, *Annona squamosal*, and *Musa* spp. in Taiwan (Ko *et al*. 2023). The previously documented conidial dimensions were 21.5 ± 1.9 × 12.5 ± 0.8 µm, L/W ratio = 1.7 ± 0.2 (Abdollahzadeh *et al*. 2010). The size and L/W ratio of conidia of B2828 were smaller than the reported dimensions, while those of the other three *L. hormozganensis* isolates were similar to the reported ones. This implies that the size and L/W ratio of conidia might differ among *L. hormozganensis* isolates. All 4 isolates did not produce a pink pigment in PDA cultures at 35℃, which was consistent with the previously reported characteristics (Abdollahzadeh *et al*. 2010).

Phylogenetic analysis revealed that eight isolates did not form a well-supported clade with any other previously identified and described *Lasiodiplodia* species; however, they were close to *L. chiangraiensis*, *L. endophytica*, and *L. ireniensis*. The morphology of *L. endophytica* has not been described, so we could not use morphology to determine if these isolates were similar to this species. The conidial dimensions of *L. chiangraiensis* were reported to be (21–)22–27(–30) × (12–)13–15(17) µm, L/W = 1.9 (Wu *et al*. 2021), while those of *L. iraniensis* were smaller, (15.3–)17–23(–29.7) × 11–14, L/W = 1.6 (Abdollahzadeh *et al*. 2010). *L. chiangraiensis* and these 8 unidentified isolates have conidia of similar sizes, but *L. chiangraiensis* is characterized by conidia remaining hyaline even after maturity (Wu *et al*. 2021), while these isolates have colored conidia after maturity, which is the same as *L. ireniensis*. Further studies are needed to decide their taxonomical position.

In conclusion, the species-specific primers developed in this study could accurately identify *L. theobromae* and *L. pseudotheobromae*, 2 of the most prevalent *Lasiodiplodia* species worldwide. These primers could facilitate monitoring of the occurrence of these 2 species in the field and investigating the species diversity of *Lasiodiplodia*. Using these primers to test the collection of *Lasiodiplodia* isolates from avocado, this study found that *L. thailandica*, *L. hormozganensis*, and some unknown *Lasiodiplodia* species were also associated with avocado in Taiwan. In addition, to our knowledge, this is the first study demonstrating that *L. thailandica* is also a pathogen causing avocado stem-end rot disease.

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Appendix 1. GenBank accession numbers for DNA sequences of *Lasiodiplodia* spp. used in the phylogenetic analyses.

Species	Isolates[z]	Genbank accession number[y]		
		RPB2	*TEF1-α*	*TUB2*
L. acacia	CBS 136434*	MT592307	*MT592133*	MT592613
L. aquilariae	CGMCC 3.18471*	KY848562	*KY848600*	N/A
L. avicenniae	CMW 41467*	KU587878	*KP860680*	KP860758
L. avicenniae	LAS158	KU587879	KU587946	KU587867
L. bruguierae	CMW41470*	KU587875	*KP860677*	KP860755
L. bruguierae	CMW41614	KU587877	KP860679	KP860757
L. chiangraiensis	MFLUCC 21-0003*	N/A	MW815630	MW815628
L. chiangraiensis	GZCC 21-0003	N/A	MW815629	MW815627
L. chiangraiensis	CDZM 081	OM243836	OM243808	OM228624
L. chonburiensis	MFLUCC 16-0376*	N/A	*MH412773*	MH412742
L. chonburiensis	PSU-LST001	N/A	LC762199	LC762205
L. cinnamomi	CFCC51997*	MH236801	*MH236799*	MH236797
L. citricola	CBS 124707*	KU696351	*GU945340*	KU887505
L. citricola	CBS 124706	KU696350	GU945339	KU887504
L. crassispora	CBS 118741*	KU696353	*DQ103557*	KU887506
L. crassispora (= *L. pyriformis*)	CBS 121770	KU696378	EU101352	KU887527
L. endophytica	MFLUCC 18-1121*	N/A	*MK584572*	MK550606
L. euphorbiaciecola	CMM3609*	N/A	*KF226689*	KF254926
L. euphorbiaciecola	CMW36231	KU696347	KU887063	KU887494
L. gilanensis	IRAN1523C*	KP872462	*GU945342*	KU887511
L. gilanensis	CBS 124705	KU696356	GU945341	KU887510
L. gonubiensis	CBS 115812*	KU696359	*DQ458877*	DQ458860
L. gonubiensis	CMW14078	KU696358	DQ103567	EU673126
L. gravistriata	CMM 4564*	N/A	*KT250950*	N/A
L. hormozganensis	CBS 124709*	KU696361	*GU945343*	KU887515
L. hormozganensis	CBS 124708	KU696360	GU945344	KU887514
L. hormozganensis	MUCC 2724	LC567804	LC567744	LC567774
L. hormozganensis	MUCC 2734	LC567803	LC567743	LC567773
L. hormozganensis	TZS8-1	N/A	OR552338	OR551948
L. hormozganensis	**B2828**	**PP216064**	**PP216045**	**PP378135**
L. hormozganensis	**B2831**	**PQ846691**	**PQ846698**	**PQ846703**
L. hormozganensis	**B2847**	**PQ846692**	**PQ846699**	**PQ846704**
L. hormozganensis	**B2858**	**PQ846693**	**PQ846700**	**PQ846705**
L iraniensis	CBS124710*	KU696363	*GU945336*	KU887516
L. iraniensis	CBS124711	KU696362	GU945335	KU887517
L. iraniensis (= *L. jatrophicola*)	CMW36237	KU696348	KU886998	KU887499
L. iraniensis (= *L. jatrophicola*)	CMW36239	KU696349	KU887000	KU887501
L. lignicola	CBS 134112*	KU696364	*KU887003*	KT852958
L. lignicola	CMW54165	MT920465	MT920442	N/A

