# Ph. D. Thesis

# Taxonomical studies on Botryosphaeriales in Japan
# （日本産ボトリオスフェリア目菌類の分類学的研究）

**Graduate school of Bioresources**
**Mie University**

**Yukako Hattori**
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# Chapter 1

# General introduction

## Background information and overview of the plant diseases caused by Botryosphaeriales

Fungi in the order Botryosphaeriales include pathogens that parasitize the leaves, fruits, and branches of plants and cause various diseases (Crous et al. 2006; Liu et al. 2012; Phillips et al. 2013, 2019). In particular, some taxa cause fruit rot, leaf blight, and branch blight in useful trees, such as fruit trees and trees that are used for urban greening and afforestation, and consequently have an economic impact globally (Slippers & Wingfield 2007). Among the Botryosphaeriales, *Botryosphaeria dothidea* has been reported in 1085 records from 62 countries, *Lasiodiplodia theobromae* in 844 records from 68 countries, *Neofusicoccum parvum* in 709 records from 43 countries (Farr & Rossman 2020), and several other species that are important plant pathogens have global distributions.

It has also been reported that some Botryosphaeriales are opportunistic pathogens that only causes disease symptoms when the host plants are exposed to stress conditions, while others are endophytic fungi that do not cause any disease symptoms (Slippers & Wingfield 2007). These reports suggest that the importance of these fungi may increase in situations where plant communities are under pressure from global environmental changes due to climate change or anthropogenic influences (Desprez-Loustau et al. 2007; Sturrock et al. 2011; Slippers et al. 2017).

Since the sticky spores of Botryosphaeriales are thought to be dispersed mainly by wind and rain or via insects (Van Niekerk et al. 2010; Mehl et al. 2013; Moyo et al. 2014;

Valencia et al. 2015), natural dispersal is expected to be relatively localized (Slippers et al. 2017). However, these fungi that are known as pathogen are currently spreading around the world with little restriction and are clearly increasing in frequency, likely as a result of globalization and the widespread movement of live plants and fresh plant products (Sakalidis et al. 2013; Crous et al. 2016; Burgess et al. 2017; Marsberg et al. 2017; Slippers et al. 2017). Therefore, there is a need to accurately identify these fungi and to gain a clear understanding of the diversity of this group to enable compliance with quarantine regulations and to apply effective control strategies against diseases (Slippers et al. 2017).

A number of tree diseases have been reported to be associated with Botryosphaeriales in Japan, including branch blight of *Cryptomeria japonica* and *Chamaecyparis obtusa* (Sawada 1950a; 1950b; Kobayashi 1962), shoot blight of larch (*Larix* spp.) trees (Sawada 1950b; Uozumi 1961), leaf spot of maple (*Acer pycnanthum*) trees (Yano and Motohashi 2016), and fruit rot of mango (*Mangifera indica*) (Takushi et al. 2017; Hattori et al. 2019). However, most of the Japanese species of Botryosphaeriales have been described based solely on their morphological characteristics, with their molecular phylogenetic position remaining unknown until now.

## Taxonomy of Botryosphaeriales – a brief history and issues

The family Botryosphaeriaceae was established in 1918 to accommodate the genera *Botryosphaeria*, *Dibotryon*, and *Phaeobotryon* (Theissen & Sydow 1918), and it was argued that its higher taxonomic group should be the order Pleosporales or Dothideales (Phillips et al. 2013, 2019). However, the development of molecular phylogenetic analysis techniques using DNA sequences revealed that the genera *Botryosphaeria* and *Guignardia* (Botryosphaeriaceae) were poorly related to the known orders in the class Dothideomycetes, so Botryosphaeriales C.L. Schoch, Crous & Shoemaker was established in 2006 as an order that included Botryosphaeriaceae as a single family

(Schoch et al. 2006; Phillips et al. 2019). In 2012, Planistromellaceae E. Barrha was transferred to this order (Minnis et al. 2012), and in 2013, Phyllostictaceae Fr., Aplosporellaceae Slippers, Boissin & Crous, Melanopsaceae A.J.L. Phillips, Slippers, Boissin & Crous, and Saccharataceae Slippers, Boissin & Crous became independent from Botryosphaeriaceae (Slippers et al. 2013; Wikee et al. 2013). Then, in 2016, Septorioideaceae Wyka & Broders was added (Wyka & Broders 2016), and in 2017, Endomelanopsisaceae Tao Yang & Crous and Pseudofusicoccumaceae Tao Yang & Crous were added into based on molecular phylogenetic analysis using the large subunit of the nuclear ribosomal RNA gene (LSU)+*rpb2* region (Yang et al. 2017), bringing the number of families to nine (Wijayawardene et al. 2018; Phillips et al. 2019). However, the phylogenetic relationships of Botryosphaeriales have previously been discussed based on phylogenetic analyses using different loci that are not unified and, in some cases, incomplete datasets (Phillips et al. 2019). Therefore, Phillips et al. (2019) reexamined this order by conducting a phylogenetic analysis using the internal transcribed spacer (ITS) + LSU dataset and by examining the morphological characteristics of sexual generations, the results of which showed that Botryosphaeriales is composed of six families (Aplosporellaceae, Botryosphaeriaceae, Melanopsaceae, Phyllostictaceae, Planistromellaceae, and Saccharataceae), with the remaining three families (Endomelanconiopsisaceae, Pseudofusicoccumaceae, and Septorioideaceae) being synonyms of other existing families.

Botryosphaeriaceae represents Botryosphaeriales and is the largest family within this order, containing 23 genera to date (Slippers et al. 2017). The family Phyllostictaceae is the sister group to Botryosphaeriaceae in the ITS+LSU maximum likelihood phylogenetic tree and consists of two genera, *Phyllosticta* and *Pseudofusicoccum*. As mentioned above, the phylogenetic relationships among the families and genera in Botryosphaeriales have now been clarified and are currently being organized. However, the definitions of species and phylogenetic relationships within each genus are still under debate, although new species descriptions have recently been made for genera within the

Botryosphaeriaceae globally following a review by Yang et al. (2017).

In Japan, at least 101 species of fungi in the family Botryosphaeriaceae belong to the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Fusicoccum*, *Lasiodiplodia*, *Macrophoma*, *Macrophomina*, *Sphaeropsis*, and *Tiarosporella* (Katsumoto 2007). However, until recently, the description and identification of species in *Botryosphaeria* and related genera were based on the morphological characters of Pennycook & Samuels (1985) and Crous & Palm (1999), causing much confusion in their classification as they are considered to have similar morphological characteristics. Furthermore, in a reexamination of the genus *Phyllosticta* from Japan, Motohashi et al. (2009) found that only 46/224 species belonged to this narrowly defined genus while the rest needed to be transferred to other genera. Therefore, there is an urgent need to review the Japanese Botryosphaeriales based on new criteria that reflect not only morphorogical characteristics but also molecular phylogenetic relationships, cultural characteristics, and pathogenicity.

## Objectives and outline of this thesis

In this study, the taxonomy of Botryosphaeriales in Japan was reexamined to elucidate the species diversity in Japan focusing on the families Botryosphaeriaceae and Phyllostictaceae and using new classification criteria to elucidate the Japanese mycoflora.

**Chapter 2** focuses on fungi in the genus *Phyllosticta*, which are parasitic on conifer trees (Cupressaceae) in Japan. The Japanese isolates were reexamined taxonomically based on their morphological characteristics, culture characteristics, molecular phylogenetic relationships, and pathogenicities, which resulted in them being divided into five clades: *P. cryptomeriae*, *P. hostae*, *P. spinarum*, *P. pilospora*, and *P. capitalensis*. Among these, *P. hostae* and *P. spinarum* were new additions to the Japanese mycoflora. In addition, new combinations of *P. pilospora* and its epitype and the *P. cryptomeriae* epitype were proposed. Inoculation tests confirmed the pathogenicity of *P. spinarum* to

*Thujopsis dolabrata.*

**Chapter 3** considers the Japanese species in the genus *Lasiodiplodia*. Thirty Japanese strains of *Lasiodiplodia* were reclassified based on their morphological characteristics, culture characteristics, and molecular phylogenetic analysis using multilocus regions, which resulted in them being divided into 11 clades that corresponded to *L. theobromae*, *L. brasiliensis*, *L. swieteniae*, *L. pseudotheobromae*, and *Lasiodiplodia* spp. Among these, *L. brasiliensis* and *L. swieteniae* were new additions to the Japanese mycoflora. In addition, *L. latispora*, *L. parvispora*, *L. ryukyuensis*, and *L. yaguchii* were proposed as new species.

**Chapter 4** examines the genus *Botryosphaeria* in Japan. The taxonomy of 23 Japanese strains was reexamined based on their morphological characteristics, culture characteristics, and molecular phylogenetic positions, which resulted in them being divided into five clades that corresponded to *B. dothidea*, *B. sinensis*, *B. qinguanensis*, and *Botryosphaeria* spp. Of these, *B. qinsuanensis* was a new addition to the Japanese mycoflora. In addition, *B. tenuispora* was proposed as a new species.

**Chapter 5** focuses on the genus *Neofusicoccum* in Japan. A total of 14 conserved strains from Japan that were identified as Botryosphaeriaceae based on their morphological characteristics were used for molecular phylogenetic analysis using a multilocus region and their morphological characteristics, which resulted in them being divided into five clades. Three new species were proposed (*N. hypelici*, *N. miyakoensis*, and *N. okinawaense*) and *Physalospora laricina*, which is the causative agent of shoot blight fungus in larch trees (*Larix* spp.), was transferred to the genus *Neofusicoccum* and an epitype was established.

Finally, **Chapter 6** discusses the reexamined Botryospheriales fungi from Japan. 26 species of Japanese Botryosphaeriales which were confirmed in this study suggested the high diversity of tree-parasitic Botryosphaeriales in Japan. On the other hand, since the taxonomic species criteria that are used for Botryospheriales globally are still unstable, it is necessary to search for genetic regions and phenotypes that are better indicators of

phylogenetic relationships.

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## Chapter 2

# Taxonomical re-examination of the genus *Phyllosticta* – parasitic fungi on Cupressaceae trees in Japan


## Summary

Taxonomical re-examination of the *Phyllosticta* species parasitic fungi on coniferous trees (Cupressaceae) in Japan was conducted based on current criteria, such as morphological and cultural characteristics, phylogenetic relationship, and pathogenicity. Phylogenetic analyses revealed several clades composed of plant pathogens isolated from a specific host as well as clades composed of endophytic species isolated from various Cupressaceae trees. Each clade was recognized as a species from the morphological characteristics and other features, respectively. Five species of *Phyllosticta* sensu stricto were recognized, and two of them were newly recorded in Japanese mycoflora. Furthermore, new comibination *P. pilospora* and its epitype are proposed. The epitype and ex-type strain are also proposed for *P. cryptomeriae* in this study.



**Keywords:** *Phyllosticta*, Cupressaceae, Japan, taxonomy, pathogenicity

## Introduction

In 1818, Persoon established the fungal genus *Phyllosticta* sensu stricto and the teleomorph *Guignardia* (Botryosphaeriaceae) (Van der Aa, 1973). *Phyllosticta* species are distributed worldwide and are known as endophytic or plant pathogenic fungi causing leaf spots, leaf blight, or fruit spots (van der Aa & Vanev, 2002, Motohashi, 2008b, Wikee et al., 2011, 2013a). Diseases of fruits and useful trees caused by these species are reported from all over the world; Leaf spot of *Cornus kousa* (Cornaceae) is caused by *Phyllosticta* sp. (Sinclair and Lyon, 2006), Leaf spot of *Cinnamomum burmannii* (Lauraceae) is caused by *P. cirsii* (Yuelian and Fengqiu, 2014), *Guignardia* blotch of horse-chestnut and buckeye (Sapindaceae) is caused by *Guignardia aesculi* (Sinclair and lyon, 2006), citrus black spot disease of citrus (Rutaceae) is caused by *P. citricarpa* (Baayen et al., 2002, Glienke et al., 2011, Guarnaccia et al., 2017, Wikee et al., 2013a), black rot of grapevine (Vitaceae) is caused by *P. ampelicida* complex (Van der Aa & Vanev, 2002, Wicht et al., 2012, Zhou et al., 2015), and *Phyllosticta* leaf spot of *Acer saccharum* and *A. rubrum* (Sapindaceae) is caused by *P. minima* (Anderson, 1919, Conners, 1967, Bissett & Darbvshire, 1984). In Japan, *P. minima* is known as a pathogen that causes leaf spot in *A. pycnanthum* and *A. maximowiczianum* (Motohashi et al., 2013, Nakashima et al., 2015, Yano & Motohashi, 2016). Likewise, *P. hamamelidis,* which is a leaf blight pathogen, caused a devastating decline in the natural habitat of *Hamamelis japonica* (Hamamelidaceae) (Motohashi et al., 2008b, 2008c). From the results of the inoculation tests in previous studies (Stewart, 1916, Luttrell, 1946, 1948), *Phyllosticta* species generally exhibit host specificity, as each species has only one host genus or family. Conversely, endophytic species, such as *P. capitalensis*, have a broad range of hosts (Wikee et al., 2011, 2013b). These species inhabit various host plant leaves without symptoms, and it behaves as the endophyte throughout their life cycles or the saprophyte. *Phyllosticta capitalensis* has been reported as an endophyte from 66 plant species of 54 genera, belonging to 38 families (Okane et al., 2001, 2003). Van der Aa (1973) redefined the genus concept of *Phyllosticta* based on the morphological characteristics of the

host plants and the host specificity expected. This concept was widely accepted among plant pathologists and mycologists and is known as sensu stricto (s. str.), which is a narrower species concept. Eventually, 2,936 *Phyllosticta* species were re-examined, and the types of specimens were observed. Moreover, it was reported that only 143 species were recognized as *Phyllosticta* s. str. (Van der Aa & Vanev, 2002). In recent years, both of morphological characters and molecular phylogenetic relationships using multi-locus sequences was introduced into the taxonomy of the genus *Phyllosticta*. The comprehensive studies of this genus using multi loci have contributed to solving cryptic species problems and discovering new taxa (Wikee et al., 2011). Each phytopathogenic species and endophytic species formed independent clades on the phylogenetic trees, which exhibited differences in cultural characteristics and growth rates on agar media (Motohashi et al., 2008a, Motohashi, 2011). Motohashi et al. (2008a) and Motohashi (2011) re-described 46 species as *Phyllosticta* s. str. from 224 hitherto known Japanese species, based on the morphological and cultural characteristics, and their phylogenetic relationship. The total forest area of Japan is 25.5 million ha, about 40 % of which (10.2 million ha) is plantations. The major tree species in planted forests are *Cryptomeria japonica* (44%), *Chamaecyparis obtusa* (25%), *Larix kaempferi* (10%), *Pinus species* (8%), *Abies sachalinensis* (8%), and species of broadleaf trees (3%) (Forestry Agency 2020). In spite of the economic and ecological importance of Cupressaceae trees, there is no update of the diseases caused by *Phyllosticta* from previous studies by Kawamura (1913) and Sawada (1950) in Japan. We have identified several species of the genus *Phyllosticta* that are parasitic on Cupressaceae (Hattori et al. 2016). However, the taxonomic position of these species on Cupressaceae plants (such as *P. cryptomeriae*, *P. capitalensis*, and *P. thujopsidis*) in Japan has not yet been elucidated. In this study, *Phyllosticta* species that are parasitic on Cupressaceae were investigated based on morphological and cultural characteristics, molecular phylogeny, and pathogenicity in an effort to reveal species diversity and speciation.

## Materials and Methods

### Sample Collections

Between May 2015 and February 2018, Symptomatic or asymptomatic leaves were collected from seven cites (Nagoya (Aichi); Hirosaki, Aomori (Aomori); Kawasaki, Sagamihara (Kanagawa), Chofu, Setagaya (Tokyo)) in Japan. The leaves of the genus *Chamaecyparis*, *Cryptomeria*, *Thuja*, and *Thujopsis*, which showed symptoms such as leaf blight and shoot blight, were collected. Healthy leaves of Cupressaceae trees were collected. *Phyllosticta* spp. were isolated from symptomatic samples by single conidia, which were germinated on modified cedar decoction medium (Motohashi et al., 2008a). All isolates were cultured at room temperature (22 °C) for 5–7 days. The tip of each hypha was transferred onto oatmeal agar (OMA) (Crous et al., 2019) and was used for experiments in this research. *Phyllosticta* spp. were isolated from asymptomatic leaves using the next surface sterilization method. Leaves (5 mm) were cut into pieces and immersed in 70% ethanol for 30 s, in 1% hypochlorous acid for 30 s, in 70% ethanol for 30 s, and in sterilized water for 60 s. These sterilized pieces were placed on 2% water agar. Following culture at room temperature (22 ℃) for 1 week, only a tip of hyphae growing from the leaf piece was transferred by a flame-sterilized needle under the microscope onto OMA plates. The specimens were stored in the Mycological Herbarium, at Mie University (TSU-MUMH), and the established isolates were maintained in the Culture Collection of MUMH (MUCC). Several types of specimens were borrowed from the Iwate University Museum (IUM), Iwate, Japan, for the comparative studies.

### Molecular and phylogenetic analysis

Genomic DNA was extracted from mycelial disks following 14 days of culturing on OA plates with Prepman®Ultra (Applied Biosystems, Massachusetts, USA) according to the manufacturer's instructions. Targeted sequences of the internal transcribed spacer (ITS), large subunit ribosomal ribonucleic acid (LSU rRNA), translation elongation factor 1-alpha

(tef1-α), and actin (act) gene regions were amplified using a TP600 Thermal Cycler (Takara Bio, Shiga, Japan) in a total volume of 12.5 μL. The PCR mixtures consisted of 1–10 ng of genomic DNA, 0.063 μL of 5 Unit Ex Taq DNA Polymerase (Takara Bio), 1.25 μL of 10× Ex Taq buffer (Takara Bio), 1 μL of 2.5 mM for dNTP mixture (Takara Bio), 0.625 μL of each primer, and 8.937 μL of sterilized distilled water. The PCR conditions and primer sets are presented in Table 2–1. The amplicon was cleaned using the GenEluteTM PCR Clean-Up Kit (Sigma-Aldrich®, St. Louis, USA) according to the manufacturer's instructions. These were sequenced by Macrogen Japan (Kyoto, Japan) using the same primers. The sequences were assembled and aligned with 47 sequences obtained from the DDBJ GenBank (Table 2–2) and with MAFFT version 7 (Katoh et al., 2019). Maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) analyses were applied to estimate the phylogenetic relationships. MP analyses were conducted using PAUP* v. 4.0 b10 (Swofford, 2002). All characters were unordered and of equal weight, and gaps were treated as missing data. The heuristic search option with 1,000 random taxon additions and TBR used as the branch-swapping algorithm were applied. The strength of the internal branches from the resultant trees was tested by bootstrap analysis (Felsenstein, 1985) using 1000 replications in both ML and MP analyses. Tree scores, including tree length (TL), consistency index (CI), retention index (RI), and a rescaled consistency index (RC), were also calculated. ML analyses were performed with raxmlHPC-PTHREADS (Stamatakis 2006). BI analyses were conducted using MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003) and used to estimate the posterior probabilities (PPs) of the tree topologies based on the Metropolis-coupled Markov chain Monte Carlo (MC3) searches which used an MCMC algorithm consisting of four chains that were parallel, derived from a random tree topology. The MCMC analysis lasted 1,000,000 generations. Trees were sampled and saved every 500 generations. The first 25% of the saved trees were discarded, and the "burn-in" phase and PPs were determined from the remaining trees. Kakusan4 (Tanabe, 2011) was used to determine the best nucleotide substitution model settings for each data partition in order to perform an optimized model. Representative sequences for all taxa were uploaded to DDBJ (Table 2–2).

The alignments were also deposited in TreeBASE (www.treebase.org, submission no.: S26199).

### Pathogenicity

Cultures of *Phyllosticta spinarum* (MUCC 2914) isolated from *Thujopsis dolabrata* var. *hondae*, *P. pilospora* (MUCC 2912) from *Chamaecyparis pisifera* '*Plumosa Aurea*', and *P. hostae* (MUCC 2934) and *P. capitalensis* (MUCC 2926) from the intact shoot of *T. dolabrata* were cultivated on OMA plates for 2 weeks and were used for the inoculation tests. The formed conidiomata and conidia on colonies were scraped with a flame-sterilized needle into sterile water. The conidial suspensions were filtered through sterilized gauze and adjusted to 105 spores/ml for each isolate using a hemocytometer. Healthy stocks of T. dolabrata var. hondae, with a height of about 30 cm, were also used for inoculation tests. The stocks of the wounded (with a knife) and woundless plots were sprayed with a conidia suspension. Control plots were inoculated with sterile water. Following inoculation, all trees were covered in plastic bags and kept in humid conditions for 3 days.

## Results

### Phylogeny

The alignment consisted of 69 strains with a total of 1612 characters (ITS: 479, LSU: 553, tef1-α: 321, act: 259), including alignment gaps. A total of 361 characters were phylogenetically informative for parsimony analysis. Out-group taxon for phylogenetic tree was *Melanops tulasnei* (CBS 116805). The MP analyses generated 160 equally parsimonious trees with 908 steps. An MP tree was selected from the equally parsimonious trees based on the results of the Kishino–Hasegawa (KH) test (TL = 1753, CI = 0.486, RI = 0.74, RC = 0.36) (Kishino & Hasegawa, 1989). The topologies of the resultant trees from MP, ML, and BI analyses were congruent, and Fig. 2–1 presents the ML tree. The results of the phylogenetic

Table 2–1. The PCR conditions and primer sets.

| Region | primer F | primer R | PCR condition |
|---|---|---|---|
| ITS | V9G (de Hoog & Gerrits van den Ende, 1998) | ITS4 (White et al., 1990) | Glienke et al. (2011) |
| LSU | LR5 (Vilgalys & Hester, 1990) | LR0R (Vilgalys & Hester, 1990) | Glienke et al. (2011) |
| *tef1-α* | EF1-728F (Carbone & Kohn, 1999) | EF2 (O'Donnell et al., 1998) | Glienke et al. (2011) |
| *act* | ACT512F (Carbone & Kohn, 1999) | ACT783R (Carbone & Kohn, 1999) | 94℃ 4min |
| | | | 94℃ 45sec |
| | | | 50℃ 30sec 40 cyc |
| | | | 72℃ 90sec |
| | | | 72℃ 2min |

Table 2–2. The list of the genus *Phyllosticta* used for phylogenetic analysis.

| **Fungal species** | **Accession No.** | **Host species** | **Host family** | **Country** | **Accession numbers** | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | **ITS** | **28S** | **tef1** | **act** |
| *Melanops tulasnei* | CBS 116805 | *Quercus* sp. | Fagaceae | Germany | FJ824769 | KF766365 | KF766423 | - |
| *Guignardia mangiferae* | IMI 260576$^{T}$ | *Mangifera indica* | Anacardiaceae | India | JF261459 | KF206222 | JF261501 | JF343641 |
| *G. rhodorae* | CBS 901.69 | *Rhododendron* sp. | Ericaceae | Netherlands | KF206174 | KF206292 | KF289230 | KF289256 |
| *P. aloeicola* | CBS 136058$^{T}$ | *Aloe ferox* | Asphodelaceae | South Africa | KF154280 | KF206214 | KF289193 | KF289311 |
| *P. beaumarisii* | CBS 535.87$^{T}$ | *Muehlenbekia adpressa* | Polygonaceae | Australia | AY042927 | KF306229 | KF289170 | KF306232 |
| *P. bifrenariae* | CBS 128855$^{T}$ | *Bifrenaria harrisoniae* | Orchidaceae | Brazil | JF343565 | KF206209 | JF343586 | JF343649 |
| *P. braziliana* | CBS 126270$^{T}$ | *Malus domestica* | Rosaceae | Germany | JF343572 | KF206217 | JF343593 | JF343656 |
| *P. capitalensis* | CBS 128856$^{T}$ | *Stanhopea graveolens* | Orchidaceae | Brazil | JF261465 | KF206304 | JF261507 | JF343647 |
| ***P. capitalensis*** | **MUCC 2916** | ***Wollemia nobilis*** | **Araucariaceae** | **Japan** | **LC542595** | **LC543421** | **LC543443** | **LC543464** |
| ***P. capitalensis*** | **MUCC 2926** | ***Thujopsis dolabrata* var. *hondae*** | **Cupressaceae** | **Japan** | **LC542594** | **LC543420** | **LC543442** | **LC543463** |
| ***P. capitalensis*** | **MUCC 2927** | ***Metasequoia glyptostroboides*** | **Cupressaceae** | **Japan** | **LC542591** | **LC543417** | **LC543439** | **LC543460** |
| ***P. capitalensis*** | **MUCC 2930** | ***Sequoia sempervirens*** | **Cupressaceae** | **Japan** | **LC542593** | **LC543419** | **LC543441** | **LC543462** |
| ***P. capitalensis*** | **MUCC 2933** | ***Taxodium distichum*** | **Cupressaceae** | **Japan** | **LC542610** | **LC543436** | **LC543457** | **LC543476** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ***P. capitalensis*** | **MUCC 2935** | ***Cedrus deodara*** | **Pinaceae** | **Japan** | **LC542592** | **LC543418** | **LC543440** | **LC543461** |
| ***P. capitalensis*** | **MUCC 2937** | ***Glyptostrobus pensilis*** | **Cupressaceae** | **Japan** | **LC542609** | **LC543435** | **LC543456** | **-** |
| *P. citriasiana* | CBS 120486 [T] | *Citrus maxima* | Rutaceae | Thailand | FJ538360 | KF206314 | FJ538418 | FJ538476 |
| *P. citribraziliensis* | CBS 100098 [T] | *Citrus* sp. | Rutaceae | Brazil | FJ538352 | KF206221 | FJ538410 | FJ538468 |
| *P. citricarpa* | CBS 127454 [T] | *Citrus limon* | Rutaceae | Australia | JF343583 | KF206306 | JF343604 | JF343667 |
| *P. citrichinaensis* | CBS 130529 [T] | *Citrus maxima* | Rutaceae | China | JN791597 | KY855795 | JN791452 | JN791526 |
| *P. citrimaxima* | CBS 136059 [T] | *Citrus maxima* | Rutaceae | Thailand | KF170304 | KF206229 | KF289222 | KF289300 |
| *P. concentrica* | CBS 937.70 [T] | *Hedera helix* | Araliaceae | Italy | FJ538350 | KF206291 | FJ538408 | KF289257 |
| *P. cordylinophila* | CBS 136244 [T] | *Cordyline fruticosa* | Asparagaceae | Thailand | KF170287 | KF206242 | KF289172 | KF289295 |
| *P. cryptomeriae* | MUCC 0028 [T] | *Cryptomeria japonica* | Cupressaceae | Japan | AB454271 | AB454271 | - | - |
| ***P. cryptomeriae*** | **MUCC 2917** | ***Cryptomeria japonica*** | **Cupressaceae** | **Japan** | **LC542596** | **LC543422** | **LC543444** | **-** |
| *P. cussoniae* | CPC 14875 | *Cussonia* sp. | Araliaceae | South Africa | JF343579 | KF206278 | JF343600 | JF343663 |
| *P. elongata* | CBS 126.22 [T] | *Oxycoccus macrocarpos* | Ericaceae | USA | FJ538353 | AB095508 | FJ538411 | FJ538469 |
| *P. ericarum* | CBS 132534 [T] | *Erica gracilis* | Ericaceae | South Africa | KF206170 | KF206253 | KF289227 | KF28291 |
| *P. eugeniae* | CBS 445.82 [T] | *Eugenia aromatica* | Myrtaceae | Indonesia | AY042926 | KF206288 | KF289208 | KF289246 |
| *P. foliorum* | CBS 447.68 [T] | *Taxus baccata* | Taxaceae | Netherlands | KF170309 | KF206287 | KF289201 | KF289247 |
| *P. gaultheriae* | CBS 447.70[T] | *Gaultheria humifusa* | Ericaceae | USA | JN692543 | KF206298 | JN692531 | KF289248 |
| *P. hamamelidis* | MUCC 0149 | *Hamamelis japonica* | Hamamelidaceae | Japan | AB454321 | AB454321 | - | KF289309 |
| *P. hostae* | CGMCC 3.14355 [T] | *Hosta plantaginea* | Asparagaceae | China | JN692535 | - | JN692523 | JN692511 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ***P. hostae*** | **MUCC 2913** | ***Thuja occidentalis*** | **Cupressaceae** | **Japan** | **LC542603** | **LC543429** | **LC543450** | **LC543470** |
| ***P. hostae*** | **MUCC 2920** | ***Abies homolepis*** | **Pinaceae** | **Japan** | **LC542606** | **LC543432** | **LC543453** | **LC543473** |
| ***P. hostae*** | **MUCC 2923** | ***Juniperus chinensis* var. *sargentii*** | **Cupressaceae** | **Japan** | **LC542611** | **LC543437** | **LC543458** | **LC543477** |
| ***P. hostae*** | **MUCC 2928** | ***Fokienia hodginsii*** | **Cupressaceae** | **Japan** | **LC542604** | **LC543430** | **LC543451** | **LC543471** |
| ***P. hostae*** | **MUCC 2929** | ***Fokienia hodginsii*** | **Cupressaceae** | **Japan** | **LC542607** | **LC543433** | **LC543454** | **LC543474** |
| ***P. hostae*** | **MUCC 2931** | ***Juniperus chinensis* '*Jacobian*'** | **Cupressaceae** | **Japan** | **LC542608** | **LC543434** | **LC543455** | **LC543475** |
| ***P. hostae*** | **MUCC 2934** | ***Thujopsis dolabrata* var. *hondae*** | **Cupressaceae** | **Japan** | **LC542605** | **LC543431** | **LC543452** | **LC543472** |
| *P. hubeiensis* | CGMCC 3.14986 [T] | *Viburnum odoratissimim* | Adoxaceae | China | JX025037 | - | JX025042 | JX025032 |
| *P. hymenocallidicola* | CBS 131309 [T] | *Hymenocallis littoralis* | Amaryllidaceae | Australia | JQ044423 | JQ044443 | KF289211 | KF289242 |
| *P. hypoglossi* | CBS 434.92 [T] | *Ruscus aculeatus* | Asparagaceae | Italy | FJ538367 | KF206299 | FJ538425 | FJ538483 |
| *P. ilicis-aquifolii* | CGMCC 3.14358 [T] | *Ilex aquifolium* | Aquifoliaceae | China | JN692538 | - | JN692526 | JN692514 |
| *P. leucothoicola* | CBS 136073 [T] | *Leucothoe catesbaei* | Ericaceae | Japan | AB454370 | AB454370 | - | KF289310 |
| *P. mangiferae-indicae* | CBS 136061 [T] | *Mangifera indica* | Anacardiaceae | Thailand | KF170305 | KF206240 | KF289190 | KF289296 |
| *P. minima* | CBS 585.84 [T] | *Acer rubrum* | Sapindaceae | USA | KF206176 | KF206286 | KF289204 | KF289249 |
| *P. neopyrolae* | CBS 134750 [T] | *Pyrola asarifolia* subsp. *incarnata* | Ericaceae | Japan | AB454318 | AB454318 | - | AB704233 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *P. owaniana* | CBS 776.97$^{T}$ | *Brabejum stellatifolium* | Proteaceae | South Africa | FJ538368 | KF206293 | FJ538426 | KF289254 |
| *P. pachysandricola* | MUCC 0124$^{T}$ | *Pachysandra terminalis* | Buxaceae | Japan | AB454317 | AB454317 | - | AB704232 |
| *P. paracapitalensis* | CBS 141353$^{T}$ | *Citrus × floridana* | Rutaceae | Italy | KY855622 | KY855796 | KY855951 | KY855677 |
| *P. paracitricarpa* | CBS 141357$^{T}$ | *Citrus limon* | Rutaceae | Greece | KY855635 | KY855809 | KY855964 | KY855690 |
| *P. paxistimae* | CBS 112527$^{T}$ | *Paxistima myrsinites* | Cerastraceae | USA | KF206172 | KF206320 | KF289209 | KF289239 |
| *P. philoprina* | CBS 587.69 | *Ilex aquifolium* | Aquifoliaceae | Netherlands | KF154278 | KF206297 | KF289206 | KF289250 |
| ***P. pilospora*** | **MUCC 2912$^{T}$** | ***Chamaecyparis pisifera* var. *plumose*** | **Cupressaceae** | **Japan** | **LC542600** | **LC543426** | **LC543448** | **LC543468** |
| ***P. pilospora*** | **MUCC 2915** | ***Chamaecyparis pisifera* var. *filifera*** | **Cupressaceae** | **Japan** | **LC542597** | **LC543423** | **LC543445** | **LC543465** |
| ***P. pilospora*** | **MUCC 2922** | ***Juniperus chinensis*** | **Cupressaceae** | **Japan** | **LC542598** | **LC543424** | **LC543446** | **LC543466** |
| *P. podocarpicola* | CBS 728.79$^{T}$ | *Podocarpus maki* | Podocarpaceae | New Zealand | KF206173 | KF206295 | KF289203 | KF289252 |
| *P. pseudotsugae* | CBS 111649 | *Pseudotsuga menziesii* | Pinaceae | USA | KF154277 | KF206321 | KF289231 | KF289236 |
| *P. rubra* | CBS 111635$^{T}$ | *Acer rubrum* | Sapindaceae | USA | KF206171 | EU754194 | KF289198 | KF289233 |
| *P. sphaeropsoidea* | CBS 756.70 | *Aesculus hippocastanum* | Sapindaceae | Germany | AY042934 | KF206294 | KF289202 | KF289253 |
| *P. spinarum* | CBS 292.90$^{T}$ | *Chamaecyparis pisifela* | Cupressaceae | France | JF343585 | KF206301 | JF343606 | JF343669 |
| ***P. spinarum*** | **MUCC 2914** | ***Thujopsis dolabrata* var. *hondae*** | **Cupressaceae** | **Japan** | **LC542601** | **LC543427** | **LC543449** | **-** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ***P. spinarum*** | **MUCC 2918** | ***Thujopsis dolabrata* var. *hondae* '*Nana*'** | **Cupressaceae** | **Japan** | **LC542612** | **LC543438** | **LC543459** | **LC543478** |
| ***P. spinarum*** | **MUCC 2919** | ***Thuja occidentalis*** | **Cupressaceae** | **Japan** | **LC542602** | **LC543428** | **-** | **LC543469** |
| ***Phyllosticta* sp.** | **MUCC 2925** | ***Thuja occidentalis*** | **Cupressaceae** | **Japan** | **LC542599** | **LC543425** | **LC543447** | **LC543467** |
| *P. styracicola* | CGMCC 3.14985[T] | *Styrax gradiflorus* | Styracaceae | China | JX052040 | - | JX025045 | JX025035 |
| *P. telopeae* | CBS 777.97[T] | *Telopea speciosissima* | Proteaceae | Tasmania | KF206205 | KF206285 | KF289210 | KF289255 |
| *P. vacciniicola* | CBS 136062[T] | *Vaccinium macrocarpum* | Ericaceae | USA | KF170312 | KF206257 | KF289229 | KF289287 |
| *P. yuccae* | CBS 112065 | *Yucca elephantipes* | Asparagaceae | USA | KF206175 | - | - | KF289237 |

CBS: CBS Fungal Biodiversity Centre, Utrecht, the Netherlands, CGMCC: China General Microbiological Culture Collection Center Institute of Microbiology Chinese Academy. [T] Ex-type, ex-neotype, and ex-epitype strains are indicated. Japanese isolates from Cupressaceae and Pinaceae in this study are indicated in bold.

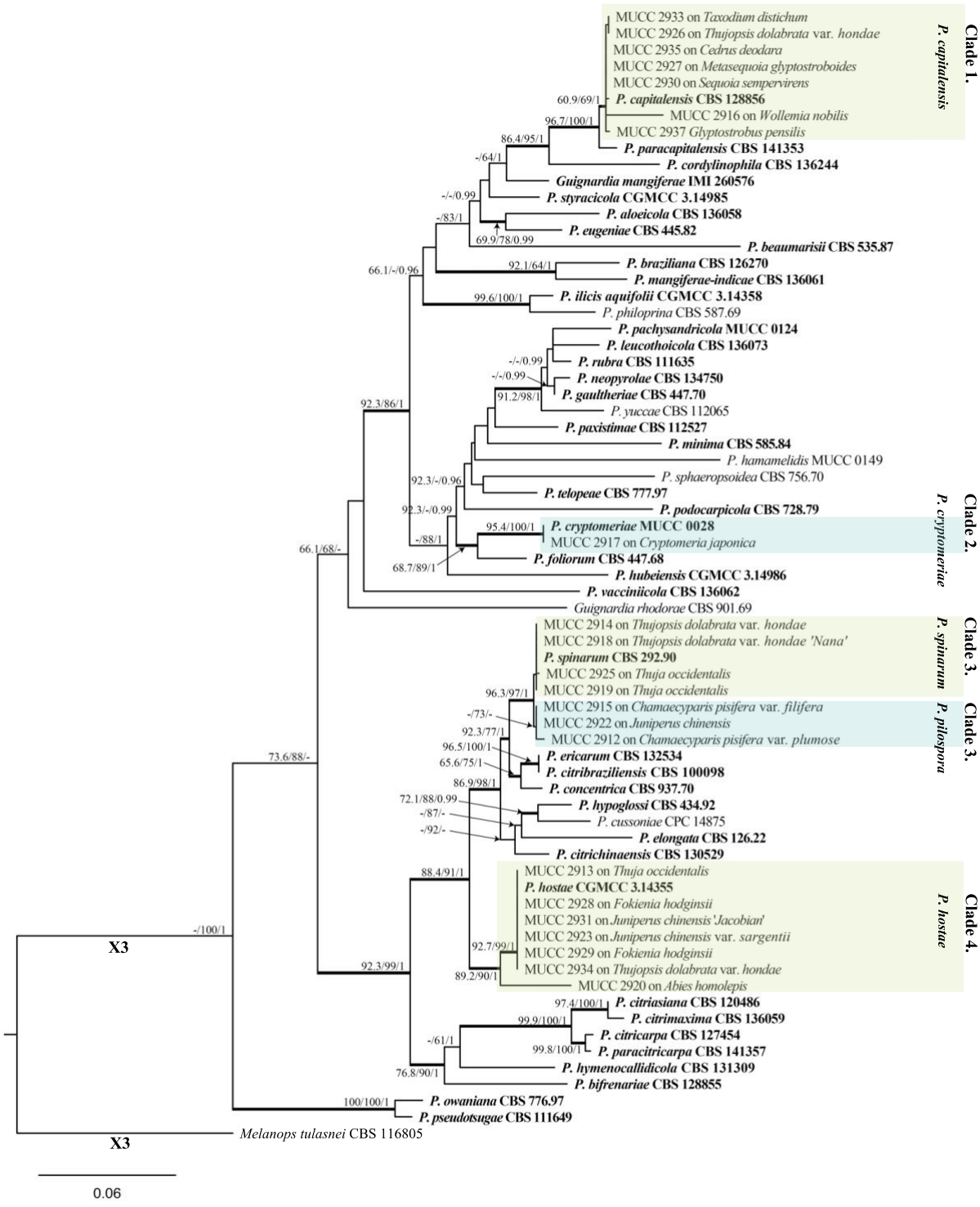


Fig. 2–1. Phylogenetic tree of the *Phyllosticta* species by constructed maximum likelihood

using the combined internal transcribed spacer (ITS), large subunit ribosomal ribonucleic acid (LSU rRNA), translation elongation factor 1-alpha (tef1-α), and actin (act) gene region dataset. MP and ML bootstrap values and Bayesian posterior probabilities (PP) are given near the branches (MP/ML/PP). Thickened nodes indicated significant support by MP/ML/PP (>60/60/0.96). Ex-type, ex-neotype, and ex-epitype strains are indicated in bold.

analysis revealed that *Phyllosticta* species were divided into two large clades: the *Phyllosticta* group, which was composed of isolates from 20 plant families, and another group, with isolates from 9 families. Of them, the *Phyllosticta* spp. found on Cupressaceae were divided into four clades. The first clade was composed of *P. capitalensis* (CBS128856) and isolates from *Wollemia* (Araucariaceae), *Cedrus* (Pinaceae), *Glyptostrobus*, *Metasequoia*, *Sequoia*, *Taxodium*, and *Thujopsis*. The second clade was *P. cryptomeriae* (MUCC 0028) on *Cryptomeria* (Cupressaceae). The third clade consisted of *P. spinarum* on the genus *Chamecyparis* (CBS 292.90), *Thuja*, and *Thujopsis*, and *P. pilospora* on *Chamaecyparis*, *Juniperus* (Cupressaceae). The fourth clade consisted of *P. hostae* (CGMCC 3.14355) on *Hosta* (Asparagaceae), *Abies* (Pinaceae), *Fokienia*, *Juniperus*, *Sequoia*, *Thuja*, and *Thujopsis* (Cupressaceae). Each clade was strongly supported by MP and ML BS values and Bayesian PPs.

**Pathogenicity**

Thirty-six days after inoculation, an injured tree with *Phyllosticta spinarum* (MUCC 2914) exhibited brown blight at the edge of the leaves (Fig. 2–5, J). *P. spinarum* was identified based on morphological characteristics and the ITS region nucleotide sequence and then re-isolated from symptomatic leaves (Fig. 2–5, J–L). Therefore, pathogenicity of *P. spinarum* was confirmed based on Koch's principle. These results indicated that *P. spinarum* exhibited infectivity and pathogenicity to *Thujopsis*. All trees inoculated with *P. capitalensis* (MUCC 2926), *P. hostae* (MUCC 2934), and *P. pilospora* (MUCC 2912) did not exhibit these

symptoms.

**Taxonomy**

***Phyllosticta capitalensis*** Henn, Hedwigia 48:43,1908.

Conidiomata on PNA, pycnidial, solitary, black, 80–101 × 131–164 μm in diam; pycnidial wall composed of depressed or irregular cells in 2–5 layers, brown to dark brown, melaeneous around the ostiole, paler towards the conidiogenous region; conidiogenous cells hyaline, cylindrical, phialidic conidiogenesis, smooth, 3.6–14.4 × 2.9–3.2 μm. Conidia unicellular, hyaline, ellipsoid, or globose, truncate at the base when young, later rounded at both ends, 10.7–13.7 × 5.5–6.6 μm, containing numerous guttules, surrounded by a hyaline slimy layer, with a slender apical appendage, 5.10–13.8 μm long.

Isolates examined: MUCC 2926, MUCC 2927, MUCC 2930 (Table 2–2).

**Host:** *Stanhopea graveolens* (Okane et al., 2001, 2003), *Cedrus deodara*, *Glyptostrobus pensilis*, *Metasequoia glyptostroboides*, *Taxodium distichum*, *Thujopsis dolabrata* var. *hondae*, *Sequoia sempervirens*, and *Wollemia nobilis* (this study).

**Note:** *Phyllosticta capitalensis* is known as an endophyte of various plants around the world. In this study, it was isolated from the genera *Metasequoia*, *Sequoia* (Cupressaceae), *Wollemia* (Araucariaceae), and *Cedrus* (Pinaceae). The growth rate of the mycerial colony on the OMA was faster than in other species, and the conidial sporulation on media was also observed (Motohashi 2011).

***Phyllosticta cryptomeriae*** Kawam., Bull. Cov. Eor. Exp. Stn. Tokyo 10: 97, 1913. (Fig. 2–2)

**Symptoms:** reddish brown to brown, small at the edge of the scale leaf, later enlarged and coalescent, expanded towards the whole of shoot and branch. Pycnidia amphigenous, epidermal, submerged, solitary, scattered, black, ellipsoid, 118–162 × 97–146 μm in diam; pycnidial wall composed of depressed or irregular cells in 2–5 layers, brown to dark brown, melaeneous around the ostiole, paler towards the conidiogenous region; conidiogenous cells hyaline, cylindrical, phialidic conidiogenesis, smooth, 3.1–12.2 × 2.3–3.4 μm. Conidia

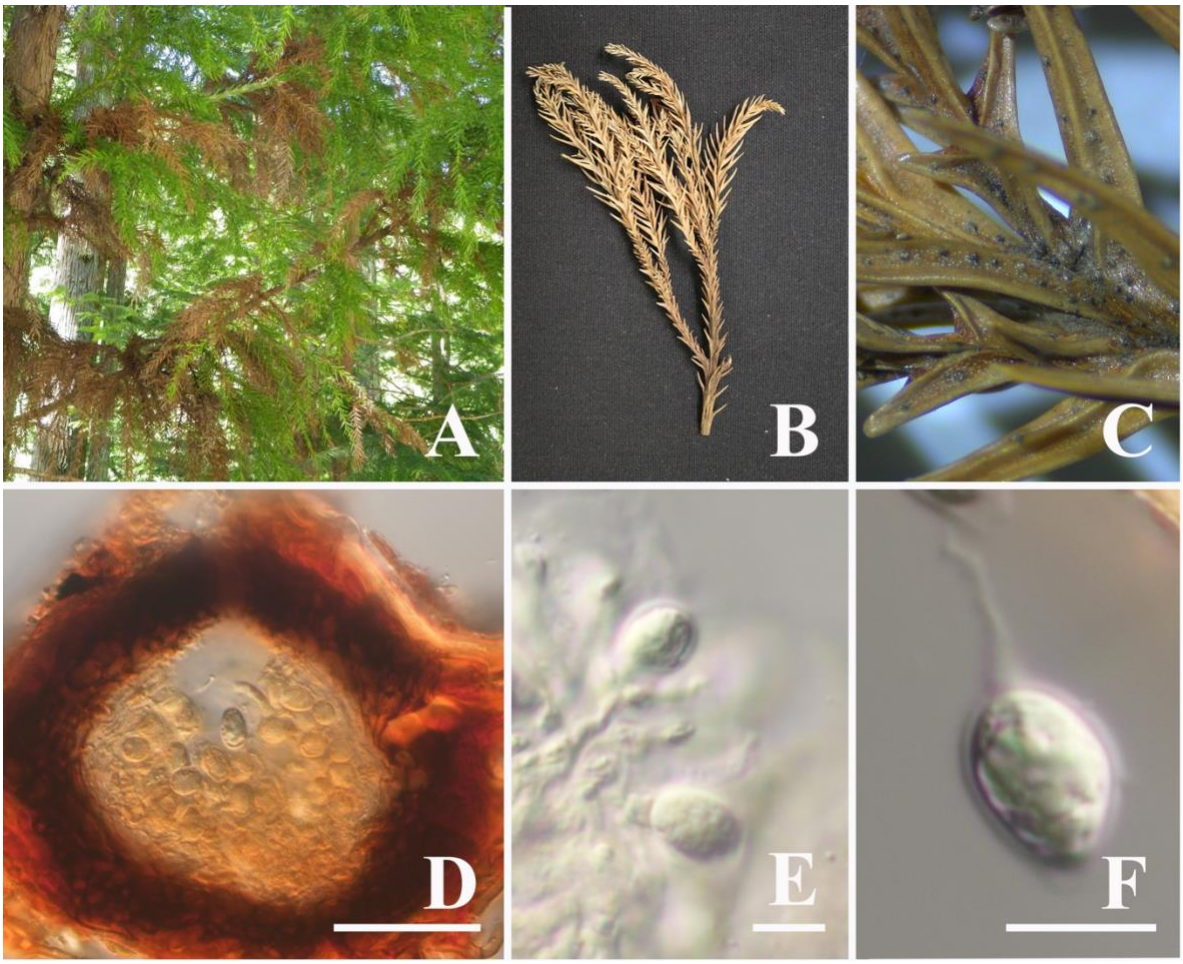


Fig. 2–2. Morphological features of *Phyllosticta cryptomeriae* (MUCC 0028 and MUCC 2917) on *Cryptomeria japonica*. A. Natural symptoms found on *Cryptomeria japonica*. B. Specimen MUMH 11913. C. Symptoms with pycnidia forming on the leaves of *Cryptomeria japonica*. D. Vertical section of pycnidium in the leaf tissue. E. Conidia and conidiophores. F. Conidium. Scale bars = C: 50 μm; D–E: 10 μm.

unicellular, hyaline, globose, or ellipsoid, truncate at the base when young, later rounded at both ends, 9.5–12.5 × 7.3–9.1 μm, containing numerous guttules, surrounded by a hyaline slimy layer, with an apical appendage 3–10μm long.