Appendix 1. GenBank accession numbers for DNA sequences of *Lasiodiplodia* spp. used in the phylogenetic analyses. (continued)

Species	Isolates[z]	Genbank accession number[y]		
		RPB2	*TEF1-α*	*TUB2*
L. macrospora	CMM 3833*	N/A	*KF226718*	KF254941
L. mahajangana	CMW27801*	KU696365	*FJ900641*	KU887518
L. mahajangana (= *L. exigua*)	CBS137785	KU696355	KJ638336	KU887509
L. margaritacea	CBS122519*	KU696367	*EU144065*	KU887520
L. mediterranea	CBS137783*	KU696368	*KJ638331*	KU887521
L. mediterranea	CBS137784	KU696369	KJ638330	KU887522
L. microcondia	CGMCC 3.18485	KY848561	*KY848614*	N/A
L. parva	CBS 456.78*	KU696372	*EF622063*	KU887523
L. parva	CBS 494.78	KU696373	EF622064	EU673114
L. plurivora	CBS 120832*	KU696374	*EF445395*	KU887524
L. pontae	CMM 1277*	N/A	*KT151791*	KT151797
L. pseudotheobromae	CBS 116459*	KU696376	*EF622057*	EU673111
L. pseudotheobromae	ADN23	**PP396034**	*MN921242*	MT095042
L. pseudotheobromae	AFL8	**PP396035**	*MT086519*	MT095036
L. pseudotheobromae	AZC12	**PP396036**	*MN921241*	MT095041
L. pseudotheobromae	AZC38	**PP396037**	*MN921243*	MT095043
L. pseudotheobromae	**B2852**	N/A	**PV107514**	**PV107500**
L. pseudotheobromae	**B2854**	**PP216065**	**PP216046**	**PP378136**
L. pseudotheobromae	**B2855**	N/A	**PV107515**	**PV107501**
L. pseudotheobromae	**B2857**	N/A	**PV107516**	**PV107502**
L. pseudotheobromae	**B2864**	N/A	**PV107517**	**PV107503**
L. pseudotheobromae	**B2866**	N/A	**PV107518**	**PV107504**
L. pseudotheobromae	**B2871**	N/A	**PV107519**	**PV107505**
L. pseudotheobromae	**B2876**	N/A	**PV107520**	**PV107506**
L. pseudotheobromae	**B2880**	N/A	**PV107521**	**PV107507**
L. pseudotheobromae	**B2885**	PP216066	**PP216047**	**PP378137**
L. pseudotheobromae	**B2888**	N/A	**PV107522**	**PV107508**
L. pseudotheobromae	**B2894**	N/A	**PV107523**	**PV107509**
L. pseudotheobromae	**B2895**	N/A	**PV107524**	**PV107510**
L. pseudotheobromae	**B2942**	N/A	**PV107525**	**PV107511**
L. pseudotheobromae	**B2944**	N/A	**PV107526**	**PV107512**
L. pseudotheobromae	**B2946**	N/A	**PV107527**	**PV107513**
L. rubropurpurea	WAC 12535*	KP872485	*DQ103571*	EU673136
L. rubropurpurea	WAC 12536	KP872486	DQ103572	KU887530
L. subglobosa	CMM3872*	N/A	*KF226721*	KF254942
L. syzygii	MFLUCC 19-0257*	N/A	*MW016943*	MW014331
L. thailandica	**ASS65**	**PP216067**	**PP216048**	**PP378138**
L. thailandica	MUCC2738	LC567810	LC567750	LC567780
L. thailandica	BJFU DZP160119-9	KY676791	KY676797	KY676794