**Lectotype:** Illustration by S. Kawamura, Bull. Cov. Eor. Exp. Stn. Tokyo 10: 108, 1913.

**Epitype (designated here):** *Cryptomeria japonica*, **Japan**, Toyama, Nakaniikawa, 20 Oct 2005, by C. Nakashima & K. Motohashi, TFM: FPH-7884 = MUMH 10451 (isolate ex-epitype, MUCC 0028 = MAFF 240057), MBT 391950.

**Host:** Cryptomeria japonica (Kawamura, 1913, Kobayashi, 1977).

**Specimens examined:** *Cryptomeria japonica*, **Japan**, Tokyo, Chofu, 22 Jun. 2015, by Y. Hattori & K. Motohashi, MUMH 11913 (isolate, MUCC 2917).

**Note:** The growth rate of the mycerial colony of this species is very slow on OMA, generally only growing about 5 mm in 15 days. Symptoms associated with this species were observed in park and planted forest trees.

***Phyllosticta hostae*** Y.Y. Su & L. Cai, Persoonia 28, 76–84, 2012. (Fig. 2–3)

**Symptoms:** brown to yellow brown, small at the edge of the scale leaf, later enlarged and coalescent, expanded towards the whole of shoot and branch. Pycnidia amphigenous, epidermal, merged, solitary or rarely gregarious, scattered, dark brown to black, ellipsoid, 69.2 × 92.7 μm in diam; pycnidial wall composed of depressed or irregular cells in 2–3 layers, brown to dark brown, melaeneous around the ostiole, paler towards the conidiogenous region; conidiogenous cells hyaline, cylindrical, phialidic conidiogenesis, smooth, 7.9–12.8 × 1.1–2.9 μm. Conidia unicellular, hyaline, ellipsoid, truncate at the base when young, later rounded at both ends, 7.5–11.1 × 6.6–7.1 μm, containing numerous guttules, surrounded by a hyaline slimy layer, with an apical appendage, 5.5–7.1 μm long.

**Host:** *Hosta plantaginea* (Su & Cai, 2012), *Hymenocallis littoralis* (Yu et al., 2015), *Thuja occidentalis*, *Abies homolepis*, *Juniperus chinensis* var. *sargentii*, *Fokienia hodginsii*, *Juniperus chinensis 'Jacobian'*, T*hujopsis dolabrata* var. *hondae* (this study)

Specimens examined: *Thuja occidentalis*, **Japan**, Kanagawa, 10 Oct. 2014, by M. Kanda, MUMH 11909 (isolate, MUCC 2913)

**Isolates examined:** MUCC 2920, MUCC2923, MUCC2928, MUCC2931, MUCC2934 (Table 2–2).

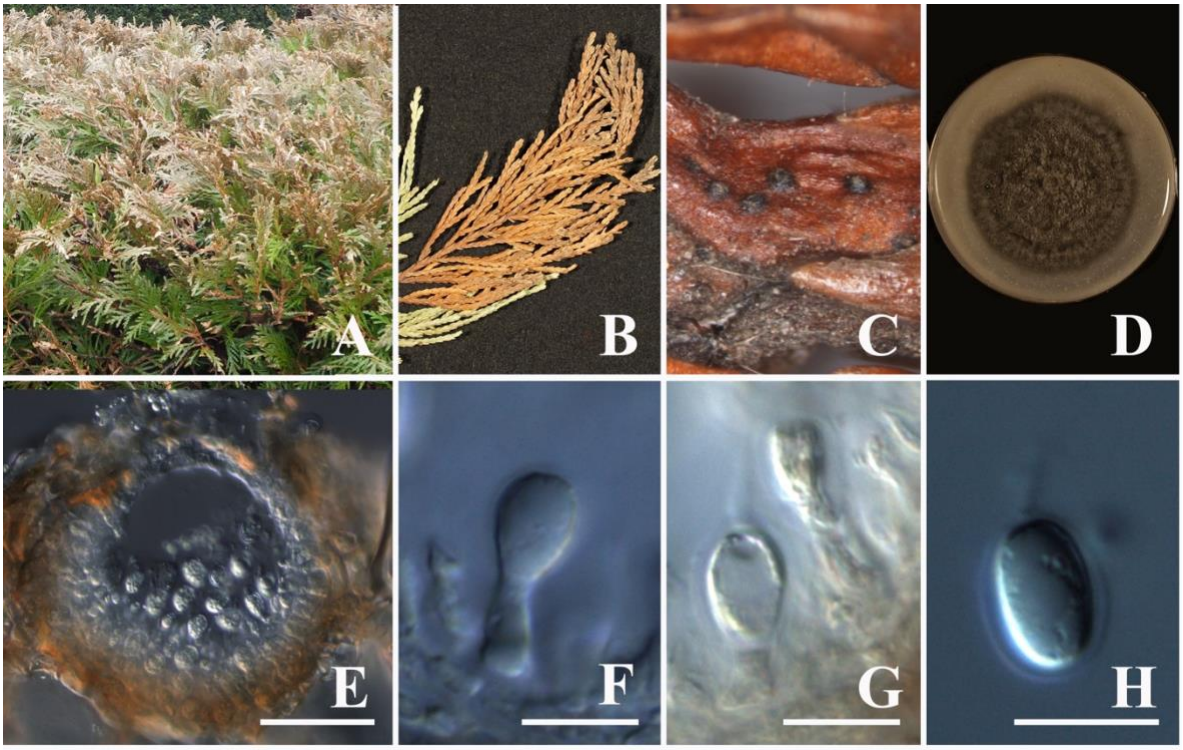


Fig. 2–3. Morphological features of the Japanese isolates of *Phyllosticta hostae* (MUCC 2913) on Thuja occidentalis. A. Natural symptoms on the *Thuja occidentalis* (MUMH 11909). B. Specimen MUMH 11909. C. Symptoms with pycnidia forming on the leaves of *Thuja occidentalis*. D. Colony on OMA, 28 °C, after 15 days, E. Vertical section of pycnidium in the leaf tissue. F–G. Conidia and conidiophores. H. Conidium. Scale bars = E: 50 μm; F–H: 10 μm.

**Note:** *Phyllosticta hostae* was added to Japanese mycoflora in this study. The size of the pycnidia and conidia collected in this study was slightly smaller than the original description of *P. hostae*. However, a clade of Japanese isolates and *P. hostae* was strongly supported by the bootstrap scores (MP BS 92.7, ML BS 97, BI PP 1) on the phylogenetic tree. The optimum growth temperature of *P. hostae* on OMA was 28 ℃–30 ℃, and it could be distinguished from *P. spinarum* (25 ℃) and *P. pilospora* (22 ℃). *Phyllosticta thujae* constituted a single clade with isolates from the genera *Chamaecyparis, Sequoia, Thujopsis,* and *Juniperus*. It was suggested that this species might have infested multiple genera of Cupressaceae. In this

study, all the trees from which this species was isolated were in the parks or gardens.

***Phyllosticta pilospora*** (Sawada) Y. Hattori, C. Nakash., & Motohashi., comb. nov. MB835269 (Fig. 2–4)

≡ *Phoma pilospora* Sawada, Bull. Gov. For. Exp. Stn. Tokyo 46: 144, 1950.

= *Phoma thujopsidis* Sawada, Bull. Gov. For. Exp. Stn. Tokyo 46: 145, 1950.

**Symptoms:** reddish brown to brown, small at the edge of the scale leaf, later enlarged and coalescent, expanded towards the whole of shoot and branch. Pycnidia amphigenous, epidermal, submerged, solitary or rarely gregarious, scattered, dark brown to black, ellipsoid, 132–194 × 145–186 μm in diam; pycnidial wall composed of depressed or irregular cells in 2–5 layers, brown to dark brown, melaeneous around the ostiole, paler towards the conidiogenous region; conidiogenous cells hyaline, cylindrical, phialidic conidiogenesis, smooth, 4–12 × 2–2.5 μm. Conidia unicellular, hyaline, ellipsoid, or obovoid, truncate at the base when young, later rounded at both ends, 9.5–12 × 7–10 μm, containing numerous guttules, surrounded by a hyaline slimy layer, with a slender and curved apical appendage, 3–10 μm long.

**Type:** *Thujopsis dolabrata*, Japan, Aomori, Shinjyo, 1 Oct. 1949, by K. Sawada & S. Murai, IUM-FS435

**Syntype:** *Thujopsis dolabrata*, **Japan**, Akita, Yurihonjo, 16 Nov. 1947, by K. Satou, IUM-FS430, **Japan**, Aomori, Ippongi, 19 Aug. 1949, IUM-FS431, by K. Sawada, **Japan**, Aomori, Yokohama, 6 Sep. 1949, IUM-FS433, by K. Sawada, **Japan**, Aomori, Soegi, 4 Oct. 1949, IUM-FS437, by K. Sawada & S. Murai, **Japan**, Aomori, Soegi, 4 Oct. 1949, IUM-FS438, by K. Sawada & S. Murai.

**Epitype (designated here):** *Chamaecyparis pisifera* '*Plumosa Aurea*', **Japan**, Kanagawa, Kawasaki, 9 Jun. 2014, by M. Kanda, MUMH 11908 (isolate ex-type, MUCC 2912).

Host: *Chamaecyparis obtuse*, *Thujopsis dolabrata* var. *hondae* (Sawada, 1950), *Juniperus*

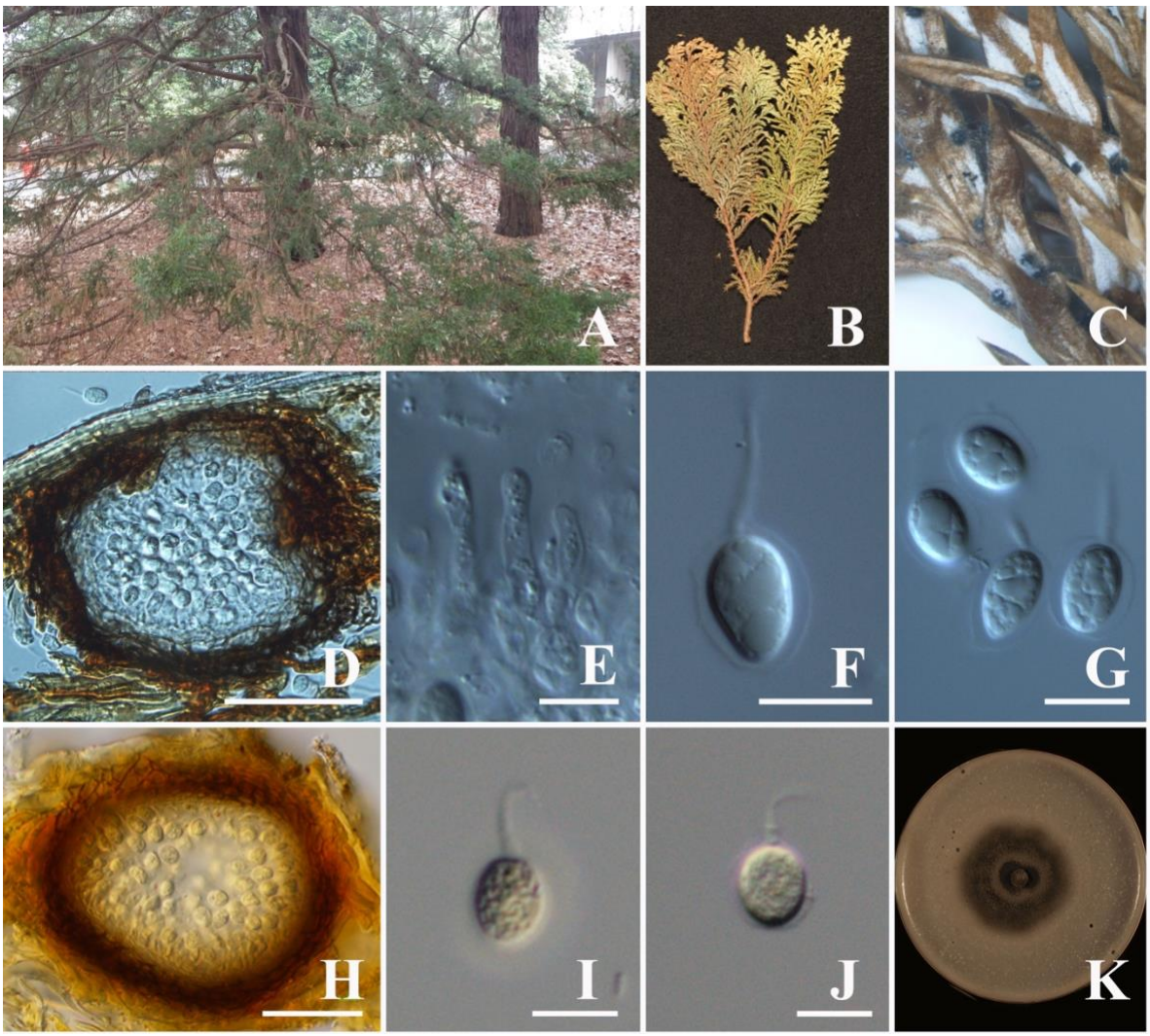


Fig. 2–4. Morphological features of *Phyllosticta pilospora* (MUCC 2912 and 2915) on *Chamaecyparis pisifera*. A. Natural symptoms on the *Chamaecyparis pisifera* var. *filifera*. B. Epitype specimen MUMH 11908. C. Symptoms with pycnidia forming on the leaves of *Chamaecyparis pisifera* '*Plumosa Aurea*'. D. Vertical section of pycnidium in the leaf tissue. E. Conidia and conidiophores. F–G. Conidia. H. Vertical section of pycnidium of *Phoma pilospora* (IUM-FS435), I. Conidium of *Phoma pilospora* (IUM-FS435), J. Conidium of *Phoma thujopsidis* (IUM-FS512). K. Colony on OMA, 25 °C. Scale bars = D, H: 50 µm; E-G, I, and J: 10 µm.

*chinensis* (this study).

**Specimens examined:** *Chamaecyparis pisifera* var. *filifera*, **Japan**, Tokyo, Chofu, 22 Jun. 2015, by Y. Hattori & K. Motohashi, MUMH 11911, (isolate, MUCC 2915), on *Juniperus chinensis*, **Japan**, Tokyo, Chofu, 27 Jul. 2015, by Y. Hattori & K. Motohashi, MUMH 11918 (isolate, MUCC 2922).

**Note:** *Phyllosticta pilospora* is transferred from the genus *Phoma* based on the morphological characteristics and phylogenetic position. Although this species has syntypes in the protologue, Sawada (1950) did not specify the holotype. From the phylogenetic tree (Fig. 2–1), *P. pilospora* is located in the same clade with *P. spinarum*, which were isolated from the genera *Chamaecyparis*, *Thuja*, and *Thujopsis*; however, these two species differed in the morphology of the conidial appendages and the optimum growth temperature (*P. pilospora* 22 ℃−25 ℃, *P. spinarum* 25 ℃−28 ℃) (Table 2–3). Although the protologue of *Phoma thujopsidis* does not describe the conidial appendage, one of the syntypes (IUM-FS438) indicated that the conidia had an apical appendage. In this study, this species was isolated from the ornamental or garden tree.

***Phyllosticta spinarum*** (Diedick) Nag Raj & Morelet, Can. J. Bot. 57, 1297. (Fig. 2–5)

**Symptoms:** reddish brown to brown, small at the edge of the scale leaf, later enlarged and coalescent, expanded towards the whole of shoot and branch. Pycnidia amphigenous, epidermal, merged, solitary, scattered, black, ellipsoid, 98–125 × 94–113 μm in diam; pycnidial wall composed of depressed or irregular cells in 2–4 layers, brown to dark brown, melaeneous around the ostiole, paler towards the conidiogenous region; conidiogenous cells hyaline, cylindrical, phialidic conidiogenesis, smooth, 4.4–7.4 × 3.1–4.1 μm. Conidia unicellular, hyaline, ellipsoid, or obovoid, truncate at the base when young, later rounded at both ends, 9.2–10.2 × 7.3–8.3 μm, containing numerous guttules, surrounded by a hyaline slimy layer, with a ligulate apical appendage, 6–8.7 μm long.

**Ex-type:** On *Chamaecyparis pisifera*, France (ex-epitype, CBS 292.90).

**Host:** *Juniperus* spp., *Chamaecyparis pisifera* (Nag Raj & Morelet, 1979), *Thujopsis*

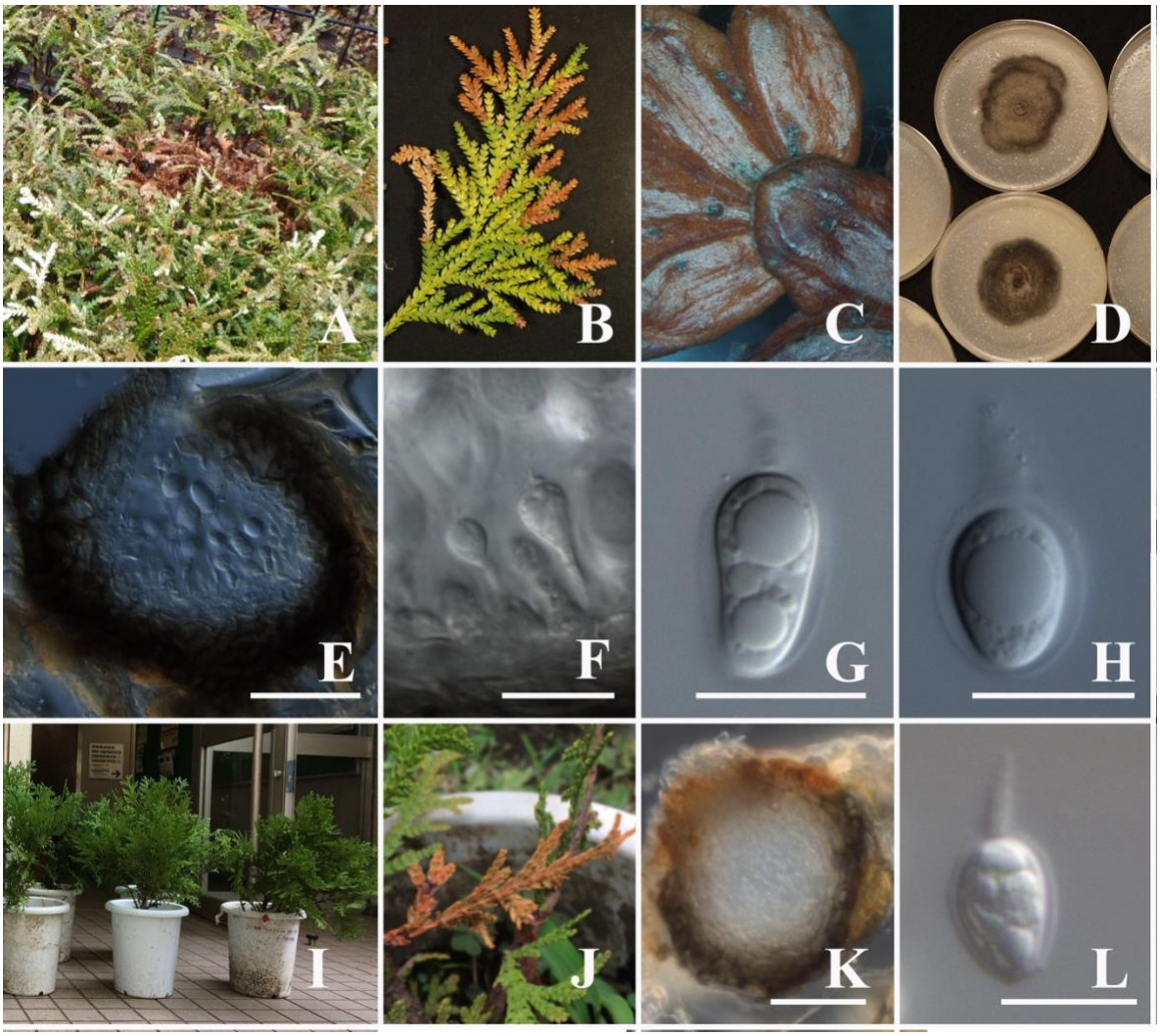


Fig. 2–5. Morphological features of the Japanese isolates of *Phyllosticta spinarum* (MUCC 2914) on *Thujopsis dolabrata* var. *hondae* and pathogenicity of *P. spinarum* (MUCC 2914). A. Natural symptoms on *Thujopsis dolabrata* var. *hondae 'Nana'*. B. Specimen MUMH 11910. C. Symptoms with pycnidia forming on the leaf of *Thujopsis dolabrata* var. *hondae*. D. Colony on OMA, 25 °C. E. Vertical section of pycnidium in the leaf tissue. F. Conidia and conidiophores. G–H. Conidia. I. Trees of *Thujopsis dolabrata* var. *hondae* for inoculation test. J. On *Thujopsis dolabrata* var. *hondae* at 36 days post-inoculation. K. Pycnidium in the leaf tissue of inoculated leaf. L. Conidium in K. 25 °C. Scale bars = E, K: 50 μm; F–H, and L: 10 μm.

*dolabrata* var. *hondae*, *Thuja occidentalis* (this study).

**Specimens examined:** *Thujopsis dolabrata* var. *hondae*, **Japan**, Tokyo, Setagaya, 10 Oct. 2014, by M, Kanda, MUMH 11910 (isolate, MUCC 2914), on *Thujopsis dolabrata* var. *hondae 'Nana'*, **Japan**, Tokyo, Chofu, 22 Jun. 2015, by Y. Hattori & K. Motohashi, MUMH 11914 (isolate, MUCC 2918), *Thuja occidentalis*, **Japan**, Tokyo, Chofu, 9 May 2016, by Y. Hattori & K. Motohashi, MUMH 11915 (isolate, MUCC 2919), **Japan**, Tokyo, Chofu, 22 Jun. 2015, by Y. Hattori & K. Motohashi, MUMH 11921(isolate, MUCC 2925).

**Note:** *Phyllosticta spinarum* is added to Japanese mycoflora in this study. The plant genera *Thujopsis*, *Chamaecyparis*, and *Juniperus* are new hosts for this species. In addition, the pathogenicity of *P. spinarum* against *Thujopsis dolabrata* was confirmed. The optimum growth temperature for the mycerial colony on OMA is 25 °C. This species could be distinguished from *P. pilospora* and *P. thujae* because the morphology of the conidial appendage of *P. spinarum* is wider and almost tongue-shaped. In this study, this species was isolated from ornamental trees.

## Discussion

In this study, I evaluated *Phyllosticta* species inhabiting Cupressaceae trees and then elucidated species diversity based on morphological characteristics, phylogenetic relationship, and pathogenicity. In previous studies, *Phyllosticta* species on specific host genera, such as species of citrus, were evaluated. Those species were composed of pathogenic and endophytic species (Glienke et al., 2011; Guarnaccia et al., 2019, Wang et al., 2011). Furthermore, each species was found to have (12%) unique species-specific genes from the entire genomic study (Guarnaccia et al., 2019). These results suggested a high species diversity rate for the genus *Phyllosticta* on the Cupressaceae plants. In the phylogenetic analyses, Japanese isolates were divided into four clades in the molecular phylogenetic analysis (Fig. 2–1).

Clade 1 is composed of *P. capitalensis*. The optimal growth temperature of the

Table 2–3. The optimum growth temperature for the mycerial colony on OMA and the size of *Phyllosticta* species on Cupressaceae.

| Fingal Species | Isolates or Typus | Host Plants | Optimum temperatures (℃) | Colony diameters after 15 days (cm) | Conidial bodies (μm) | Appendages (μm) | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Length × Width | Length | Width at base | Width at tip |
| *P. capitalensis* | MUCC 2926 | *Thujopsis* | 28 | 77.96 | 10.7–13.7 × 5.5–6.6 | 5.10–13.80 | 0.94–1.77 | 0.52–0.84 |
| | MUCC 2927 | *Metasequoia* | 28–30 | 78.70 | 10.2–13.6 ×6.7–7.1 | 4.77–13.30 | 1.05–1.71 | 0.53–1.01 |
| | MUCC 2930 | *Sequoia* | 30 | 79.25 | 10–20.1 × 6.0–7.1 | 2.22–19.73 | 0.54–2.09 | 0.55–1.21 |
| *P. capitalensis* | CBS 111655 | *Thuja* | 28–30 | 78.37 | 9.0–11.5 × 4.7–6.2 | 3.82–8.43 | 1.03–1.85 | 0.71–1.46 |
| (*P. thujae*) | | | | | | | | |
| *P. hostae* | MUCC 2913 | *Thuja* | 30 | 57.24 | 9.4–13.5 × 5.9–7.9 | 5.50–7.10 | 1.17–2.14 | 0.37–2.43 |
| | MUCC 2920 | *Abies* | 28–30 | 78.23 | 9.2–12.7 × 5.6–9.1 | 5.19–17.54 | 0.53–3.50 | 0.35–1.80 |
| | MUCC 2923 | *Juniperus* | 28–32 | 56.76 | - | - | - | - |
| | MUCC 2928 | *Fokienia* | 28–32 | 75.27 | - | - | - | - |
| | MUCC 2931 | *Juniperus* | 28–32 | 76.84 | 10.8–12.9 × 6.7–7.5 | 6.55–11.59 | 1.06–2.24 | 0.76–1.06 |
| | MUCC 2934 | *Thujopsis* | 30 | 62.81 | - | - | - | - |
| *P. pilospora* | IUM-FS435 | *Thuopsis* | - | - | 9.5–12 × 7–10 | 3.00–10.00 | - | - |
| | MUCC 2912 | *Chamaecyparis* | 22 | 44.25 | 9.9–14.6 × 5.6–8.8 | 4.10–14.00 | 0.85–2.56 | 0.54–1.43 |
| | MUCC 2915 | *Chamaecyparis* | 25 | 51.65 | 10.7–14.0 × 6.4–9.3 | 2.30–12.80 | 1.01–2.50 | 0.40–1.49 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MUCC 2922 | *Juniperus* | 22–25 | 46.30 | 10.1–15.0 × 7.1–8.2 | 4.78–15.15 | 1.18–2.17 | 0.39–1.90 |
| *P. spinarum* | CBS 292.90 | *Chamaecyparis* | 25 | 52.68 | 9.9–13.4 × 6.7–7.6 | 6.47–14.80 | 1.02–3.23 | 0.71–1.53 |
| | MUCC 2914 | *Thujopsis* | 28 | 42.34 | 9.0–12.0 × 7.3–9.3 | 3.10–18.70 | 1.02–3.23 | 0.72–1.53 |
| | MUCC 2918 | *Thujopsis* | 25 | 39.82 | - | - | - | - |
| *Phyllosticta* sp. | MUCC 2925 | *Thuja* | 25–28 | 44.93 | 9.6–15.3 × 6.2–10.9 | 4.86–22.63 | 0.73–2.34 | 0.58–1.58 |

mycelial colony was 28–30 °C (Table 2–3). No significant differences in morphological characteristics were observed between the isolates of clade 1. However, *P. capitalensis* (MUCC 2926, MUCC 2927, and MUCC2930) in clade 1 could be distinguished from other lineage groups based on the width of the conidial appendage (tip, base (n = 50); MUCC 2926 0.52–0.84, 0.94–1.77; MUCC 2930 0.55–1.21, 0.54–2.09; *P. hostae* (MUCC 2913) 0.37–2.43, 1.17–2.14); *P. pilospora* (MUCC 2912) 0.54–1.43, 0.85–2.56; *P. spinarum* (CBS29.290) 0.71–1.53, 1.02–3.23 (Table 2–3).

Clade 2 was consisted of two *P. cryptomeriae* isolates. This species grew very slowly on the OMA medium.

Clade 3 included *P. spinarum* (ex-type CBS 292.90) and *P. pilospora* (ex-epitype MUCC 2912) on *Chamaecyparis*. In addition, it included isolates obtained from *Juniperus*, *Thuja*, and *Thujopsis*. These species were differentiated by optimum growth temperatures of the mycelial colony and morphological characteristics of the apical conidial appendage (Table 2–3). *P. pilospora* and *P. spinarum* had optimum temperatures of 22 °C and 25–28 °C, respectively. The widths at the base and tip of the appendage were 0.85–2.57 μm and 0.54–1.48 μm for P. pilospora and 1.02–3.23 μm and 0.72–1.53 μm for P. spinarum. Besides, the former species had a filamentous appendage (Fig. 2–4F), and the latter had a ligulate appendage (Fig. 2–5H; Table 2–3). *Phyllosticta* species were often distinguished by the differences in the shape and size of the apical appendage of the conidia (Bissett, 1979, Baayen et al., 2002). The observations in this study supported taxonomic importance of these criteria for species identification. The results of the inoculation tests indicated that only *P. spinarum* caused pathogenicity to the genus *Thujopsis* which exhibited scale leaf blight symptoms (Fig. 2–5J). However, *P. capitalensis*, *P. hostae*, and *P. pilospora*, obtained from the intact scale leaves of the Cupressaceae trees, did not exhibit symptoms on the inoculated trees. These results suggested that the fungi located in clade 3 had infectability to Cupressaceae trees and exhibited variable ecological behavior. For instance, some exhibited pathogenicity causing scale leaf blight, and some behaved as endophytes. Moreover, the isolates located

in clade 3, which were obtained from various host plants with symptoms, formed small inner clades (Fig. 2–1), which suggested the existence of several cryptic species from the morphological characteristics and an optimum growth temperature for the mycelial colony.

Clade 4 included *P. hostae* (CGMCC 3.14355) and the isolates inhabiting *Abies* (Pinaceae), *Fokienia*, *Juniperus*, *Sequoia*, *Thuja*, and *Thujopsis* (Cupressaceae). MUCC 2913, which was isolated from the genus *Thuja*, was previously mis-identified as *P. thujae* based on the host plants and the similarity of the morphological characteristics (Hattori et al. 2016). *P. thujae* was known as an endemic species in North America (Bissett & Palm 1989). However, an isolate of *P. thujae* (CBS 111655) was judged as *P. capitalensis* from the homology of the rDNA ITS region sequence deposited in the GenBank. The phylogenetic position of *P. thujae* was still unclear. *P. hostae* was known as a pathogen causing leaf spots on the *Hosta* and *Hymenocallis* plants in China (Su & Cai, 2012, Yu et al., 2015). According to the original description of *P. hostae*, the size of the conidia was 8–15 × 5–9 μm, which was almost the same as MUCC 2913: 9.4–13.5 × 5.9–7.9 μm. Furthermore, the length of the appendage was almost the same (*P. hostae*; 4–8 × 1–3 μm., MUCC 2913; 5.5–7.1 × 0.3–2.4 μm). The isolates of MUCC 2913, 2929, 2923, 2928, 2929, 2931, and 2934 were clustered together with *P. hostae* (CGMCC3.14355) based on the MP, ML, and BI trees, and this was also strongly supported by the bootstrap scores (Fig. 2–1). Therefore, the Japanese isolates from the Cupressaceae trees were identified as *P. hostae*. Only MUCC 2913 was isolated from a scale leaf with symptoms; all of the others were isolated as endophytes from the intact trees. The optimal growth temperature of the species located in clade 4 was 28–32 °C (Table 2–3), and it was higher than that of clade 3. *Phyllosticta* spp., which were isolated from the intact scale leaf of the Juniperus grew at 22–30 °C, with an optimum growth temperature of 22 °C (Table 2–3).

In this study, there were two isolates of the genus *Phyllosticta* from *Chamaecyparis*, four from *Thujopsis*, three from *Thuja*, and three from *Juniperus*. More than one *Phyllosticta* species was found from the same host genus of Cupressaceae. Those species

belonged to respective lineages and had broad ranges of host. *Phyllosticta* species were traditionally thought to have a host range that was limited to one plant genus. It was based on the conventional species concept, and a lot of species had been described by this concept (van der Aa, 1973, Wikee et al., 2013a). As a rare species, *P. capitalensis*, which is an endophytic species, had a broad host range and could be isolated from a variety of plants (Wikee et al., 2013b). The endophytic species *P. capitalensis* exhibited a difference in the growth rate on the agar media, as it grew faster than that of the pathogenic species (Motohashi et al., 2013). In addition, each *Phyllosticta* species was recognized on the phylogenetic tree and exhibited differences in the optimum temperatures for mycelial growth in this study. These differences might be used as criteria for the primary identification of *Phyllosticta* species by a combination of information, such as the host plant and cultural characteristics.

*Phyllosticta spinarum* on Cupressaceae is located within a clade of *P. concentrica*. This suggested that *P. spinarum* had evolved from the endophytic niche to the pathogenic niche via maintenance of the polyxeny (Fig. 2–1). *Chamaecyparis* and *Thuja* plants were distributed in North America and East Asia, and *Thujopsis* plants were endemic to Japan. The splitting time was estimated between the genera *Thuja* and *Thujopsis* and occurred 58.46 million years ago, *Juniperus* and *Cupressus* was 46.29 million years ago (Yu et al. 2020). Moreover, Accordingly Motohashi et al. (2009), *Phyllosticta* species were estimated to have emerged about 43.05 to 29.8 million years ago. These perceptions suggested that each *Phyllosticta* species on *Cupressaceae* had been differentiated independently and repeatedly from the endophytic species with polyxeny, or other specific hosts via monoxeny by host-jumping, for example, *P. foliorum* on *Taxus* as shown in clade 2 (Fig. 2–1).

*Phyllosticta* species on Cupressaceae trees in Japan were examined in this study. The results revealed that *P. pilospora* was transferred from *Phoma*, and two species, *P. hostae* and *P. spinarum*, were found in Japan. As mentioned above, the habitat of the Cupressaceae family is in the northern hemisphere and also in the southern hemisphere.

Further studies of the relationship between *Phyllosticta* and Cupressaceae might reveal the evolutionary process involved in the parasitism of *Phyllosticta* species.

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# Chapter 3

## Species Diversity of Genus *Lasiodiplodia* in Japan


## Abstract

To clarify the species diversity of *Lasiodiplodia* in Japan, I examined 30 Japanese isolates based on their morphological, cultural characteristics, and phylogenetic relationships. Phylogenetic analyses using a matrix composed of an internal transcribed spacer, *tef1-α*, *tub2*, and *rpb2* sequences revealed that the examined *Lasiodiplodia* isolates were divided into eleven clades, which corresponds to *L. theobromae*, *L. brasiliensis, L. swieteniae*, *L. pseudotheobromae*, and *Lasiodiplodia* spp. These *Lasiodiplodia* species were also identified based on their morphological characteristics and the optimum growth temperature of each isolate. Moreover, two of the *Lasiodiplodia* isolates were newly added to the Japanese mycoflora, while four other species—*L. latispora, L. parvispora, L. ryukyuensis* and *L. yaguchii* were proposed.



**Keywords:** *Botryosphaeriaceae, DNA phylogeny, new species, Ryukyu islands, Bonin islands*

## Introduction

The genus *Lasiodiplodia* (*Botryosphaeriaceae*) was established by Ellis & Everheart in 1896. This genus is known for the plant pathogenic fungi associated with fruit rots, dieback, and shoot blights of fruit trees, mainly in subtropical and tropical regions (Phillips *et al.* 2013). These species have elliptical to long ellipsoidal conidia, a single septum, light to dark brown, and longitudinal striations following maturation, they also form pycnidial paraphyses (Phillips *et al.* 2013, De Silva *et al.* 2019). However, these characters are not diversified for species limitation. The lack of morphological features caused the uncertain identification of the species, and the inflation of the number of host plants for *L. theobromae*, which is a typical plant pathogenic species in the temperate and tropics, though this is without detailed studies. Crous *et al.* (2006) showed the phylogenic overview of *Botryosphaeriacease* using large subunit rDNA sequences, and they suggested the existence of cryptic genera and species. After that, many cryptic species were found and described from hitherto known host plants (Burgess *et al.* 2006, Alves *et al.* 2008, Abdollahzadeh *et al.* 2010). The type species of this genus, *L. theobromae*, was shown as a species complex, including numerous potential species, from the molecular phylogenetic study using the internal transcribed spacer (ITS) and *tef1-a* regional sequences (Alves *et al.* 2008). It also showed the multi-locus matrix, which was composed of ITS and *tef1-a* coded regions, was insufficient for the identification of *Lasiodiplodia* species (Cruywagen *et al.* 2017, Slippers *et al.* 2017). Subsequently, molecular phylogenetic analysis based on the combined data matrix composed of three regions, ITS, *tef1-a,* and *tub2* proved that it was useful for species classification (Phillips *et al.* 2019, Jayasiri *et al.* 2019).

Therefore, species identification based on molecular phylogenetic analyses is required (Slippers *et al.* 2014). Nowadays, both molecular phylogenetic relationships using multi-locus and morphological features have been the mainstream for the grasp of species diversity within the *L. theobromae* species complex. Moreover, phylogenetic studies revealed several species from a specific host plant. Four species of *Lasiodiplodia* were isolated from *Magnolia*

*candolii* (De Silva *et al.* 2019). In Laos, 12 strains of the genus *Lasiodiplodia* were isolated from *Aquilaria crassna* (Wang *et al.* 2019). Alternatively, in Japan, only *L. theobromae* has been reported as a plant pathogen inflicting various crop diseases, causing disease that affects thirty-three plant genera of twenty-three families (the database of common names of plant disease in Japan, https://www.gene.affrc.go.jp/databases-micro_pl_diseases.php (July 2020). Previous studies have reported *L. parva*, *L. pseudotheobromae*, and *L. theobromae* from a comprehensive fungal survey of the Ogasawara Islands or Miyako Islands, but all of them are identifications that combine ITS or 28S region with morphological information (Sato et al. 2010, Kageyama 2010, Sato et al. 2016, Tanaka et al. 2017). Although the diversity of host plants in Asian countries suggests the richness of the diversity of *Lasiodiplodia* species, it is still unclear due to the lack of comprehensive reexamination of *Lasiodiplodia* species reported from Asian countries.

Therefore, this study identified and reclassified the Japanese isolates of *Lasiodiplodia* species obtained from various hosts to evaluate the taxonomic criteria using their morphological, cultural characteristics, and molecular phylogenetic relationships, and to clarify the diversity of *Lasiodiplodia* species in Japan.

## Materials and Methods

### Sample Collections

Twenty-three strains of the genus *Lasiodiplodia* were stored in the Microbiological Genebank, National Institute of Agrobiological Science (MAFF), five strains isolated from the symptomatic twigs, fruits, and leaves of mangoes in Miyako island, Okinawa Prefecture, and two strains were isolated from mangoes imported from the Philippines showing symptoms. A total of 30 isolates were used (Table 3–1). These strains were cultivated on a potato dextrose agar medium (PDA: Nissui Pharmaceutical Co., Ltd., Japan) or malt agar (MA: Becton Dickinson, USA) at room temperature under room light diffusion. To observe conidiomata and conidia, these were transferred to pine needles agar medium (PNA). The

isolates were stored and maintained in the Culture Collection of Mycological Herbarium, Mie University (MUCC), Tsu, Mie, Japan.

**Morphological and Cultural Characteristics**

For observing the conidiomata and conidia, isolates were cultivated on PNA at room temperature under room light. Fungal structures were examined by light microscopy, using a Zeiss Axio Imager A1 microscope (Zeiss, Oberkochen, Germany). The length and width of mature conidia were measured and recorded, and the L/W (length/width ratio) was calculated. The length and width of the conidiogenous cells were also measured and recorded. The growth temperature test was conducted to know the optimum temperature for colony growth. These strains were cultivated on a PDA at 25°C under dark conditions. After 48 h, the major and minor diameters of the mycelial colony were measured. The initial size of the mycelial disk was subtracted from the grown size of the colony. The same procedure was performed for test sections at temperatures of 25°C, 30°C, and 35°C. The average value of the colony size was calculated for each test section.

**Molecular and phylogenetic analysis**

Genomic DNA was extracted from the mycelial disks after 14 d of culturing on PDA plates with DNeasy UltraClean Microbial Kit (MO QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Targeted sequences of the internal transcribed spacer (ITS), translation elongation factor 1-alpha (*tef1-α*), and DNA-directed RNA polymerase II subunit (*rpb2*) gene region were amplified on T100 Thermal Cycler (BIORAD, Tokyo, Japan) in a total volume of 12.5 µL. The polymerase chain reaction (PCR) mixtures consisted of genomic DNA 1–10 ng, 0.25 unit Bioline *Taq* DNA Polymerase (Bioline, London, UK) 0.05 µL (*tef1-α, rpb2*: 0.1 µL), 10× $NH_4$ reaction buffer (Bioline) 1.25 µL, 2.5 mM $MgCl_2$ (Bioline) 0.5 µL (*tub2*: 0.38 µL), 2.5 mM each of deoxyribonucleotide triphosphate mixture (Bioline) 0.25 µL (*tef1-α, tub2, rpb2*: 0.5 µL), each primer 0.25 µL, 0.7 µL Dimethyl sulfoxide (Sigma-Aldrich, St. Louis, USA) was added for only *tef1-α* amplification, and sterilized distilled water up to

Table 3–1. List of *Lasiodiplodia* isolates used in this study.

| Fungal species | MUCC No. | MAFFT No. | Host Family | Host species | Prefecture | Regions | Identified by previous study | Reference |
|---|---|---|---|---|---|---|---|---|
| *L. brasiliensis* | 2553 | − | Anacardiaceae | *Mangifera indica* | − | − | − | − |
| *L. brasiliensis* | 2725 | 241241 | Caricaceae | *Carica papaya* | Ibaraki | − | *L. theobromae* | − |
| *L. brasiliensis* | 2729 | 242316 | Anacardiaceae | *Mangifera indica* | Tokyo | Ogasawara island | *Lasiodiplodia* sp. | − |
| *L. latispora* | 2739 | 306027 | Rutaceae | *Citrus sinensis* | Tokyo | Ogasawara island | *L. theobromae* | Sato et al. 2010 |
| *L. parvispora* | 2723 | 239129 | Pandanaceae | *Pandanus odoratissimus* | Kagoshima | − | *L. theobromae* | Yumiki et al. 2008 |
| *L. parvispora* | 2724 | 240591 | Xanthorrhoeaceae | *Aloe vera* | Okinawa | Miyako island | *L. theobromae* | Kageyama 2010 |
| *L. pseudotheobromae* | 2726 | 241277 | Rasaceae | *Rosa* sp. | Wakayama | − | *L. theobromae* | − |
| *L. ryukyuensis* | 2732 | 243701 | Asparagaceae | *Agave sisalana* | Tokyo | Ogasawara island | *L. parva* | − |
| *L. ryukyuensis* | 2733 | 243706 | Cannabaceae | *Trema orientalis* | Tokyo | Ogasawara island | *L. parva* | Sato et al. 2016 |
| *L. ryukyuensis* | 2734 | 243707 | Araceae | *Monstera* sp. | Tokyo | Ogasawara island | *L. parva* | − |
| *L. ryukyuensis* | 2737 | 244451 | Malvaceae | *Hibiscus rosa-sinensis* | Tokyo | Ogasawara island | *L. parva* | − |
| *L. ryukyuensis* | 2741 | 306514 | Nymphalidae | *Idea leuconoe* (insects) | Okinawa | Main island | *L. theobromae* | Nago & Matsumoto 1994 |
| *L. ryukyuensis* | 2742 | 306515 | Hernandiaceae | *Hernandia nymphaeifolia* | Okinawa | Ishigaki island | *L. theobromae* | Nago & Matsumoto 1994 |
| *L. ryukyuensis* | 2743 | 306516 | Apocynaceae | *Parsonsia alboflavescens* | Okinawa | Iriomote island | *L. theobromae* | Nago & Matsumoto 1994 |
| *L. ryukyuensis* | 2883 | 237946 | Arecaceae | *Cocos nucifera* | Okinawa | Miyako island | *L. theobromae* | − |
| *L. thailandica* | 2738 | 244514 | Crassulaceae | *Bryophyllum pinnatum* | Tokyo | Ogasawara island | *L. pseudotheobromae* | Tanaka et al. 2017 |
| *L. theobromae* | 2577 | − | Anacardiaceae | *Mangifera indica* | Okinawa | Miyako island | − | − |
| *L. theobromae* | 2590 | − | Anacardiaceae | *Mangifera indica* | Okinawa | Miyako island | − | − |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *L. theobromae* | 2604 | – | Anacardiaceae | *Mangifera indica* | – | – | – | – |
| *L. theobromae* | 2632 | – | Anacardiaceae | *Mangifera indica* | Okinawa | Miyako island | – | – |
| *L. theobromae* | 2740 | 306028 | Annonaceae | *Annona squamosa* | Tokyo | Ogasawara island | *L. theobromae* | Sato et al. 2010 |
| *L. theobromae* | 2746 | 243205 | Malvaceae | *Theobroma cacao* | Tokyo | Ogasawara island | *L. theobromae* | – |
| *L. yaguchii* | 2587 | – | Anacardiaceae | *Mangifera indica* | Okinawa | Miyako island | – | – |
| *L. yaguchii* | 2728 | 241893 | Anacardiaceae | *Mangifera indica* | Tokyo | Ogasawara island | *L. pseudotheobromae* | Sato et al. 2010 |
| *L. yaguchii* | 2735 | 243711 | Asteraceae | *Wollastonia dentata* | Tokyo | Ogasawara island | *L. pseudotheobromae* | Sato et al. 2016 |
| *L. yaguchii* | 2736 | 244435 | Amaryllidaceae | *Crinum asiaticum* | Tokyo | Ogasawara island | *L. pseudotheobromae* | Sato et al. 2016 |
| *Lasiodiplodia* sp. | 2730 | 242320 | Passifloraceae | *Passiflora edulis* | Tokyo | Ogasawara island | *L. theobromae* | – |
| *Lasiodiplodia* sp. | 2731 | 242322 | Arecaceae | *Phoenix roebelenii* | Tokyo | Hachijyo island | *L. theobromae* | – |
| *Lasiodiplodia* sp. | 2744 | 306517 | Apocynaceae | *Anodendron affine* | Okinawa | Main island | *L. theobromae* | Nago & Matsumoto 1994 |
| *Lasiodiplodia* sp. | 2745 | 411002 | Fabaceae | *Laburnum anagyroides* | Tochigi | – | *L. theobromae* | – |

12.5 μL. The conditions of polymerase chain reaction were 2 min (*tub2*: 4 min) of 94 °C, followed by 40 cycles of 94 °C for 45 s (*tef1-α, tub2*: 30 s), the annealing temperature for 30 s, 72 °C for 90 s (*tef1-α*: 45 s, *tub2*: 60 s), and a final elongation at 72 °C for 2 min. The condition for *rpb2* were 5 min of 95 °C, followed by 5 cycles of 95 °C for 45 s, 60 °C for 45 s, 72 °C for 120 s, followed by 5 cycles of 95 °C for 45 s, 58 °C for 45 s, 72 °C for 120 s, followed by 5 cycles of 95 °C for 45 s, 54 °C for 45 s, 72 °C for 120 s, and a final elongation at 72 °C for 8 min. Each primer set and annealing temperature are shown in Table (Table 3–2). The amplicon was sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) on an Applied Biosystems 3730xl DNA Analyzer, which is a DNA sequencing system. The sequences were assembled and aligned with 82 sequences of the genus *Lasiodiplodia* recollected from the DNA Data Bank of Japan GenBank using multiple alignment using fast fourier transform version7 (Katoh *et al.* 2019). Maximum Likelihood (ML) and Bayesian Inference (BI) analyses were used to estimate phylogenetic relationships. ML analyses were performed using raxmlHPC-PTHREADS (Stamatakis 2006). The strength of the internal branches from the resultant trees was tested by bootstrap analysis (Felsenstein, 1985) using 1000 replications in the ML analyses. BI analyses were performed using BEAST v. 2.5.1 (Bouckaert *et al.* 2019) to estimate the posterior probabilities (PPs) of tree topologies based on the metropolis-coupled Markov chain Monte Carlo searches, which used the Markov chain Monte Carlo (MCMC) algorithm of four chains in parallel from a random tree topology. The MCMC analysis lasted until 10,000,000 generations. Trees were sampled and saved every 1,000 generations. The first 25% of saved trees were discarded representing the “burn-in” phase, and the PPs determined from the remaining trees. Representative sequences for all taxa were uploaded to GenBank (Table 3–3). Sequence alignments were deposited in TreeBASE number S26808.