Appendix 1. GenBank accession numbers for DNA sequences of *Lasiodiplodia* spp. used in the phylogenetic analyses. (continued)

Species	Isolates[z]	Genbank accession number[y]		
		RPB2	*TEF1-α*	*TUB2*
L. thailandica	BJFU DZP160123-13	KY751298	KY751304	KY751301
L. thailandica (= *L. hyaline*)	CGMCC 3.17975*	KX499955	*KX499917*	KX499992
L. thailandica (= *L. hyalina*)	CGMCC 3.18383	KY751296	KY751302	KY751299
L. thailandica	**B2844**	**PP216068**	**PP216049**	**PP378139**
L. thailandica	**B2848**	**PP216069**	**PP216050**	**PP378140**
L. thailandica	**B2870**	**PP216070**	**PP216051**	**PP378141**
L. thailandica	**B2913**	**PP216071**	**PP216052**	**PP378142**
L. thailandica	**B2932**	**PP216073**	**PP216054**	**PP378144**
L. thailandica	**B2941**	**PP216074**	**PP216055**	**PP378145**
Lasiodiplodia sp.	**AZP74**	**PP216075**	**PP216056**	**PP378146**
Lasiodiplodia sp.	**B2850**	**PQ846694**	**PQ846701**	**PQ846706**
Lasiodiplodia sp.	**B2881**	**PQ846695**	**PQ846702**	**PQ846707**
Lasiodiplodia sp.	**B2903**	**PP216076**	**PP216057**	**PP378147**
Lasiodiplodia sp.	**B2915**	**PP216077**	**PP216058**	**PP378148**
Lasiodiplodia sp.	**B2917**	**PP216078**	**PP216059**	**PP378149**
Lasiodiplodia sp.	**B2928**	**PP216079**	**PP216060**	**PP378150**
Lasiodiplodia sp.	**B2930**	**PP216080**	**PP216061**	**PP378151**
L. theobromae	ACY4	**PP396038**	*MN921239*	MT095039
L. theobromae	ADN21	**PP396039**	*MN921237*	MT095037
L. theobromae	AFL1	**PP396040**	*MT086516*	MT095033
L. theobromae	AZC42	**PP396041**	*MN921240*	MT095040
L. theobromae	AZP58	**PP396042**	*MT086514*	MT095031
L. theobromae	AZP69	**PP396043**	*MT086515*	MT095032
L. theobromae	**B2825**	N/A	**PV167144**	**PV134345**
L. theobromae	**B2827**	N/A	**PV167145**	**PV134346**
L. theobromae	**B2843**	N/A	**PV167146**	**PV134347**
L. theobromae	**B2849**	N/A	**PV167147**	**PV134348**
L. theobromae	**B2862**	N/A	**PV167148**	**PV134349**
L. theobromae	**B2863**	N/A	**PV167149**	**PV134350**
L. theobromae	**B2865**	N/A	**PV167150**	**PV134351**
L. theobromae	**B2873**	N/A	**PV167151**	**PV134352**
L. theobromae	**B2875**	N/A	**PV167152**	**PV134353**
L. theobromae	**B2877**	N/A	**PV167153**	**PV134354**
L. theobromae	**B2886**	N/A	**PV167154**	**PV134355**
L. theobromae	**B2887**	N/A	**PV167155**	**PV134356**
L. theobromae	**B2891**	**PP216081**	**PP216062**	**PP378152**
L. theobromae	**B2900**	**PP216082**	**PP216063**	**PP378153**
L. theobromae	**B2926**	N/A	**PV167156**	**PV134357**
L. theobromae	**B2937**	N/A	**PV167157**	**PV134358**

Appendix 1. GenBank accession numbers for DNA sequences of *Lasiodiplodia* spp. used in the phylogenetic analyses. (continued)

Species	Isolates[z]	Genbank accession number[y]		
		RPB2	*TEF1-α*	*TUB2*
L. theobromae	**B2940**	N/A	**PV167158**	**PV134359**
L. theobromae	**B2943**	N/A	**PV167159**	**PV134360**
L. theobromae	**B2953**	N/A	**PV167160**	**PV134361**
L. theobromae	CBS 164.96*	KU696383	*AY640258*	KU887532
L. theobromae	CBS 111530	KU696382	*EF622054*	KU887531
L. tropica	CGMCC 3.18477*	KY848574	*KY848616*	KY848540
L. venezuelensis	CBS 118739*	KU696384	*DQ103568*	KU887533
L. viticola	CBS 128313*	KU696385	*HQ288269*	HQ288306
L. vitis	CBS 124060*	KX463994	KX464642	KX464917
Diplodia seriata	CBS 112555	KX463962	AY573220	DQ458856

[z] Isolates in bold were obtained in this study; isolates with asterisks are ex-type cultures.

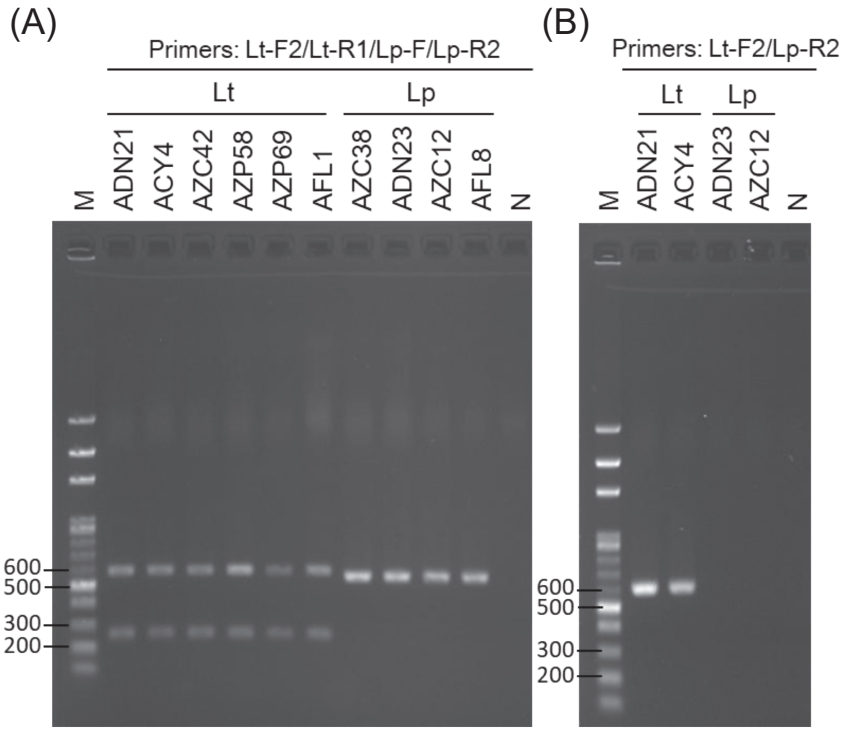
[y] ITS: internal transcribed spacer regions 1 and 2, including the 5.8S ribosomal RNA gene; *RPB2*: RNA polymerase II largest subunit; *TEF1-α*: translation elongation factor 1-α; *TUB2*: β-tubulin; accession numbers in bold indicate sequences obtained in this study; accession numbers in italics indicate sequences used for the design of primers.

Appendix 2. The isolates of *Lasiodiplodia* spp. used in the tests of species-specific primers.