## Results

### Phylogeny

The ITS + *tef-1a* + *tub2* + *rpb2* combined the data matrices of 80 sequences, which consisted of 1575 characters. Out taxa were *Diplodia seriata* (CBS 112555), *D. mutila* (CMW 7060), and *Neodeightonia phoenicum* (CBS 122528). Thirty strains of the *Lasiodiplodia* isolates from Japan have successfully obtained the sequences for ITS, *tef1-a*, *tub2*, and *rpb2* in all four regions. These 30 strains were used for analysis using ML and Bayes methods. The analysis included the sequences of various *Lasiodiplodia* type strains and the isolates obtained in this study. The tree topology of the ML-tree and BI-tree were similar. Fig. 3–1 shows the tree created by the BI method. The *Lasiodiplodia* isolates in this study were classified into eight clades: *L. theobromae* (MUCC 2577, MUCC 2590, MUCC 2604, MUCC 2632, MUCC 2740, and MUCC 2746), *L. brasiliensis* (MUCC 2553, MUCC 2725, and MUCC 2729), *Lasiodiplodia* spp. (MUCC 2723, MUCC 2724, MUCC 2732, MUCC 2733, MUCC 2734, MUCC 2737, MUCC 2741, MUCC 2742, MUCC 2743, and MUCC 2883), *L. swieteniae* (MUCC2738), *Lasiodiplodia* spp. (MUCC 2587, MUCC 2728, MUCC 2730, MUCC 2735, MUCC 2736, MUCC 2730, and MUCC 2744), *Lasiodiplodia* sp. (MUCC 2745), *Lasiodiplodia* sp. (MUCC 2731), *L. pseudotheobromae* (MUCC 2726). In this study, I propose four new species of *Lasiodiplodia* based on molecular phylogenetic analysis and phenotypic characteristics.

Table 3–2. The PCR conditions and primer sets.

| Region | primer F | primer R | Anealing temperature |
|---|---|---|---|
| ITS | V9G (De Hoog & Gerrits van den Ende, 1998) | ITS4 (White et al., 1990) | 48 |
| *tef1-α* | EF1-728F (Carbone & Kohn, 1999) | EF1-986R (Carbone & Kohn, 1999) | 52 |
| *tub2* | BT2A (Glass & Donaldson, 1995) | BT2B (Glass & Donaldson, 1995) | 55 |
| *rpb2* | RPB2-5f2 (Liu et al., 1999) | fRPB2-7cR (Liu et al., 1999) | 60→58→54 |

Table 3–3. List of the genus *Lasiodiplodia* used for phylogenetic analysis.

| Fungal species | Isolates No. | Country | Accession numbers | | | |
|---|---|---|---|---|---|---|
| | | | ITS | tef1 | tub2 | rpb2 |
| *Diplodia mutila* | CMW 7060 | Netherlands | AY236955 | AY236904 | AY236933 | EU339574 |
| *Diplodia seriata* | CBS 112555$^{T}$ | Portugal | AY259094 | AY573220 | DQ458856 | – |
| *L. aquilariae* | CGMCC 3.18471$^{T}$ | Laos | KY783442 | KY848600 | – | KY848562 |
| *L. avicenniae* | LAS 199 | South Africa | KU587957 | KU587947 | KU587868 | KU587880 |
| *L. avicenniae* | CMW 41467$^{T}$ | South Africa | KP860835 | KP860680 | KP860758 | KU587878 |
| *L. avicenniarum* | MFLUCC17–2591$^{T}$ | Thailand | MK347777 | MK340867 | – | – |
| *L. brasiliensis* | CGMCC 3.18480 | Laos | KY783475 | KY848612 | KY848556 | KY848595 |
| *L. brasiliensis* | CMM 4015$^{T}$ | Brazil | JX464063 | JX464049 | – | – |
| ***L. brasiliensis*** | **MUCC 2553** | **Philippines** | **LC567299** | **LC567728** | **LC567758** | **LC567788** |
| ***L. brasiliensis*** | **MUCC 2725** | **Japan** | **LC567307** | **LC567736** | **LC567766** | **LC567796** |
| ***L. brasiliensis*** | **MUCC 2729** | **Japan** | **LC567300** | **LC567729** | **LC567759** | **LC567789** |
| *L. bruguierae* | CMW 41470$^{T}$ | South Africa | KP860833 | KP860678 | KP860756 | KU587875 |
| *L. bruguierae* | CMW 42480 | South Africa | KP860832 | KP860677 | KP860755 | KU587876 |
| *L. caatinguensis* | CMM 1325$^{T}$ | Brazil | KT154760 | KT008006 | KT154767 | – |
| *L. caatinguensis* | IBL 381 | Brazil | KT154757 | KT154751 | KT154764 | – |
| *L. chinensis* | CGMCC 3.18061$^{T}$ | China | KX499889 | KX499927 | KX500002 | KX499965 |
| *L. chinensis* | CGMCC 3.18066 | China | KX499899 | KX499937 | KX500012 | KX499974 |
| *L. citricola* | IRAN 1522C$^{T}$ | Iran | GU945354 | GU945340 | KU887505 | KU696351 |
| *L. citricola* | CBS 124706 | Iran | GU945353 | GU945339 | KU887504 | KU696350 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *L. crassispora* | CBS 118741$^{T}$ | Australia | DQ103550 | DQ103557 | KU887506 | KU696353 |
| *L. crassispora* | CMW 13488 | Venezuela | DQ103552 | DQ103559 | KU887507 | KU696352 |
| *L. curvata* | CGMCC 3.18456$^{T}$ | Laos | KY783437 | KY848596 | KY848529 | KY848557 |
| *L. curvata* | CGMCC 3.18476 | Laos | KY783443 | KY848601 | KY848532 | KY848563 |
| *L. euphorbicola* | CMM 3609$^{T}$ | Brazil | KF234543 | KF226689 | KF254926 | – |
| *L. euphorbicola* | CMW 33350 | Brazil | KU887149 | KU887026 | KU887455 | KU696346 |
| *L. exigua* | BL 184 | Tunisia | KJ638318 | KJ638337 | – | – |
| *L. exigua* | CBS 137785$^{T}$ | Tunisia | KJ638317 | KJ638336 | KU887509 | KU696355 |
| *L. gilanensis* | IRAN 1501C | Iran | GU945352 | GU945341 | KU887510 | KU696356 |
| *L. gilanensis* | IRAN 1523C$^{T}$ | Iran | GU945351 | GU945342 | KU887511 | KU696357 |
| *L. gonubiensis* | CMW 14077$^{T}$ | South Africa | AY639595 | DQ103566 | DQ458860 | KU696359 |
| *L. gonubiensis* | CMW 14078 | South Africa | AY639594 | DQ103567 | EU673126 | KU696358 |
| *L. gravistriata* | CMM 4564$^{T}$ | Brazil | KT250949 | KT250950 | – | – |
| *L. gravistriata* | CMM 4565 | Brazil | KT250947 | KT266812 | – | – |
| *L. hormozganensis* | IRAN 1498C | Iran | GU945356 | GU945344 | KU887514 | KU696360 |
| *L. hormozganensis* | IRAN 1500C$^{T}$ | Iran | GU945355 | GU945343 | KU887515 | KU696361 |
| *L. hyalina* | CGMCC 3.17975$^{T}$ | China | KX499879 | KX499917 | KX499992 | KX499955 |
| *L. iraniensis* | IRAN 1502C | Iran | GU945347 | GU945335 | KU887517 | KU696362 |
| *L. iraniensis* | IRAN 1520C$^{T}$ | Iran | GU945348 | GU945336 | KU887516 | KU696363 |
| *L. irregularis* | CGMCC 3.18468$^{T}$ | Laos | KY783472 | KY848610 | KY848553 | KY848592 |
| *L. jatrophicola* | CMM3610$^{T}$ | Brazil | KF234544 | KF226690 | KF254927 | – |
| *L. laeliocattleyae* | BOT 29 | Egypt | JN814401 | JN814428 | – | – |

| | | | | | | |
|---|---|---|---|---|---|---|
| *L. laeliocattleyae* | CBS 130992$^{T}$ | Egypt | JN814397 | JN814424 | KU887508 | KU696354 |
| *L. laosensis* | CGMCC 3.18464$^{T}$ | Laos | KY783471 | KY848609 | KY848552 | KY848591 |
| *L. laosensis* | CGMCC 3.18473 | Laos | KY783450 | KY848603 | KY848536 | KY848570 |
| *L. latispora* | MUCC 2739$^{T}$* | Japan | LC567320 | LC567749 | LC567779 | LC567809 |
| *L. lignicola* | CBS 134112$^{T}$ | Laos | JX646797 | KU887003 | JX646845 | KU696364 |
| *L. lignicola* | MFLUCC 11-0656 | Thailand | JX646798 | – | JX646846 | – |
| *L. lingnicola* | CGMCC 3.18460 | Thailand | KY783462 | – | – | KY848582 |
| *L. macroconidia* | CGMCC 3.18479$^{T}$ | Laos | KY783438 | KY848597 | KY848530 | KY848558 |
| *L. macrospora* | CMM 3833$^{T}$ | Brazil | KF234557 | KF226718 | KF254941 | – |
| *L. mahajangana* | CMW 27801$^{T}$ | Madagascar | FJ900595 | FJ900641 | FJ900630 | KU696365 |
| *L. mahajangana* | CMW 27818 | Madagascar | FJ900596 | FJ900642 | FJ900631 | KU696366 |
| *L. margaritacea* | CBS 122519$^{T}$ | Australia | EU144050 | EU144065 | KU887520 | KU696367 |
| *L. margaritacea* | CBS 122065 | Australia | EU144051 | EU144066 | – | – |
| *L. mediterranea* | CBS 137783$^{T}$ | Italy | KJ638312 | KJ638331 | KU887521 | KU696368 |
| *L. mediterranea* | CBS 137784 | Italy | KJ638311 | KJ638330 | KU887522 | KU696369 |
| *L. microcondia* | CGMCC 3.18485$^{T}$ | Laos | KY783441 | KY848614 | – | KY848561 |
| *L. missouriana* | UCD 2193MO$^{T}$ | USA | HQ288225 | HQ288267 | HQ288304 | KU696370 |
| *L. missouriana* | UCD 2199MO | USA | HQ288226 | HQ288268 | HQ288305 | KU696371 |
| *L. mitidjana* | MUM 19.90 | Algeria | MN104115 | MN159114 | – | – |
| *L. parva* | CBS 456.78$^{T}$ | Colombia | EF622083 | EF622063 | KU887523 | KU696372 |
| *L. parva* | CBS 494.78 | Colombia | EF622084 | EF622064 | EU673114 | KU696373 |
| ***L. parvispora*** | **MUCC 2723** | **Japan** | **LC567316** | **LC567745** | **LC567775** | **LC567805** |

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|---|---|---|---|---|---|---|
| ***L. parvisporus*** | **MUCC 2724$^{T}$** | **Japan** | **LC567315** | **LC567744** | **LC567774** | **LC567804** |
| *L. plurivora* | CBS 120832$^{T}$ | South Africa | EF445362 | EF445395 | KP872421 | KP872479 |
| *L. pontae* | CMM 1277$^{T}$ | Brazil | KT151794 | KT151791 | KT151797 | – |
| *L. pseudotheobromae* | CBS 116459$^{T}$ | Costa Rica | EF622077 | EF622057 | EU673111 | KU696376 |
| *L. pseudotheobromae* | CGMCC 3.18466 | Laos | KY783444 | KY848615 | KY848533 | KY848564 |
| ***L. pseudotheobromae*** | **MUCC 2726** | **Japan** | **LC567327** | **LC567756** | **LC567786** | **LC567816** |
| *L. pyriformis* | CBS 121770$^{T}$ | Namibia | EU101307 | EU101352 | KU887527 | KU696378 |
| *L. pyriformis* | CBS 121771 | Namibia | EU101308 | EU101353 | KU887528 | KP872484 |
| *L. rubropurpurea* | WAC 12535$^{T}$ | Australia | DQ103553 | DQ103571 | EU673136 | KU696380 |
| *L. rubropurpurea* | WAC 12536 | Australia | DQ103554 | DQ103572 | KU887530 | KU696381 |
| ***L. ryukyuensis*** | **MUCC 2732** | **Japan** | **LC567317** | **LC567746** | **LC567776** | **LC567806** |
| ***L. ryukyuensis*** | **MUCC 2733** | **Japan** | **LC567313** | **LC567742** | **LC567772** | **LC567802** |
| ***L. ryukyuensis*** | **MUCC 2734** | **Japan** | **LC567314** | **LC567743** | **LC567773** | **LC567803** |
| ***L. ryukyuensis*** | **MUCC 2737** | **Japan** | **LC567308** | **LC567737** | **LC567767** | **LC567797** |
| ***L. ryukyuensis*** | **MUCC 2741** | **Japan** | **LC567310** | **LC567739** | **LC567769** | **LC567799** |
| ***L. ryukyuensis*** | **MUCC 2742** | **Japan** | **LC567309** | **LC567738** | **LC567768** | **LC567798** |
| ***L. ryukyuensis*** | **MUCC 2743** | **Japan** | **LC567311** | **LC567740** | **LC567770** | **LC567800** |
| ***L. ryukyuensis*** | **MUCC 2883$^{T}$** | **Japan** | **LC567312** | **LC567741** | **LC567771** | **LC567801** |
| *L. sterculiae* | CBS 342.78$^{T}$ | Germany | KX464140 | KX464634 | KX464908 | KX463989 |
| *L. subglobosa* | CMM 3872$^{T}$ | Brazil | KF234558 | KF226721 | KF254942 | – |
| *L. subglobosa* | CMM 4046 | Brazil | KF234560 | KF226723 | KF254944 | – |
| *L. swieteniae* | MFLUCC 18–0244$^{T}$ | Thailand | MK347789 | MK340870 | MK412877 | – |

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|---|---|---|---|---|---|---|
| ***L. swieteniae*** | **MUCC 2738** | **Japan** | **LC567321** | **LC567750** | **LC567780** | **LC567810** |
| *L. tenuiconidia* | CGMCC 3.18449[T] | Laos | KY783466 | KY848619 | – | KY848586 |
| *L. thailandica* | CBS 138653 | Thailand | KM006433 | KM006464 | – | – |
| *L. thailandica* | CBS 138760[T] | Thailand | KJ193637 | KJ193681 | – | – |
| *L. theobromae* | CBS 111530 | Unknown | EF622074 | EF622054 | KU887531 | KU696382 |
| *L. theobromae* | CBS 164.96[T] | Papua New Guinea | AY640255 | AY640258 | KU887532 | KU696383 |
| ***L. theobromae*** | **MUCC 2577** | **Japan** | **LC567302** | **LC567731** | **LC567761** | **LC567791** |
| ***L. theobromae*** | **MUCC 2590** | **Japan** | **LC567305** | **LC567734** | **LC567764** | **LC567794** |
| ***L. theobromae*** | **MUCC 2604** | **Philippines** | **LC567303** | **LC567732** | **LC567762** | **LC567792** |
| ***L. theobromae*** | **MUCC 2632** | **Japan** | **LC567304** | **LC567733** | **LC567763** | **LC567793** |
| ***L. theobromae*** | **MUCC 2740** | **Japan** | **LC567306** | **LC567735** | **LC567765** | **LC567795** |
| ***L. theobromae*** | **MUCC 2746** | **Japan** | **LC567301** | **LC567730** | **LC567760** | **LC567790** |
| *L. tropica* | CGMCC 3.18477[T] | Laos | KY783454 | KY848616 | KY848540 | KY848574 |
| *L. venezuelensis* | WAC 12540 | Venezuela | DQ103548 | DQ103569 | KP872427 | KP872491 |
| *L. venezuelensis* | CBS 118739[T] | Venezuela | DQ103547 | DQ103568 | KU887533 | KU696384 |
| *L. viticola* | UCD 2553AR[T] | USA | HQ288227 | HQ288269 | HQ288306 | KU696385 |
| *L. viticola* | UCD 2604MO | USA | HQ288228 | HQ288270 | HQ288307 | KU696386 |
| *L. vitis* | CBS 124060[T] | Italy | KX464148 | KX464642 | KX464917 | KX463994 |
| ***L. yaguchii*** | **MUCC 2587[T]** | **Japan** | **LC567322** | **LC567751** | **LC567781** | **LC567811** |
| ***L. yaguchii*** | **MUCC 2728** | **Japan** | **LC567323** | **LC567752** | **LC567782** | **LC567812** |
| ***L. yaguchii*** | **MUCC 2735** | **Japan** | **LC567326** | **LC567755** | **LC567785** | **LC567815** |

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|---|---|---|---|---|---|---|
| ***L. yaguchii*** | **MUCC 2736** | **Japan** | **LC567325** | **LC567754** | **LC567784** | **LC567814** |
| *Lasiodiplodia* sp. | CGMCC 3.18450 | Laos | KY783439 | KY848598 | – | KY848559 |
| *Lasiodiplodia* sp. | CGMCC 3.18459 | Laos | KX871219 | KX871228 | KX871225 | KX871222 |
| *Lasiodiplodia* sp. | CGMCC 3.18462 | Laos | KY783463 | KY848607 | KY848547 | KY848583 |
| *Lasiodiplodia* sp. | CGMCC 3.18463 | Laos | KY783456 | KY848606 | KY848542 | KY848576 |
| ***Lasiodiplodia* sp.** | **MUCC 2730** | **Japan** | **LC567318** | **LC567747** | **LC567777** | **LC567807** |
| ***Lasiodiplodia* sp.** | **MUCC 2731** | **Japan** | **LC567328** | **LC567757** | **LC567787** | **LC567817** |
| ***Lasiodiplodia* sp.** | **MUCC 2744** | **Japan** | **LC567319** | **LC567748** | **LC567778** | **LC567808** |
| ***Lasiodiplodia* sp.** | **MUCC 2745** | **Japan** | **LC567324** | **LC567753** | **LC567783** | **LC567813** |
| *Neodeightonia phoenicum* | CBS 122528$^{T}$ | Spain | EU673340 | EU673309 | EU673116 | – |

$^{T}$ Ex-type, ex-neotype, and ex-epitype strains are indicated. Japanese isolates examined in this study are indicated in bold.

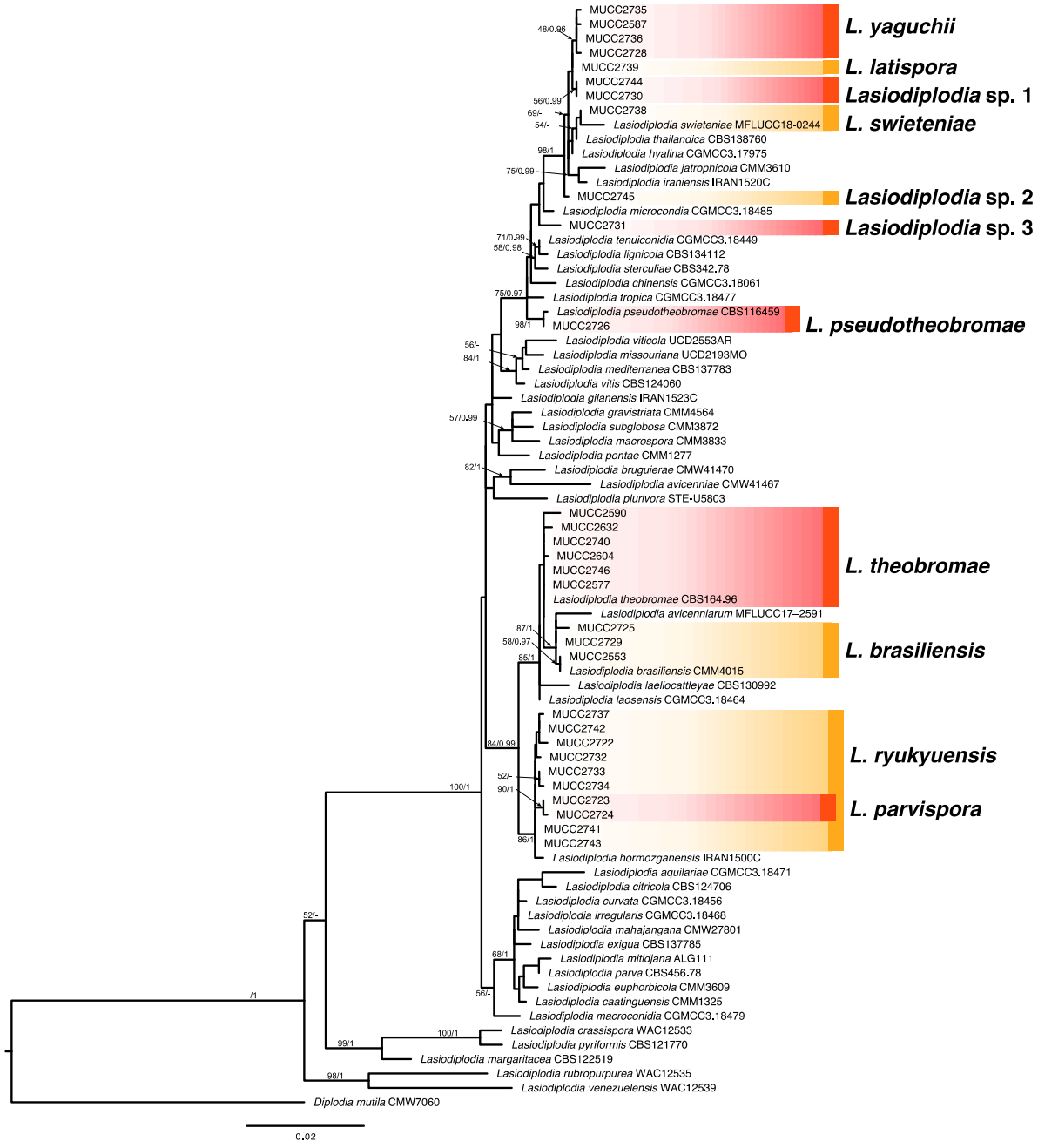


Fig. 3–1. Phylogenetic tree of the *Lasiodiplodia* species constructed by BI using the combined ITS, tef1-α, tub2, and rpb2 gene region datasets. ML bootstrap values and Bayesian posterior probabilities (PP) are given near the branches (ML/PP). Thickened

nodes indicate significant support by ML/PP (>60/0.96). Ex-type and ex-epitype strains are boldly indicated.

**Taxonomy**

***Lasiodiplodia brasiliensis*** M.S.B. Netto, M.W. Marques & A.J.L. Phillips, Fungal Diversity 67: 134, 2014.

Conidiomata formed on PNA within 21−35 days, pycnidial, uniloculate, light gray to dark gray, covered with hyphae; conidiogenous cells were not observed. Conidia oblong or rarely ellipsoidal, rounded at both ends, initially hyaline, aseptate, becoming light brown to dark brown and one-septate after release from the conidiomata, with longitudinal striations, 19–33 × 12–16 µm, L/W=1.93 (Min 1.35, Max 2.45; n=119).