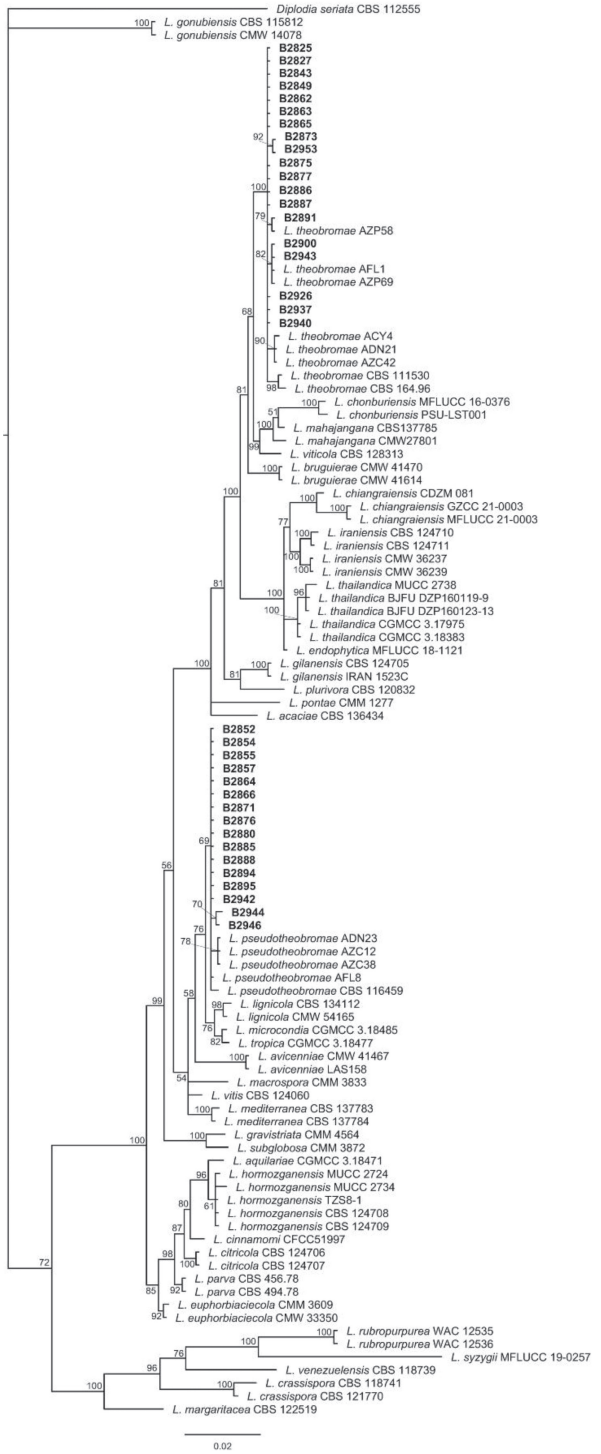
Species	Isolate	Tissue type	Location	Collection date
L. pseudotheobromae	ADN23	branch	Danei, Tainan	2019/1/22
	AFL8	branch	Fanlu, Chiayi	2019/9/30
	AZC12	branch	Zhuqi, Chiayi	2018/9/21
	AZC38	branch	Zhuqi, Chiayi	2019/4/2
	B2852	fruit	Shanshang, Tainan	2020/8/13
	B2854	fruit	Shanshang, Tainan	2020/8/18
	B2855	fruit	Zhuqi, Chiayi	2020/8/24
	B2857	fruit	Zhuqi, Chiayi	2020/8/24
	B2864	fruit	Zhuqi, Chiayi	2020/8/24
	B2866	fruit	Shanshang, Tainan	2020/8/18
	B2871	fruit	Zhuqi, Chiayi	2020/8/24
	B2876	fruit	Zhuqi, Chiayi	2020/8/24
	B2880	fruit	Zhuqi, Chiayi	2020/8/24
	B2885	fruit	Zhuqi, Chiayi	2020/8/24
	B2888	fruit	Shanshang, Tainan	2020/8/18
	B2894	fruit	Zhuqi, Chiayi	2020/8/24
	B2895	fruit	Zhuqi, Chiayi	2020/8/24
	B2942	fruit	Zhuqi, Chiayi	2020/12/3
	B2944	fruit	Zhuqi, Chiayi	2020/12/3
	B2946	fruit	Zhuqi, Chiayi	2020/12/3
L. theobromae	ADN21	branch	Danei, Tainan	2019/1/22
	ACY4	branch	Chiayi City	2019/5/10
	AFL1	branch	Fanlu, Chiayi	2019/9/30
	AZC42	branch	Zhuqi, Chiayi	2019/4/2
	AZP58	branch	Zhongpu, Chiayi	2019/11/1
	AZP69	branch	Zhongpu, Chiayi	2019/11/27
	B2825	fruit	Zhuqi, Chiayi	2020/7/21
	B2827	fruit	Zhuqi, Chiayi	2020/7/31
	B2843	fruit	Zhuqi, Chiayi	2020/8/10
	B2849	fruit	Zhuqi, Chiayi	2020/8/10
	B2862	fruit	Zhuqi, Chiayi	2020/8/24
	B2863	fruit	Shanshang, Tainan	2020/8/21
	B2865	fruit	Zhuqi, Chiayi	2020/8/24
	B2873	fruit	Zhuqi, Chiayi	2020/8/24
	B2875	fruit	Zhuqi, Chiayi	2020/8/24
	B2877	fruit	Zhuqi, Chiayi	2020/8/24
	B2886	fruit	Zhuqi, Chiayi	2020/8/24
	B2887	fruit	Zhuqi, Chiayi	2020/8/24
	B2891	fruit	Shanshang, Tainan	2020/8/23
	B2900	fruit	Zhuqi, Chiayi	2020/8/24
	B2926	fruit	Zhuqi, Chiayi	2020/11/12

Appendix 2. The isolates of *Lasiodiplodia* spp. used in the tests of species-specific primers. (continued)

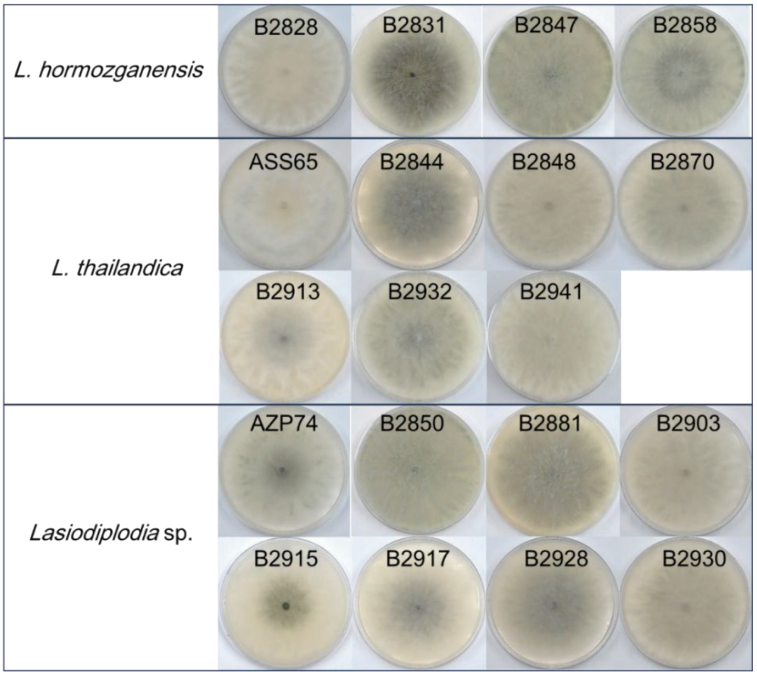
Species	Isolate	Tissue type	Location	Collection date
	B2937	fruit	Zhuqi, Chiayi	2020/12/3
	B2940	fruit	Zhuqi, Chiayi	2020/12/3
	B2943	fruit	Zhuqi, Chiayi	2020/12/3
	B2953	fruit	Zhuqi, Chiayi	2020/12/3
L. thailandica	B2844	fruit	Zhuqi, Chiayi	2020/8/17
	B2848	fruit	Shanshang, Tainan	2020/8/14
	B2870	pedicel	Zhuqi, Chiayi	2020/8/31
	B2913	fruit	Zhuqi, Chiayi	2020/11/12
	B2932	pedicel	Zhuqi, Chiayi	2020/12/1
	B2941	fruit	Zhuqi, Chiayi	2020/11/30
	ASS65	branch	Shanshang, Tainan	2020/9/9
L. hormozganensis	B2828	fruit	Zhuqi, Chiayi	2020/8/7
	B2831	fruit	Zhuqi, Chiayi	2020/7/31
	B2847	fruit	Zhuqi, Chiayi	2020/8/10
	B2858	fruit	Zhuqi, Chiayi	2020/8/24
Lasiodiplodia sp.	AZP74	branch	Zhongpu, Chiayi	2019/12/19
	B2850	fruit	Shanshang, Tainan	2020/8/7
	B2881	fruit	Zhuqi, Chiayi	2020/8/24
	B2903	fruit	Zhuqi, Chiayi	2020/8/24
	B2915	fruit	Zhuqi, Chiayi	2020/11/12
	B2917	fruit	Zhuqi, Chiayi	2020/11/12
	B2928	pedicel	Zhuqi, Chiayi	2020/12/1
	B2930	pedicel	Zhuqi, Chiayi	2020/12/1