**Cultural Characteristics:** on PDA: Aerial mycelia were dense with light gray colonies, white at the center, olive to dark gray around the edge, or olive at the center and light gray around the edge, reaching 90 mm at 7 d after inoculation (MUCC2553; MUCC2725), and growing between 20°C and 35°C. The optimum temperature for growth was 25–35°C.

**Host:** *Annona muricata*, *Aquilaria crassna*, *Gossypium hirsutum* (Machado *et al.* 2019, Wang *et al.* 2019, Tan *et al.* 2019), *Mangifera indica*, and *Carica papaya* (this study).

**Materials Examined:** on an imported fruit of *Mangifera indica* from the Philippines, July 2, 2018, by *Y. Hattori,* culture MUCC2553; on the fruit of *Carica papaya*, **Japan**, Ibaraki, Tsukuba, December 2008, by *Y. Otani,* culture MAFF 241241 (MUCC2725); on a twig of *Mangifera indica*, **Japan**, Tokyo, Ogasawara, August 2002, by *T. Ono,* culture MAFF 242316 (MUCC2729).

**Note:** From the results of molecular phylogenetic analysis, three examined isolates were located in the same clade as an ex-type isolate of *L. brasiliensis* (CMM4015) and *L. avicenniarum* (MFLUCC17–2591). *L. brasiliensis* has been reported from *Annona muricata* in Brazil, *Aquilaria crassna* in Laos, and *Gossypium hirsutum* in Mexico and

Australia (Machado *et al.* 2019, Wang *et al.* 2019, Tan *et al.* 2019). However, this species had not been reported in Japan. This study is the first report of a new habitat for *L. brasiliensis* and of new host plants, Mango and Papaya. The conidia size of the three examined isolates was shorter than that in the previous study (22.7–29.2 × 11.7–17.0 um in Netto *et al.* 2014), while there was no difference in width. The L/W ratio was also high (1.8 in Netto *et al*. 2014). The optimum growth temperature of this species was at 31.9°C in Netto *et al.* (2014), and it was from 30°C to 35°C in this study. The conidia size of てthe isolates in this study were shorter and wider than *L. avicenniarum* (26–32 × 11–14 um in Jayasiri *et al.* 2019).

***Lasiodiplodia latispora*** L. Nakano, Y. Hattori & C. Nakash., sp. nov. [MB836595], Fig. 3–2.

**Etymology:** Named after the shape of a wider conidia.

Sexual state unknown, Conidiomata formed on PNA within 66 days, pycnidial, uniloculate, light brown to dark brown; paraphyses hyaline, apex rounded, aseptate; conidiogenous cells hyaline, rod shape, holoblastic, 3–3.5 × 10.5–15.5 µm. Conidia ellipsoidal, rounded at both ends, initially hyaline, aseptate or 1-septate, becoming brown and one-septate after release from the conidiomata, without longitudinal striations, rarely with warts on the conidial surface, 18–26 × 11–17 µm, L/W=1.56 (Min 1.26, Max 2.14; n=100).

**Cultural Characteristics:** on PDA: Aerial mycelia were dense with light gray or light brown colonies, the appearance of black spots, reaching 90 mm 15 d after inoculation (MUCC 2739), and growing between 20°C and 35°C. The optimum temperature for growth was 20°C–30°C.

**Host:** *Citrus sinensis* (this study).

**Materials Examined:** on *Citrus sinensis*, **Japan**, Tokyo, Ogasawara, 1986, by *T. Sato*., culture MAFF 306027 (MUCC 2739).

**Holotypus:** The dried culture of MUCC2739 isolated from *Citrus sinensis*, **Japan**,

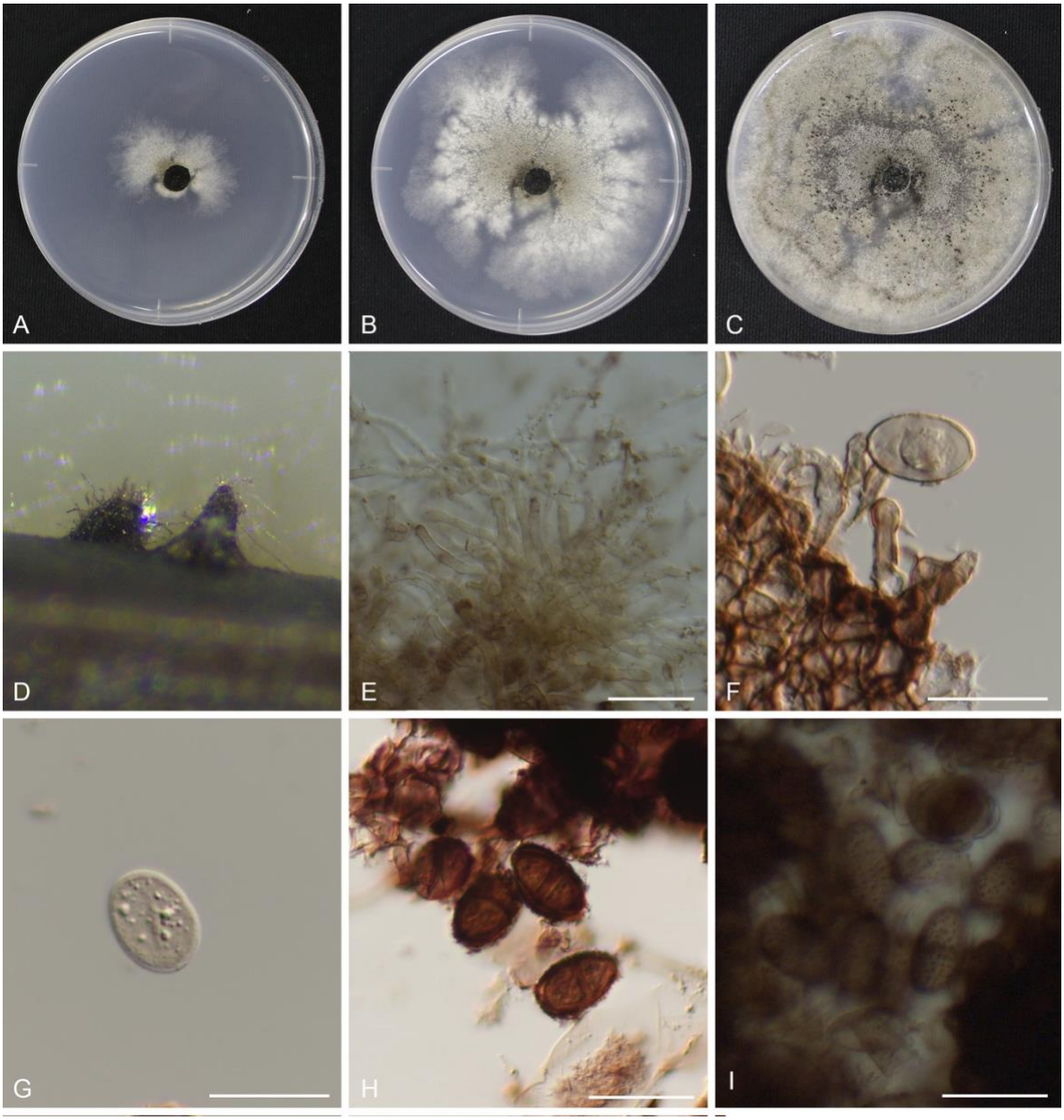


Fig. 3–2: Morphological features of *Lasiodiplodia latispora* (MUCC 2739). **A–C**. Colony on PDA; **A.** after 3 d, **B**. after 7 d, **C**. after 15 d, **D**. Conidiomata formation on the PNA, **E**. Paraphyses, **F.** Conidiogenous cell **G**. Immature conidia, **H**. Mature conidia, and **I**. Warts on the conidia surface. Scale bars = E–H: 20 µm.

Tokyo, Ogasawara, 1986, by *T. Sato*., ex-holotype MAFF 306027 (MUCC 2739).

**Note:** From molecular phylogenetic analysis, the ex-type strain of the present species formed a sister group with a statistically-moderate supported species, which included *L. yaguchii* (BI pp: 0.98), and *Lasiodiplodia* sp. 1 (ML bs: 57; BI pp: 0.99). Morphological features revealed the conidia with no septum even when they mature, no longitudinal striations, also, black spots on the culture were observed. Moreover, the L/W ratio of conidia reached a value (1.56), which is significantly lower than *L. yaguchii* (1.79). From these characters and the host plant, they were considered a new species.

***Lasiodiplodia parvispora*** L. Nakano, Y. Hattori & C. Nakash., sp. nov. [MB836596], Fig. 3–3.

**Etymology**：Named after the small conidia.

Sexual state unknown, Conidiomata formed on PNA within 21–48 days, pycnidial, uniloculate, light gray to light brown, covered with hyphae; paraphyses hyaline, apex rounded, aseptate; conidiogenous cells hyaline, cylindrical or rod shape, holoblastic, 4.90–15.03 × 1.73–7.75 µm (n=24). Conidia ellipsoidal, rarely obovoid, rounded at both ends, initially hyaline, aseptate, becoming light brown to dark brown and one-septate after release from the conidiomata, with longitudinal striations, 15.01–23.60 × 8.84–13.33 µm, L/W=1.75 (Min 1.32, Max 2.23; n=200).

**Cultural Characteristics:** on PDA: Aerial mycelia were dense with colonies that were gray at the center, white to olive-buff around, and gray at the edge, reaching 90 mm at 7 d after inoculation (MUCC 2723; MUCC 2724), and growing between 20°C and 35°C. The optimum temperature for growth was 25°C–35°C.

**Host:** *Aloe vera*, *Pandanus odoratissimus* (this study).

**Materials Examined:** on *Pandanus odoratissimus*, **Japan**, Kagoshima, Oshima, November 2001, by *T. Kobayashi*, culture MAFF 239129 (MUCC 2723), on *Aloe vera,* **Japan**, Okinawa, Miyako, September 2007, by *Y. Hirooka*., culture MAFF 240591 (MUCC 2724).

**Holotypus**: Dried culture MUCC2724 isolated from *Aloe vera*, **Japan**, Okinawa, Miyako, September 2007, by *Y. Hirooka*., ex-holotype MAFF 240591 (MUCC 2724).

**Note:** The molecular phylogenetic analysis revealed the formation of a sister group with *L. hormozganensis* and *L. ryukyuensis*. It is different from *both species* because it forms an independent clade with high support of ML, and Bayes (ML bs: 88; BI pp: 0.99). Besides, the size of mature conidia was smaller than that of *L. hormozganensis* and *L. ryukyuensis* (Table 3–4), and the optimum temperature for colony growth was higher than that of *L. hormozganensis* (25°C–30°C).

***Lasiodiplodia pseudotheobromae*** A.J.L. Phillips, A. Alves & Crous, Fungal Diversity 28: 8, 2008.

**Cultural Characteristics:** on PDA: Aerial mycelia were dense with white colonies reaching 90 mm 7 d after inoculation (MUCC 2726), and growing between 20°C and 35°C. The optimum temperature for growth was 35°C.

**Host:** *Gmelina arborea* (Phillips *et al.* 2008), *Rosa* sp. (Alves *et al.* 2008, Tan *et al.* 2019, this study).

**Materials Examined:** on *Rosa* sp.*,* **Japan**, Wakayama, September 2008, by *Y. Otani*, culture MAFF 241277 (MUCC 2726).

**Note**: Light to dark brown mycelium covered the pine leaves at 39 d, but conidiomata was not formed. The results from molecular phylogenetic analysis revealed that MUCC2726 and the type isolate of *L. pseudotheobromae* (CBS116549) were located in the same clade. A study reported that the isolate of *L. pseudotheobromae* produced a pink pigment on PDA at 35°C (Alves *et al.* 2008). However, it was not observed on the medium using MUCC 2716 in the same conditions. *Lasiodiplodia pseudotheobromae* has been isolated from various hosts in the world, and it has also been reported from plant genus *Rosa* in the Netherlands and Australia (Alves *et al.* 2008, Tan *et al.* 2019).

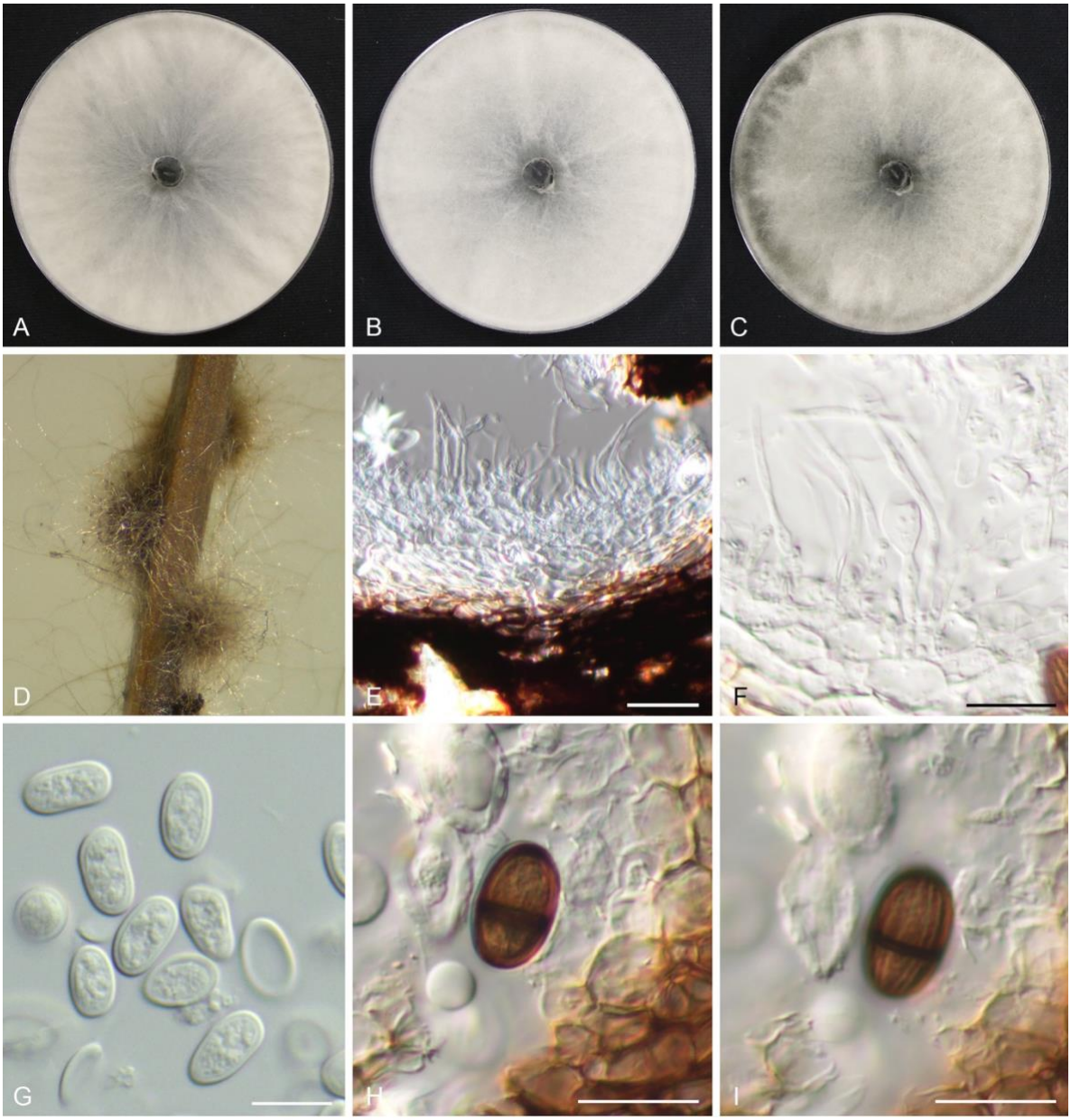


Fig. 3–3: Morphological features of *Lasiodiplodia parvispora* (MUCC 2724). **A–C**. Colony on PDA; **A.** after 3 d, **B**. after 7 d, **C**. after 15 d, **D**. Conidiomata formation on the PNA, **E**. Paraphyses, **F.** Conidiogenous cells, **G**. Immature Conidia, **H**. Mature conidium, and **I**. Longitudinal striations on mature conidium. Scale bars = E–H: 20 µm.

***Lasiodiplodia ryukyuensis*** Y. Hattori, L. Nakano & C.Nakash., sp.nov. [MB836597], Fig. 3–4.

**Etymology**: Named after the Ryukyu islands, where were collected the specimens.

Sexual state unknown, Conidiomata formed on PNA within 13–52 days, pycnidial, uniloculate, light gray to dark gray, covered with hyphae; paraphyses hyaline, rounded at both ends, aseptate; conidiogenous cells hyaline, cylindrical, holoblastic, 7.5–9.5 × 2.5–3 µm (n=2). Conidia ellipsoidal or rarely oblong, rounded at both ends, initially hyaline, aseptate, becoming light brown to dark brown and one-septate after release from the conidiomata, with longitudinal striations, 9–27.5 × 10–15 µm、L/W=1.71 (Min 1.33, Max2.07; n=188).

**Cultural Characteristics:** on PDA: Aerial mycelia were dense with colonies that were dark gray at the center, light gray around the edge, or dark gray to dark olive at the center and light gray around the edge, reaching 90 mm 7 d after inoculation (MUCC 2732; MUCC 2733; MUCC 2734; MUCC 2737; MUCC 2742; MUCC 2883), and growing between 20°C and 35°C. The optimum temperature for growth varies between 20°C–30°C (MUCC 2737), 30°C (MUCC 2742), 35°C (MUCC 2883, MUCC 2733, MUCC 2732), and 30°C–35°C (MUCC 2734).

**Host:** *Agave sisalana*, *Trema orientalis*, *Monstera* sp., *Hibiscus rosa-sinensis*, *Idea leuconoe*, *Hernandia nymphaeifolia*, *Parsonsia alboflavescens*, *Cocos nucifera* (this study).

**Materials Examined: on** *Cocos nucifera*, **Japan**, Okinawa, Miyako, November 1991, by *T. Kobayashi*, culture MAFF 237946 (MUCC2883), on *Agave sisalana*, **Japan**, Tokyo, Ogasawara, September 2012, by *T. Sato*, culture MAFF 243701 (MUCC2732); on *Trema orientalis*, **Japan**, Tokyo, Ogasawara, September 2012, by *T. Sato*, culture MAFF 243706 (MUCC2733); on *Monstera* sp., **Japan**, Tokyo, Ogasawara, August 2002, by *T. Sato*, culture MAFF 243707 (MUCC2734); on *Hibiscus rosa-sinensis*, **Japan**, Tokyo, Ogasawara, September 2013, by *T. Sato*, culture MAFF 244451 (MUCC 2737); on *Idea leuconoe*, **Japan**, Okinawa, main island of Okinawa, October 1991, by *H. Nago*,

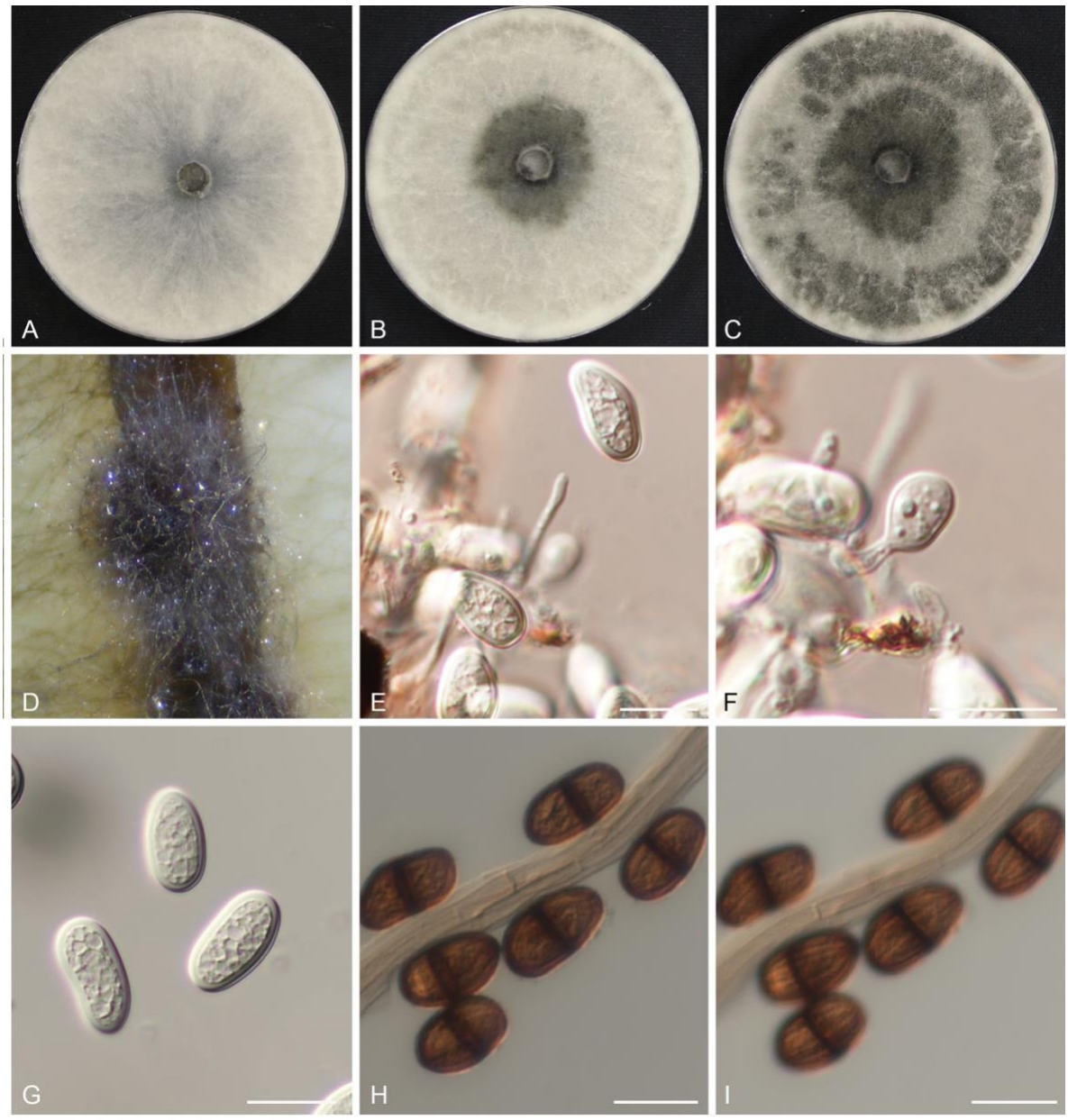


Fig. 3–4: Morphological features of *Lasiodiplodia ryukyuensis* (MUCC 2883). **A–C**. Colony on PDA; **A.** after 3 d, **B**. after 7 d, **C**. after 15 d, **D**. Conidiomata forming on the PNA, **E**. Paraphyses, **F.** Conidiogenous cell, **G**. Immature Conidia, **H**. Mature conidia, and **I**. Longitudinal striations on mature conidia. Scale bars = E–H: 20 µm.

culture MAFF 306514 (MUCC 2741); on *Hernandia nymphaeifolia*, **Japan**, Okinawa, February 1993, by *H. Nago*, culture MAFF 306515 (MUCC2742); on *Parsonsia alboflavescens*, **Japan**, Okinawa, Ishigaki, February 1993, by *H. Nago*, culture MAFF 306516 (MUCC2743).

**Holotypus**: Dried culture MUCC 2883 isolated from *Cocos nucifera*, **Japan**, Okinawa, Miyako, November 1991, by *T. Kobayashi*, ex-type culture MAFF 237946 (MUCC2883).