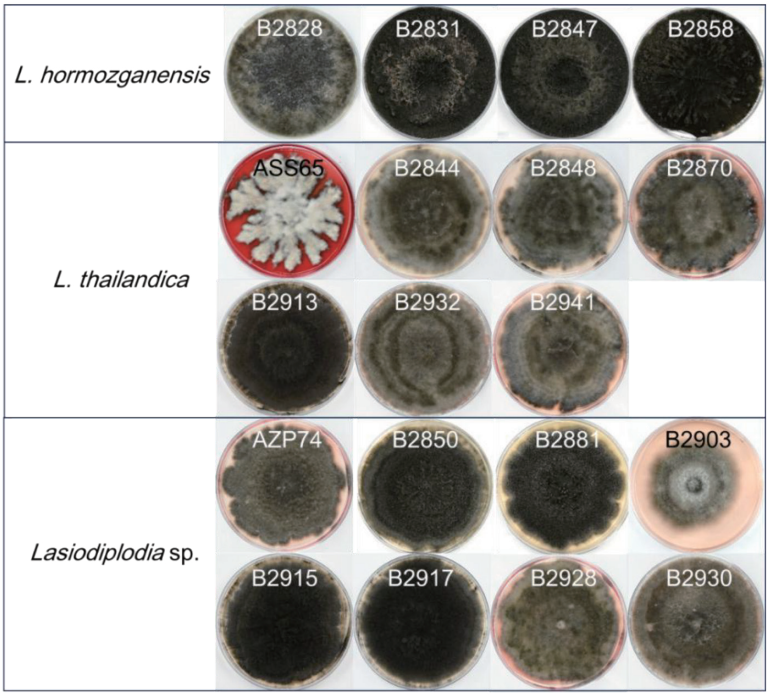
Appendix 3. The results of multiplex PCR with the two species-specific primers pairs (A) Lt-F2/Lt-R1 and Lp-F/Lp-R2 and PCR with (B) Lt-F2/Lp-R2. M: 100-bp DNA ladder (Protech Technology Enterprise, Taipei, Taiwan). N: negative control.



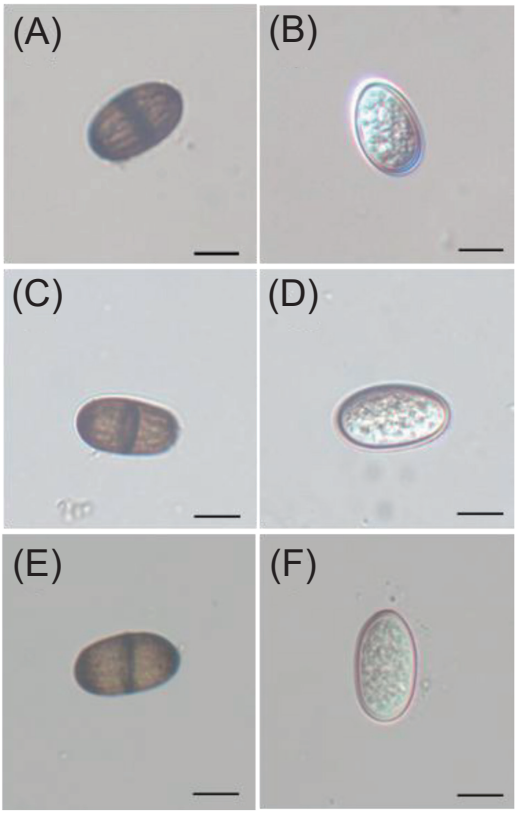
Appendix 4. Bayesian phylogenetic tree of 108 *Lasiodiplodia* isolates from avocado in Taiwan and from the GenBank database. The phylogenetic tree was built using concatenated sequences of partial *TEF1-α* and *TUB2* gene regions. The Bayesian posterior probabilities are indicated next to the nodes. The tree is rooted with *Diplodia seriata* CBS 112555. Isolates from this study are emphasized in bold.



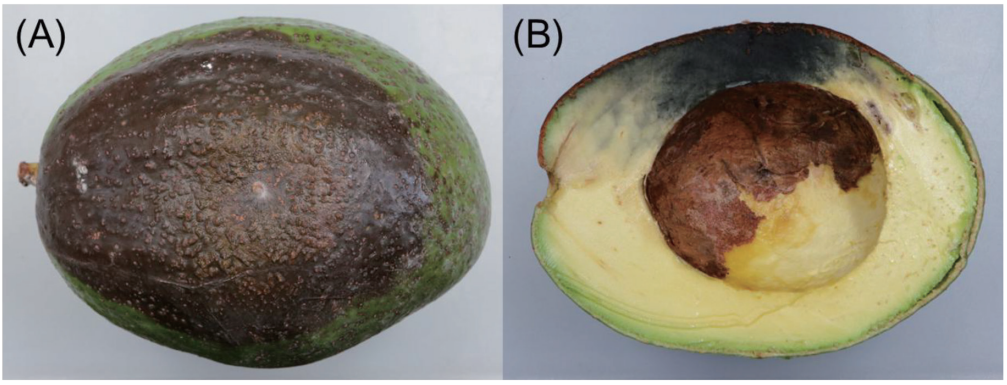
Appendix 5. Colony morphology of the isolates of *Lasiodiplodia hormozganensis*, *L. thailandica*, and unidentified *Lasiodiplodia* isolates cultivated on potato dextrose agar after 3 d at 25℃.



Appendix 6. Colony morphology of the isolates of *Lasiodiplodia hormozganensis*, *L. thailandica*, and unidentified *Lasiodiplodia* isolates cultivated on potato dextrose agar after 14 d at 35℃.



Appendix 7. Conidial morphology of *Lasiodiplodia hormozganensis*, *L. thailandica*, and an unidentified *Lasiodiplodia* isolate. (A) Mature conidia and (B) immature conidium of *L. hormozganensis* B2828. (C) Mature conidium and (D) immature conidium of *L. thailandica* B2848. (E) Mature conidium and (F) immature conidium of *Lasiodiplodia* sp. B2903. Scale bars: 10 µm.



Appendix 8. (A) External lesions and (B) internal discoloration that developed 7 d after artificial inoculation with *Lasiodiplodia thailandica* B2848 on 'Choquette' avocado fruits.

臺灣酪梨上之 *Lasiodiplodia* 屬病原菌之新穎分子鑑定技術

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摘要

梁鈺平、倪蕙芳。2025。臺灣酪梨上之 *Lasiodiplodia* 屬病原菌之新穎分子鑑定技術。台灣農業研究 74(4):459–484。

Lasiodiplodia theobromae 與 *L. pseudotheobromae* 為廣泛分布於熱帶與亞熱帶之植物病原菌，造成許多作物病害，但兩者不易透過傳統型態學方法鑑定與區分。本研究根據 *Lasiodiplodia* 屬物種間之轉譯延長因子 1-α (translational elongation factor 1-α; *TEF1-α*) 序列之差異性，開發了 2 對物種專一性引子對 (Lt-F2/Lt-R1 與 Lp-F/Lp-R2)。以基因體 DNA 進行聚合酶連鎖反應，結果顯示 Lt-F2/Lt-R1 與 Lp-F/Lp-R2 分別對 *L. theobromae* 與 *L. pseudotheobromae* 具有專一性，對於非標的物種則均無擴增反應。使用此二引子增幅分離自臺灣酪梨 (*Persea americana*) 的 54 株 *Lasiodiplodia* 菌株之 DNA，成功自其中鑑定出屬於 *L. theobromae* 與 *L. pseudotheobromae* 的菌株，然有些菌株則無法被以上二對引子增幅，顯示臺灣酪梨上存在其他未知的 *Lasiodiplodia* spp. 病原。進一步以 *TEF1-α*、β 微管蛋白 2 (β-tubulin 2; *TUB2*) 及 RNA 聚合酶 II (RNA polymerase subunit II; *RPB2*) 之基因序列進行以上未知菌株之親緣分析，結果顯示另有 *L. thailandica*、*L. hormozganensis* 及目前尚無法以此親緣關係鑑定的 *Lasiodiplodia* 物種存在於臺灣之罹病酪梨果實、枝條及果梗，經病原性試驗後顯示以上三者對酪梨果實均有病原性。本研究首次報導 *L. thailandica* 為酪梨之病原菌，且本研究開發之專一性引子將可於未來作為鑑別常見病原 *L. theobromae* 與 *L. pseudotheobromae* 的有效工具，有助於研擬正確防治策略。

關鍵詞：*Lasiodiplodia hormozganensis*、*Lasiodiplodia thailandica*、*Lasiodiplodia theobromae*、*Lasiodiplodia pseudotheobromae*、專一性引子。

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