**Note**: From the molecular phylogenetic tree, eight isolates examined in this study formed sister groups with the ex-type culture of *L. hormozganensis* (IRAN1500C). The clade composed of two cultures of *L. hormozganensis* with high support from both ML and Bayes (ML bs: 74%; pp: 0.98) differing from the other eight cultures, after which the cultures were judged as different species. Besides, *L. hormozganensis* had its optimum growth temperature at 25°C–30°C (Abdollahzadeh *et al.* 2010). However, many of the eight cultures have their optimum growth temperature at 30°C and higher. *Lasiodiplodia hormozganensis* was described from Iran and subsequently reported from the Middle East countries, Brazil, and Australia (Abdollahzadeh *et al.* 2010, Urbez-Torres *et al.* 2012, Marques *et al.* 2013, Burgess *et al.* 2019). Only two cases from China and Malaysia have been reported in East Asia (Li *et al.* 2015, Kee *et al.* 2019).

***Lasiodiplodia swieteniae*** Jayasiri, E.B.G. Jones & K.D. Hyde, Mycosphere10: 143, 2019.

Conidiomata formed on PNA within 39 days, pycnidial, uniloculate, dark brown, covered with hyphae; Conidiogenous cells hyaline, cylindrical or rod shape, holoblastic, 4.5–14.5 × 1.8–4.5 µm, L/W=3.21 (n=16). Conidia ellipsoidal, rounded at both ends, initially hyaline, aseptate, becoming light brown to dark brown and one-septate after release from the conidiomata, with longitudinal striations, 19.5–28 × 12–16 µm, L/W = 1.71 (Min 1.43, Max 2.04; n=40).

**Cultural Characteristics:** on PDA: Aerial mycelia were dense with light brown to olive colonies reaching 90 mm 7 d after inoculation (MUCC), and growing between 20°C and

35°C. The optimum temperature for growth was 25°C–30°C.

**Host:** *Swietenia* sp. (Jayasiri et al. 2019), *Kalanchoe pinnata* (this study).

**Materials Examined:** on *Kalanchoe pinnata*, **Japan**, Tokyo, Ogasawara, September 2012, by *K. Tanaka*, culture MAFF 244514 (MUCC2738).

**Note**: From the molecular phylogenetic analysis, MUCC2738 was located in the same clade as the type culture of *L. swieteniae* (MFLUCC 18–0244). Morphologically, the size of mature conidia of MUCC2738 was smaller than *L. swieteniae*. Mature conidia of *L. swieteniae* had 1–3 septa; however, the examined culture had one septum (Table 3–4). So far, *L. swieteniae* has been reported only from woody plants such as *Swietenia* sp. in Thailand (Jayasiri et al. 2019). This study is the first to report the present species from herbaceous plants.

***Lasiodiplodia theobromae*** (Pat.) Griffon & Maubl., Bulletin de la Société Mycologique de France 25: 57, 1909.

Conidiomata formed on PNA within 12−64 days, pycnidial, uniloculate, light gray to dark gray, covered with hyphae; Conidiogenous cells hyaline, cylindrical or rod shape, holoblastic, 6.46–27.61 × 2.01–6.08 µm (n=21). Conidia ellipsoidal or ovoid, rounded at both ends, initially hyaline, aseptate or rarely one-septate, becoming dark brown and one-septate after release from the conidiomata, with longitudinal striations, 19.24–29.98 × 11.23–17.79 µm, L/W=1.79 (Min 1.28, Max 2.33; n=341).

**Cultural Characteristics:** on PDA: Aerial mycelia were dense with colonies, which were light-dark gray, light gray, olive-buff, or dark gray at the center, white around the edge, or white at the center and light brown around the edge, reaching 90 mm 7 d after inoculation (MUCC2553; MUCC2725) and growing between 20°C and 35°C. The optimum temperature for growth was 30°C–35°C.

**Host:** *Mangifera indica*, *Annona squamosa*, T*heobroma cacao* (this study).

**Materials Examined**: on fruit of *Mangifera indica*, **Japan**, Okinawa, Miyako, July 31, 2018, by *Y. Hattori*, culture MUCC2577; on stem of *Mangifera indica*, **Japan**, Okinawa,

Miyako, August 2, 2018, by *Y. Hattori*, culture MUCC 2590; on branch of *Mangifera indica*, **Japan**, Okinawa, Miyako, October 23, 2018, by *Y. Hattori*, culture MUCC 2632; on *Mangifera indica* imported from the Philippines, **Japan**, May 16, 2018, by *S. Kitabata*, culture MUCC2604; on *Annona squamosa*, **Japan**, Tokyo, Ogasawara, 1986, by *T. Sato*, culture MAFF 306028 (MUCC 2740); on *Theobroma cacao*, **Japan**, Tokyo, Ogasawara, October 2011, by *T. Sato*, culture MAFF 243205 (MUCC 2746).

**Note**: From the results of molecular phylogenetic analysis, the six cultures examined in this study were in the same clade as *L. theobromae*. Observation of morphological characteristics revealed that the size and L/W ratio of mature conidia of these isolates fell within the range of those of *L. theobromae* (Table 3–4). *Lasiodiplodia theobromae* has been isolated from various plants, mainly in the tropical and subtropical regions, as the pathogen of stem-end rot disease. As mentioned above, *L. theobromae* has been treated as the only species causing the stem-end rot disease in Japan without the detailed study. Therefore, the current taxonomic position of *L. theobromae* cultures deposited in the Japanese culture collection was unclear. In this study, the Japanese cultures isolated from three hosts, *M. indica*, *A. squamosa*, and *T. cacao*, were confirmed as a narrow sense *L. theobromae*.

***Lasiodiplodia yaguchii*** Y. Hattori, L. Nakano & C. Nakash., sp. nov. [MB836598], Fig. 3–5.

**Etymology:** Named after, Prof. Yukio Yaguchi who is the Japanese plant pathologist of Mango.

Sexual state unknown, Conidiomata formed on PNA within 26–39 days, pycnidial, uniloculate, light brown to dark brown, covered with hyphae; conidiogenous cells hyaline, cylindrical or rod shape, holoblastic, 7.5–12 × 1.8–4.4 µm, L/W=3.09 (n=16). Conidia ellipsoidal or rarely oblong, rounded at both ends, initially hyaline, aseptate, becoming light brown to dark brown and one-septate after release from the conidiomata, with longitudinal striations, 19–26 × 11–15 µm, L/W=1.79 (Min 1.35, Max 2.22; n=117).

**Cultural Characteristics:** on PDA: Aerial mycelia were dense with light gray to light brown colonies, reaching 90 mm at 3 d after inoculation (MUCC 2578; MUCC 2728; MUCC 2735; MUCC2736) and growing between 20°C and 35°C. The optimum temperature for growth was 25°C–30°C.

**Host:** *Crinum asiaticum, Mangifera indica, Wollastonia dentata* (this study).

**Materials Examined:** on the fruit of *Mangifera indica*, **Japan**, Okinawa, Miyako Island, July 2018, by *Y. Hattori*, culture MUCC 2587; in the flesh water, **Japan**, Tokyo, Ogasawara, June 2009, by *S. Uzuhashi*, culture MAFF241893 (MUCC 2728); on *Wollastonia dentata*, **Japan**, Tokyo, Ogasawara, Chichijima Island, September 2012, by *T. Sato,* culture MAFF 243711 (MUCC 2735), on *Crinum asiaticum*, **Japan**, Tokyo, Ogasawara, September 2013, by *T. Sato*, culture MAFF 244435 (MUCC 2736).

**Holotypus**: Dried culture of MUCC 2587, isolated from *Mangifera indica*, **Japan**, Okinawa, Miyako Island, July 2018, by *Y. Hattori,* culture ex-type MUCC 2587.

**Note:** On the molecular phylogenetic analysis, this species formed a single clade. The clade composed of the four examined isolates was moderately supported by the statistical scores of Bayes (BI pp: 0.98). Further, as a morphological feature, *L. yaguchii* does not form the paraphyses.

***Lasiodiplodia* sp. 1**

**Cultural Characteristics:** on PDA: Aerial mycelia were dense with light to dark gray colonies reaching 90 mm 3 d after inoculation (MUCC 2730; MUCC 2744), and growing between 20°C and 35°C. The optimum temperature for growth was 25°C–30°C.

**Host:** *Passiflora edulis*, *Anodendron affine* (this study).

**Materials Examined:** on *Passiflora edulis,* **Japan**, Tokyo, Ogasawara, Chichijima, April 2003, by *T. Ono*, culture MAFF 242320 (MUCC 2730); on *Anodendron affine,* **Japan**, Okinawa, main island of Okinawa, February 1993, by *H. Nago*, culture MAFF 306517 (MUCC 2744).

**Note**: The examined cultures were kept at the culture collection as *L. theobromae*. From

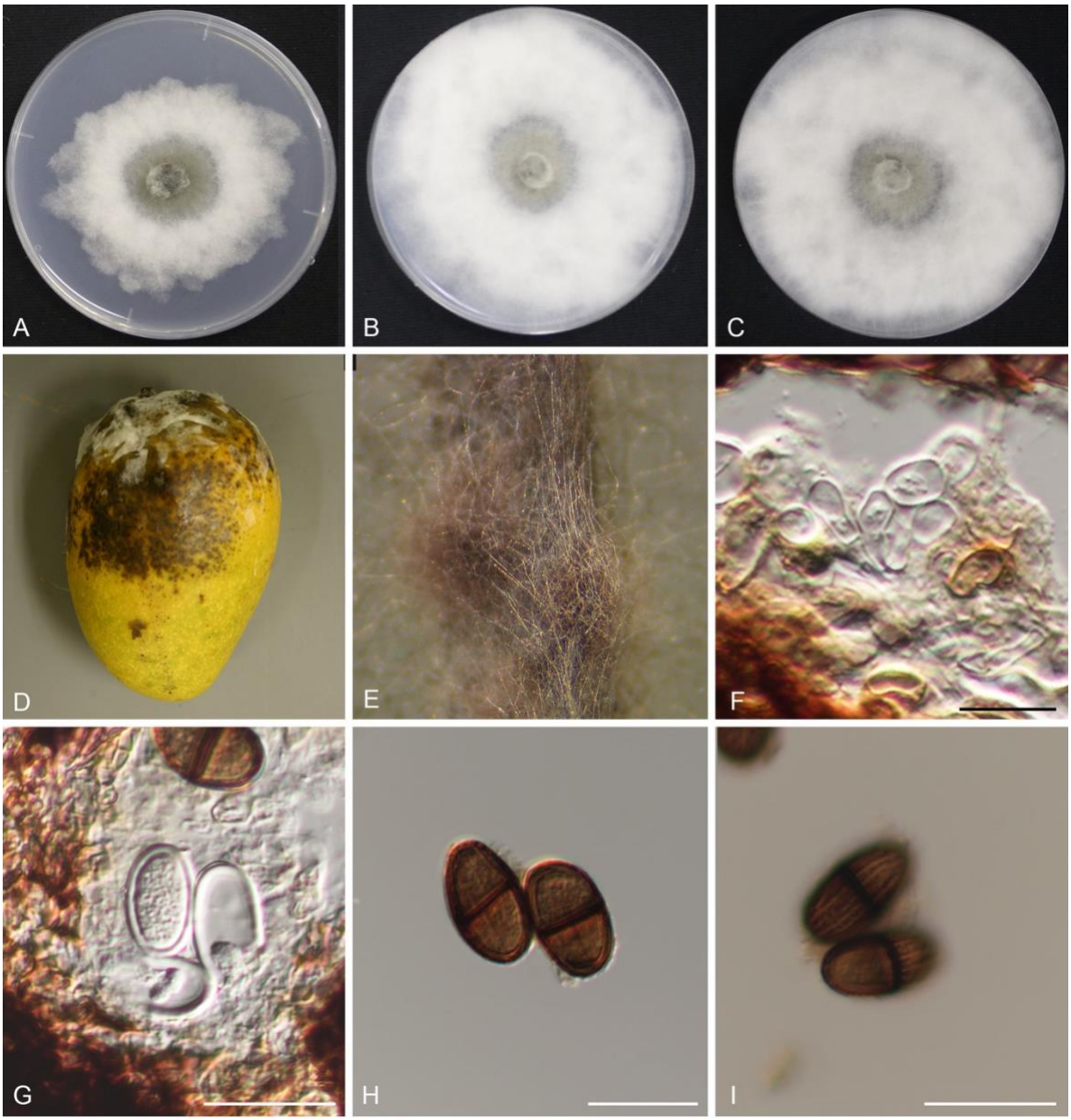


Fig. 3–5: Morphological features of *Lasiodiplodia yaguchii* (MUCC 2587 & MUCC 2735). **A–C**. Colony on PDA (MUCC 2587); **A.** after 3 d, **B**. after 7 d, **C**. after 15 d, **D**. Symptoms found on *Mangifera indica*, **E**. Conidiomata formation on the PNA (MUCC 2735), **F**. Conidiogenous cells (MUCC 2735), **G**. Immature Conidia (MUCC 2735) **H**. Mature conidia, and **I**. Longitudinal striations on mature conidia (MUCC 2735). Scale bars = E–H: 20 µm.

the molecular phylogenetic tree, these two cultures formed a moderately supported clade (ML bs: 57%; BI pp: 0.99), which differed from *L. theobromae*. It should, therefore, be treated as an independent species. Alternatively, conidiomata was not observed on the medium, whereas light to dark gray mycelium covered the pine leaves at 48–88 d. This time, it was abandoned and a new name was described.

***Lasiodiplodia* sp. 2**

**Cultural Characteristics:** on PDA: Aerial mycelia were dense with light gray colonies reaching 90 mm 3 d after inoculation (MUCC 2745), and growing between 20°C and 35°C. The optimum temperature for growth was 25°C–35°C.

**Host:** *Laburnum anagyroides* (this study).

**Materials Examined:** on *Laburnum anagyroides,* **Japan**, Tochigi, Ashikaga, December 2004, by *T. Kobayashi*, culture MAFF 411002 (MUCC 2745).

**Note**: Although conidiomata covered with light to dark gray hyphae were formed on pine leaves within 21 days, conidiogenous cells and conidia were not formed within a conidioma. From the molecular phylogenetic tree, MUCC2745 did not form clades with any other operational taxonomic units (OTUs). However, it was closely related to the clades containing *L. hyalina*, *L. jatrophicola*, *L. iraniensis*, *L. latispora*, *L. swieteniae*, *L. thailandica, L. yaguchii, and Lasiodiplodia* sp.1. The phenotypic characters of MUCC2745 suggested that this culture is an independent species, which has its optimum temperature for colony growth to be higher than that of *L. iraniensis* (MUCC2745: 25°C–35°C vs. *L. iraniensis*: 25°C–30°C) (Abdollahzadeh *et al.* 2010). Moreover, it was higher than the strains examined in this study (*L. latispora*: 20°C–30°C, *L. swieteniae*: 25°C–30°C, *L. yaguchii*: 25°C–30°C, and *Lasiodiplodia* sp.1: 25°C–30°C).

***Lasiodiplodia* sp. 3**

**Cultural Characteristics:** on PDA: Aerial mycelia were dense with dark gray colonies at the center, olive around the edge reaching 90 mm 7 d after inoculation (MUCC), and

growing between 20°C and 35°C. The optimum temperature for growth was at 30°C.

**Host:** *Phoenix roebelenii* (this study).

**Materials Examined:** on *Phoenix roebelenii,* **Japan**, Tokyo, Hachijojima Is., May 1999, by *J. Takeuchi*, culture MAFF 242322 (MUCC 2731).

**Note**: Although dark gray mycelium covered pine leaves 89 d after inoculation on PDA medium, conidiomata was not formed on it. Results of the molecular phylogenetic analysis showed that MUCC2731 did not form clades with any other OTUs, and was located on the basal position of the clade containing *L. chinensis*, *L. microcondia*, *L. lignicola*, *L. pseudotheobromae*, *L. sterculiae*, *L. tenuiconidia*, and *L. tropica*.

## Discussion

In this study, cultures belonging to the genus *Lasiodiplodia,* isolated from various plants in Japan, were re-examined by molecular phylogenetic analysis and observation of their morphological characteristics. From the result, those cultures can be divided into eleven clades. Four of the eleven clades were recognized as hitherto known species, *L. theobromae*, *L. brasiliensis*, *L. swieteniae*, and *L. pseudotheobromae*. *L. brasiliensis* and *L. swieteniae* were newly added to the Japanese mycoflora. The other seven clades were new species, *L. latispora, L. parvispora, L. ryukyuensis, L. yaguchii* and three potential new species. Each morphological characteristics of conidia on the medium of four clades were observed and described as new species.

Phylogenetic analysis with the matrix composed of multi-locus sequences revealed the species diversity of the genus *Lasiodiplodia*, which led to the reexamination of pathogens described as *L. theobromae.* It showed the existence of cryptic species. Currently, 13 species of *Lasiodiplodia* is known in East Asia (USDA Fungal databases https://nt.ars-grin.gov/fungaldatabases/fungushost/new_frameFungusHostReport.cfm) (July 2020). However, only a few species of the genus *Lasiodiplodia,* typified by *L.*

Table 3–4. List of morphological characteristics of the genus *Lasiodiplodia.*

| Species | Conidia | | | | Literature |
|---|---|---|---|---|---|
| | Septate | Conidial bodies (µm) | Average | L/W | |
| *L. aquilariae* | 1 | (23–)25–28(–29) × 12–16 | 26.9 × 14.1 | 1.8 | Wang et al. (2019) |
| *L. avicenniae* | 1 | (19–)24–26(–30) × (9–)12–12.5(–15) | – | – | Osorio et al. (2017) |
| *L. avicenniarum* | 1 | 26–32 × 11–14 | 28 × 12 | – | Jayasiri et al. (2019) |
| *L. brasiliensis* | 1 | 22.7–29.2 × 11.7–17.0 | 26.0 × 14.7 | 1.8 | Netto et al. (2014) |
| | – | 20.3–27.4 × 10.4–14.7 | – | 1.85 | Coutinho et al. (2017) |
| | **1** | **19.1–32.9 × 12.0–16.1** | **26.6 × 13.9** | **1.93** | **this study** |
| *L. bruguierae* | 1 | (19–)25–26(–32) × (11–)12–13(–15) | – | – | Osorio et al. (2017) |
| *L. caatinguensis* | 1 | 13–20.2 × 10.1–12.6 | 18.2× 11.8 | 1.75 | Coutinho et al. (2017) |
| *L. chinensis* | – | (18–)19–25 × 12–14 | 21.9 × 12.6 | 1.75 | Dou et al. (2017a) |
| *L. citricola* | 1 | (20–)22–27(–31) × (10.9–)12–17(–19) | 24.5 × 15.4 | 1.6 | Abdollahzadeh et al. (2010) |
| *L. crassispora* | 1 | 27–30 × 14–17 | 28.8 × 16.0 | 1.8 | Burgess et al. (2006) |
| *L. curvata* | 1 | (18–)20–24(–25) × 12–15 | 23.6 × 13.8 | 1.7 | Wang et al. (2019) |
| *L. egyptiacae* | 1 | (17–)20–24(–27) × (11–)11–12(–13) | 22 × 12 | 1.8 | Ismail et al. (2012) |
| | – | 22–29 × 14–17 | – | – | Machado et al. (2014) |
| *L. euphorbicola* | 1 | 15–23 × 9–12 | – | – | Machado et al. (2014) |
| | – | 15.1–22.5 × 8.6–13.7 | – | 1.7 | Coutinho et al. (2017) |
| *L. exigua* | – | (19.6–)21.8(–24.3) × (10.8–)12.3(–13.3) | – | 1.8 | Linaldeddu et al. (2015) |
| *L. gilanensis* | 1 | (25.2–)28–35(–38.8) × (14.4–)15–18(–19) | 31 × 16.6 | 1.9 | Abdollahzadeh et al. (2010) |

| | | | | | |
|---|---|---|---|---|---|
| *L. gonubiensis* | 1–3 | (28–)32–36(–39) × (14–)16–18.5(–21) | 33.8 × 17.3 | 1.9 | Pavlic et al. (2004) |
| *L. gravistriata* | 1 | 24.5–28.5 × 10.5–16 | 26.2 × 13.8 | 1.9 | Netto et al. (2017) |
| *L. hormozganensis* | 1 | (15.3–)18–24(–25.2) × 11–14 | 21.5 × 12.5 | 1.7 | Abdollahzadeh et al. (2010) |
| | – | 19–22.8 × 10.7–11.7 | – | 1.9 | Netto et al. (2014) |
| *L. hyalina* | 1 | (19–)20–27(–28) × 12–16 | 24.0 × 13.6 | 1.7 | Dou et al. (2017b) |
| *L. iraniensis* | 1 | (15.3–)17–23(–29.7) × 11–14 | 20.7 × 13 | 1.6 | Abdollahzadeh et al. (2010) |
| *L. irregularis* | 1–2 | (20–)22–29(–30) × (12–)13(–15) | 24.8 × 13.6 | 1.8 | Wang et al. (2019) |
| *L. jatrophicola* | 1 | 22–26 × 14–17 | – | – | Machado et al. (2014) |
| *L. laosensis* | 1 | (23–)24–28(–30) × (13–)14–15(–17) | 25.8 × 14.9 | 1.7 | Wang et al. (2019) |
| ***L. latispora*** | **0–1** | **18.4–25.7 × 11.5–16.7** | **21.6 × 13.9** | **1.56** | **this study** |
| *L. lignicola* | – | (15–)16–17.5 × (8–)8.5–10.5(–11) | – | 1.7 | Phillips et al. (2013) |
| *L. macroconidia* | 1 | (26–)28–34(–36) × 13–16 | 29.5 × 14.6 | 2 | Wang et al. (2019) |
| *L. macrospora* | 1 | 28–35 × 15–17 | – | – | Machado et al. (2014) |
| *L. mahajangana* | 1 | (13.5–)15.5–19(–21.5) × (10–)11.5–13(–14) | 17.5 × 11.5 | 1.4 | Begoude et al. (2010) |
| *L. margaritacea* | 1 | (12–)14–17(–19) × (10–)11–12(–12.5) | 15.3 × 11.4 | 1.3 | Pavlic et al. (2008) |
| *L. mediterranea* | – | (26.3–)30.6(–37) × (13.5–)16.1(–18) | – | 1.9 | Linaldeddu et al. (2015) |
| *L. mitidjana* | – | (22.6–)27.7(−31.9) × (13.5–)16.7(−19.6) | 27.7 × 16.7 | 1.7 | Berraf-Tebbal et al. (2020) |
| *L. microconidia* | 1 | (18–)19–22(–23) × 10–15 | 20.8 × 13.2 | 1.5 | Wang et al. (2019) |
| *L. missouriana* | 1 | (16.1–)17.4–19.6(–21) × (8.1–)8.9–10.6(–11.8) | 18.5 × 9.8 | 1.89 | Urbez-Torres et al. (2012) |
| *L. parva* | 1 | (15.5–)16–23.5(–24.5) × (10–)10.5–13(–14.5) | 20.2 × 11.5 | 1.8 | Alves et al. (2008) |
| | 1 | 15–23 × 10–13 | – | – | Machado et al. (2014) |

| | | | | | |
|---|---|---|---|---|---|
| ***L. parvispora*** | **1** | **15.0–23.6 × 8.9–13.3** | **19.5× 11.2** | **1.75** | **this study** |
| *L. plurivora* | 1 | (22–)26.5–32.5(–35) × (13–)14.5–17(–18.5) | 29.6 × 15.6 | 1.9 | Damm et al. (2007) |
| *L. pontae* | 1 | 16.3–26.4 × 9.6–15 | 21 × 12.1 | 1.74 | Coutinho et al. (2017) |
| *L. pseudotheobromae* | 1 | (22.5–)23.5–32(–33) × (13.5–)14–18(–20) | 28 × 16 | 1.7 | Alves et al. (2008) |
| | – | 21.7–26.3 × 13.4–14.8 | – | 1.7 | Abdollahzadeh et al. (2010) |
| | – | (21.5–)24.5–29.5(–31) × (13.5–)14– 16.5(–18) | – | – | Begoude et al. (2010) |
| | – | 20.8–32.5 × 11.1–18.5 | – | 1.79 | Coutinho et al. (2017) |
| | – | – | 26.7 × 12.3 | 2.1 | Ismail et al. (2012) |
| | 1 | 23–30 × 11–13 | 28 × 12 | – | Jayasiri et al. (2019) |
| | 1 | 26–31 × 13–16 | – | – | Machado et al. (2014) |
| | – | 21.2–25.8 × 12.5–13.9 | – | 1.8 | Netto et al. (2014) |
| *L. pyriformis* | 0 | (19–)21.5–25(–28) × (13.5–)15.5–19.5(–21.5) | 23.3 × 17.6 | 1.3 | Slippers et al. (2014) |
| *L. rubropurpurea* | 1 | 24–33 × 13–17 | 28.2 × 14.6 | 1.9 | Burgess et al. (2006) |
| ***L. ryukyuensis*** | **1** | **16.6–27.6 × 10.4–15.1** | **21.1 × 12.4** | **1.71** | **this study** |
| *L. sterculiae* | – | (12–)14–16(–17) × (8–)10–11(–12) | – | – | Yang et al. (2017) |
| *L. subglobosa* | – | 16–23 × 11–17 | – | – | Machado et al. (2014) |
| *L. swieteniae* | 1–3 | 24–32 × 11–14 | 30 × 13 | – | Jayasiri et al. (2019) |
| | **1** | **19.5–27.7 × 12.3–15.6** | **23.0 ×13.4** | **1.71** | **this study** |
| *L. tenuiconidia* | 1 | (18–)19–24(–26) × (11–)12–16(–17) | 22.3 × 14.7 | 1.5 | Wang et al. (2019) |
| *L. thailandica* | 1–3 | (20–)22–25(–26) × (12–)13–15(–16) | – | – | Trakunyingcharoen et al. (2015) |
| | – | 28–29 × 11–13 | – | – | De Silva et al. (2019) |

| | | | | | | |
|---|---|---|---|---|---|---|
| *L. theobromae* | 1 | | (19–)21–31(–32.5) × (12–)13–15.5(–18.5) | 26.2 × 14.2 | 1.9 | Alves et al. (2008) |
| | | – | 22.4–24.2 × 12.9–14.3 | – | 1.8 | Abdollahzadeh et al. (2010) |
| | | – | (20.5–)22.5–26(–30.5) × (11.5–)12.5–15(–17) | – | – | Begoude et al. (2010) |
| | | – | 19.7–26.7 × 10.9–15.3 | – | 1.69 | Coutinho et al. (2017) |
| | | – | – | 23.7 × 13.3 | 1.7 | Ismail et al. (2012) |
| | 1 | | 18–24 × 8–9 | 22 × 8.5 | – | Jayasiri et al. (2019) |
| | 1 | | 23–31 × 13–15 | – | – | Machado et al. (2014) |
| | | – | 20.7–22.7 × 11.7–14.1 | – | 1.8 | Netto et al. (2014) |
| | **1** | | **19.2–30.0 × 11.2–17.8** | **24.5 × 13.8** | **1.79** | **this study** |
| *L. tropica* | 1–2 | | (17–)18–24(–25) × (12–)13–14(–15) | 21.2 × 12.4 | 1.7 | Wang et al. (2019) |
| *L. venezuelensis* | 1 | | 26–33 × 12–15 | 28.4 × 13.5 | 2.1 | Burgess et al. (2006) |
| *L. viticola* | 1 | | (16.8–)18.2–20.5(–22.9) × (7.9–)8.8–10.1(–10.7) | 19.5 × 9.5 | 2.05 | Urbez-Torres et al. (2012) |
| *L. vitis* | 1 | | (–25)26–28(–32) × (12–)15–16(–17) | – | – | Yang et al. (2017) |
| ***L. yaguchii*** | **1** | | **19.0–26.1 × 11.2–15.1** | **23.6 × 13.3** | **1.79** | **this study** |

*theobromae,* have been reported from Japan (Katumoto, 2010) because the identification of this genus on diseased plants was made with insufficient and simplified taxonomical criteria, such as its symptoms and longitudinal striations on conidia. Furthermore, four species, which served as the host plant, *Agave sisalana*, *Laburnum anagyroidii*, *Monstera* sp., *Phoenix roebelen*, were newly confirmed to be part of the genus *Lasiodiplodia*.

The examined materials in this study were mainly collected in the Ryukyu (including Ishigaki, Miyako, and Okinawa) and Ogasawara islands, Japan, showing the rich species diversity of the genus *Lasiodiplodia*. The Ryukyu islands are volcanic or elevated coral islands adjacent to the Asian continent. The rise and fall of the sea level by tectonic activity and ocean current significantly affect the exchange of plant and fungal flora. Alternatively, the Ogasawara islands are known as oceanic islands, which have never connected to the continent and have no interaction with living things other than drifting by ocean currents and bringing in humans. These islands have a unique plant flora, and it suggests a unique mycoflora, too. Studies on the diversity of *Lasiodiplodia* might be a good example for understanding the speciation of plant pathogen, which includes the endemic and monoxenic species, and the cosmopolitan and polyxenic species.

Many isolates of the genus *Lasiodiplodia* were unconditionally designated as *L. theobromae* by their symptoms and simplified morphological characteristics. Therefore, although this species is the polyxenic plant pathogen and has been reported from various plants, the actual number of hosts of *L. theobromae sensu stricto* is still unclear. Similarly, in the broad sense the naming of *L. theobromae* by taxonomical reexamination of cultures is necessary.

Recently, taxonomical studies on *Lasiodiplodia* species in Asian countries became active. *L. brasiliensis,* isolated from *M. indica* and *C. papaya* in Japan, including fruits imported from the Philippines were examined in this study. This species is often isolated from the same hosts in tropical areas (Coutinho *et al.* 2017, Dou *et al.* 2017b). *L. thailandica* has characteristic conidia in size, and paraphyses, was introduced from Thailand (Trakunying charoen *et al.* 2015). *L. chinensis* was discovered as a new species

closely related to *L. pseudotheobromae* (Dou *et al.* 2017a), and *L. hyalina* was introduced as a new species closely related to *L. thailandica* and *L. iraniensis* (Dou *et al.* 2017b) in China, and *L. avicenniarum* and *L. swieteniae* were described as a new species in Thailand (Jayasiri *et al.* 2019). However, in other East Asian countries, especially in Japan, a few studies have undergone taxonomic reexamination, and many isolates are still treated as *L. theobromae* in a broad sense. To understand the diversity of *Lasiodiplodia* in East Asia, it is necessary to perform molecular phylogenetic analysis using the multi-locus matrix, also a comparative study of their morphological characteristics is required.

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# Chapter 4

## Taxonomical study of Botryosphaeria species in Japan


## Abstract

The phylogenetic position of 50 isolates of the genus *Botryosphaeria* isolated from 31 host plant species in the 19 families in Japan were analyzed using the combined sequences consisting of partial DNA sequences of rDNA ITS, rpb2, tef1-α, and tub2 regions. As a result, one isolate obtained from *Gamblea innovans* formed a clade with *B. qingyuanensis*. The other 49 isolates formed a large clade with *B. dothidea* epitype and its related species. Twenty-four isolates with morphological characteristics observed on the medium or specimen were carried out taxonomical examinations based on molecular phylogenetic and morphological characteristics. These were divided into five clades and include *Botryosphaeria dothidea*, *B. qingyuanensis*, *B. sinensis*, and *Botryosphaeria* spp. Two species, *B. qingyuanensis* and *B. sinensis* have been newly added to the Japanese mycoflora, but their host plants are not specified. *Botryosphaeria tenuispora* isolated from *Leucothoe fontanesiana* and insect galls on leaves of *Aucuba japonica* and has been proposed as a new species. However, the resolution for species distinction by the multi-region analyses adopted in the present study was seemed not to be enough to distinguish *B. dothidea* species complex. Therefore, we need alternative and additional phylogenetic approach that will divide species complex of *B. dothidea*.



***Key Words*:** *Botryosphaeriaceae, new species, phylogeny, plant pathogen, systematics*

## Introduction

Genus *Botryosphaeria* (Botryosphaeriaceae, Botryosphaeriales) was introduced by Cesati and de Notaris [1]. *Botryosphaeria* has been known to be a plant pathogenic, endophytic, and saprobic fungus [2-5]. Some species of this genus cause diseases of crops and economic impact on forests and useful trees worldwide [6]. However, some species are known to behave as opportunistic pathogens with weak symptoms or endophytes without symptoms under stressful conditions [6]. Several researchers have discussed these various niches. Marsberg et al. [7] discussed the distinction between the endophyte and the latent pathogen for parts of their life cycle and concluded that it is of little value. Moreover, symbiotic relationships among the host plants, insects inhabiting the gall, and *Botryosphaeria* spp. have been discovered [8-10].

*Botryosphaeria dothidea*, a type species of the genus *Botryosphaeria*, is known for its cosmopolitan distribution and numerous hosts [4,6,7]. Slippers et al. [11] reexamined the *B. dothidea* based on molecular phylogeny and phenotypic characteristics and proposed several species for those previously identified as *B. dothidea*. They also emended the species concept with a newly designated epitype of *B. dothidea*. Thereafter, several species have been described as follows: *Botryosphaeria agaves*, *Botryosphaeria auasmontanum*, *Botryosphaeria corticis*, *Botryosphaeria fabicerciana*, *Botryosphaeria fusispora*, *Botryosphaeria guttulata*, *Botryosphaeria kuwatsukai*, *Botryosphaeria minutispermatia*, *Botryosphaeria pseudoramosa*, *Botryosphaeria qingyuanensis*, *Botryosphaeria ramosa*, *Botryosphaeria rosaceae*, *Botryosphaeria scharifii*, *Botryosphaeria sinensis*, and *Botryosphaeria wangensis*. However, the taxonomical positions of numerous species of *Botryosphaeria* described without phylogenetic data is still unclear [12,13].

In Japan, according to the database of the common names of plant diseases in Japan

[14], 14 species of the genus *Botryosphaeria* cause diseases of 30 plant species of 21 families. In our previous studies [15], molecular and phylogenetic analyses using the large ribosomal subunit of rDNA (LSU) + DNA-directed RNA polymerase II subunit (RPB2) regions suggested that 9 of 20 isolates identified previously as isolates of the genus *Botryosphaeria* were that of the genus *Neofusicoccum* and 9 of 10 isolates of the genus *Dothiorella* were that of the genus *Botryosphaeria*. Therefore, in this study, the isolates kept as Botryosphaeriaceae in culture collections were reexamined for their taxonomical position based on multi-locus molecular and phylogenetic analyses using the internal transcribed spacer (ITS) region of rDNA, RPB2, translation elongation factor 1-α (TEF1-α), and β-tubulin (TUB2) and morphological characteristics on host plants and media.

## Materials and methods

### Sample collection and morphological study

Fifty-six isolates identified as *Botryosphaeria* and *Dothiorella* species kept at the Laboratory of Forest Pathology, Forestry and Forest Products Research Institute (Tsukuba, Ibaraki, Japan), the Institute of Fruit Tree and Tea Science, National Agriculture and Food Research Organization (Tsukuba, Ibaraki, Japan), and the Culture Collection of the Laboratory of Phytopathology, Mie University (Tsu, Mie, Japan) were examined (Table 4–1). These isolates included those from various host plants and insect galls (Table 4–2). These isolates were cultivated on potato dextrose agar (PDA) medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) or malt agar (Becton Dickinson, Franklin, NJ, USA) at room temperature under room light diffusion. To observe conidiomata and conidia, the isolates were transferred to boiled mulberry agar (BMA; [16]). In brief, mulberry leaves were cut into 5 cm squares, boiled for 30 to 60 s, and dried on a paper towel. These leaves were placed on water agar medium. Mycelial discs containing *Botryosphaeria* isolates, which had been cultivated for 1

week on PDA, were transferred onto BMA and cultivated for 1 week to 3 months at room temperature under room light diffusion. The specimens were deposited at the Mycological Herbarium at Mie University (MUMH). The examined isolates were maintained at the Culture Collection of Mycological Herbarium, Mie University (MUCC; Tsu, Mie, Japan).

**Molecular and phylogenetic analyses**

Genomic DNA was extracted from mycelial discs after 7 days of culture on PDA plates with DNeasy Ultra Clean Microbial Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Targeted sequences of the ITS region of rDNA and TEF1-α, TUB2, and RPB2 gene-coding regions were amplified using the T100 Thermal Cycler (Bio-Rad, Tokyo, Japan) via polymerase chain reaction (PCR). The total volume of the PCR mixture was 12.5 μL; it consisted of 1–10 ng of genomic DNA, 0.05 μL of 0.25 unit Taq DNA polymerase (Bioline, London, UK; TEF1-α 0.1 μL and RPB2 0.1 μL), 1.25 μL of 10× $NH_4$ reaction buffer (Bioline), 1.9 to 2.5 mM $MgCl_2$ (Bioline; ITS, RPB2, and TEF1-α 2.5 mM and TUB2 1.9 mM), 2.5 to 5.0 mM each of deoxyribonucleotide triphosphate mixture (Bioline; ITS 2.5 mM and TEF1-α, TUB2, and RPB2 5.0 mM), 0.2 μM of each primer, and 5.6% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA), which was added only for TEF1-α amplification, and sterilized distilled water up to 12.5 μL.

The PCR conditions were as follows: for ITS: initial denaturation (94°C, 5 min), 40 cycles of amplification (denaturation 94°C, 45 s; annealing 48°C, 30 s; and extension 72°C, 90 s), and final extension (72°C, 2 min); for TEF1-α: initial denaturation (94°C, 5 min), 40 cycles of amplification (denaturation 94°C, 30 s; annealing 52°C, 30 s; and extension 72°C, 45 s), and final extension (72°C, 2 min); for TUB2: initial denaturation (94°C, 5 min), 40 cycles of amplification (denaturation 94°C, 30 s; annealing 52°C, 30 s; and extension 72°C, 60 s), and final extension (72°C, 2 min); and for RPB2: initial denaturation (95°C, 5 min), touch-down amplification (5 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 120 s; 5

cycles of 95°C for 45 s, 58°C for 45 s, and 72°C for 120 s; and 30 cycles of 95°C for 45 s, 54°C for 45 s, and 72°C for 120 s), and final elongation at 72°C for 8 min. The primer sets are shown in Table 4–3. The amplicon was sequenced in both directions using the respective PCR primers and the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 3730xl DNA analyzer installed at the Mie University Advanced Science Research Promotion Center (Tsu, Mie, Japan). The sequences were assembled and aligned with 16 sequences of the *Botryosphaeria* sp. collected from GenBank using the software MAFFT version 7 [17].

Maximum likelihood (ML) and Bayesian inference (BI) analyses were used to estimate phylogenetic relationships. ML analyses were performed using raxml HPC-PTHREADS [18]. The strength of the internal branches from the resultant trees was tested by bootstrap analysis [19] using 1000 replications. BI analyses were performed using BEAST version 2.5.1 [20] to estimate the posterior probabilities (PPs) of tree topologies based on the metropolis-coupled Markov chain Monte Carlo (MCMC) searches, which used the MCMC algorithm of four chains in parallel from a random tree topology. The MCMC analysis lasted 10,000,000 generations. Trees were sampled and saved every 1000 generations. The first 25% of the saved trees were discarded, representing the “burn-in” phase, and the PPs were determined from the remaining trees. Representative sequences for all taxa were uploaded to GenBank (Table 4–4). Sequence alignments prepared in this study were deposited in TreeBASE number S26984 and S27295.

## Results

### Phylogeny

For molecular phylogenetic analysis, the data matrix of 66 OTUs (operational taxonomic units) consisted of 1825 characters (ITS: 534 bp, RPB2: 647 bp, TEF1-α: 280 bp, TUB2: 364 bp) was performed. Analyses using 24 strains for which morphological characteristics were

observed were performed separately, the ITS+TEF1-α+TUB2+RPB2 combined data matrix of 41 sequences consisted of 1756 characters (ITS: 536, TEF1-α: 280, RPB2: 576, and TUB2: 364). In both analyses, *Cophinforma atrovirens* (CBS 124934) was selected as the out taxon. The resultant ML trees are shown in Fig. 4–1 and Fig. 4–2. The topologies of the generated trees from ML and BI analyses were congruent. As a result of the phylogenetic analysis, one isolate obtained from *Gamblea innovans* formed a clade with *B. qingyuanensis*. The other 49 isolates formed a large clade with *B. dothidea* epitype and its related species. Japanese isolates with morphological characteristics observed on the medium or specimen formed five groups with the hitherto known species or newly recognized species. These are *B. dothidea* (MUCC 157, MUCC 221, MUCC 245, MUCC 248, MUCC 254, MUCC 2521, MUCC 2543, MUCC 2627, MUCC 2748–2751, and MUCC 2755), *B. tenuispora* (MUCC 237 and MUCC 2900), *B. qingyuanensis* (MUCC 321), *B. sinensis* (MUCC 2522, MUCC 2533, and MUCC 2537), and *Botryosphaeria* sp. (MUCC 2754, MUCC 2897–2899, and MUCC 2901).

**Taxonomy**

***Botryosphaeria dothidea*** (Moug. ex Fr.) Cesati & De Notaris, Commentario della Società Crittogamologica Italiana 1: 212, 1863.

**Teleomorphic state:** It has been reported.

**Cultural characteristics on PDA:** Colonies were gray to dark gray with dense aerial mycelia, reaching 90 mm at 7 days after inoculation.

**Host:** *Prunus* sp., *Rosa* sp. [11], *Castanea crenata*, *Daphniphyllum macropodum*, *Eucalyptus viminalis*, *Leucothoe catesbaei*, *Lindera obtusiloba*, *Pyrus pyrifolia*, *Prunus persica*, *Prunus* sp., *Saxifraga stolonifera* (this study).

**Materials examined:** on *Daphniphyllum macropodum*, **Japan**, Aichi, Nagoya, 14 Nov 2005, by I. Araki & K. Motohashi (MUMH 10467, culture MUCC 157); on *Leucothoe catesbaei*, ibid, June 19, 2006, by I. Araki & K. Motohashi (MUMH 10395, culture

Table 4–1: List of Japanese *Botryosphaeria* isolates used in this study.

| Species[a] | No. | Host Family | Host | Location | Identified by previous study |
|---|---|---|---|---|---|
| *B. dothidea* SC | MUCC 13 (MAFF 240043) | Fagaceae | *Quercus serrata* | Aichi | *Fusicoccum* sp. |
| *B. dothidea* SC | MUCC 157 | Daphniphyllaceae | *Daphniphyllum macropodum* | Aichi | - |
| *B. dothidea* SC | MUCC 221 | Ericaceae | *Leucothoe fontanesiana* | Aichi | - |
| *B. dothidea* SC | MUCC 222 | Ericaceae | *Rhododendron* sp. | Aichi | *Fusicoccum* sp. |
| *B. dothidea* SC | MUCC 237 | Ericaceae | *Leucothoe fontanesiana* | Aichi | - |
| *B. dothidea* SC | MUCC 245 | Daphniphyllaceae | *Daphniphyllum macropodum* | Aichi | - |
| *B. dothidea* SC | MUCC 248 | Lauraceae | *Lindera obtusiloba* | Aichi | - |
| *B. dothidea* SC | MUCC 254 | Saxifragaceae | *Saxifraga stolonifera* | Aichi | - |
| *B. dothidea* SC | MUCC 269 | Rosaceae | *Aronia* sp. | Aichi | *Fusicoccum* sp. |
| *B. dothidea* SC | MUCC 272 | Daphniphyllaceae | *Daphniphyllum macropodum* | Aichi | *Fusicoccum* sp. |
| *B. dothidea* SC | MUCC 322 | Celastraceae | *Euonymus hamiltonianus* | Aichi | *Fusicoccum* sp. |
| *B. dothidea* SC | MUCC 2506 (FFPRI 411219) | Rosaceae | *Prunus yedoensis* | Yamagata | *B. dothidea* |
| *B. dothidea* SC | MUCC 2507 | Fagaceae | *Castanea crenata* | Kanagawa | *B. dothidea* |

| | | | | | |
|---|---|---|---|---|---|
| | (FFPRI 411220) | | | | |
| *B. dothidea* SC | MUCC 2508 (MAFF 410799) | Betulaceae | *Betula pendula* | Tokyo | *Botryosphaeria* sp. |
| *B. dothidea* SC | MUCC 2510 (MAFF 410798) | Salicaceae | *Populus alba* | Kyoto | *Botryosphaeria* sp. |
| *B. dothidea* SC | MUCC 2516 (FFPRI 411221) | Cupressaceae | *Chamaecyparis obtusa* | Shiga | *Botryosphaeria* sp. |
| *B. dothidea* SC | MUCC 2520 (FFPRI 411222) | Fabaceae | *Acacia melanoxylon* | Fukuoka | *Botryosphaeria* sp. |
| *B. dothidea* SC | MUCC 2521 (MAFF 410826) | Rosaceae | *Prunus* sp. | Ibaraki | *B. dothidea* |
| *B. dothidea* SC | MUCC 2522 (MAFF 410827) | Rosaceae | *Prunus* sp. | Ibaraki | *B. dothidea* |
| *B. dothidea* SC | MUCC 2523 (MAFF 410828) | Rosaceae | *Prunus* sp. | Ibaraki | *B. dothidea* |
| *B. dothidea* SC | MUCC 2524 (MAFF 410829) | Rosaceae | *Prunus* sp. | Ibaraki | *B. dothidea* |

| | | | | | |
|---|---|---|---|---|---|
| *B. dothidea* SC | MUCC 2525 (MAFF 410833) | Rosaceae | *Prunus* sp. | Tokyo | *B. dothidea* |
| *B. dothidea* SC | MUCC 2526 (MAFF 410842) | Rosaceae | *Prunus* sp. | Ibaraki | *B. dothidea* |
| *B. dothidea* SC | MUCC 2533 (FFPRI 411202) | Aucubaceae | *Aucuba japonica* | Tokyo | *Dothiorella* sp. |
| *B. dothidea* SC | MUCC 2536 (FFPRI 411223) | Paulowniaceae | *Paulownia tomentosa* | Niigata | *Dothiorella* sp. |
| *B. dothidea* SC | MUCC 2537 (FFPRI 411203) | Paulowniaceae | *Paulownia tomentosa* | Niigata | *Dothiorella* sp. |
| *B. dothidea* SC | MUCC 2538 (FFPRI 411224) | Paulowniaceae | *Paulownia tomentosa* | Niigata | *Dothiorella* sp. |
| *B. dothidea* SC | MUCC 2539 (FFPRI 411225) | Paulowniaceae | *Paulownia tomentosa* | Fukuoka | *Dothiorella* sp. |
| *B. dothidea* SC | MUCC 2540 (FFPRI 411226) | Anacardiaceae | *Rhus verniciflua* | Niigata | *Dothiorella* sp. |
| *B. dothidea* SC | MUCC 2541 (MAFF 410141) | Fagaceae | *Castanea crenata* | Hyogo | *Dothiorella* sp. |

| | | | | | |
|---|---|---|---|---|---|
| *B. dothidea* SC | MUCC 2542 (FFPRI 411227) | Fagaceae | *Castanea crenata* | Hyogo | *Dothiorella* sp. |
| *B. dothidea* SC | MUCC 2543 (FFPRI 411204) | Myrtaceae | *Eucalyptus viminalis* | Tokyo | *Dothiorella* sp. |
| *B. dothidea* SC | MUCC 2544 (FFPRI 411228) | Myrtaceae | *Eucalyptus camaldulensis* | Saitama | *Dothiorella* sp. |
| *B. dothidea* SC | MUCC 2627 | Rosaceae | *Pyrus pyrifolia* | Mie | - |
| *B. dothidea* SC | MUCC 2661 (FFPRI 411229) | Pinaceae | *Larix kaempferi* | Tokyo | *Guignardia cryptomeriae* |
| *B. dothidea* SC | MUCC 2670 (MAFF 410182) | Betulaceae | *Alnus glutinosa* | Iwate | *Guignardia alnigena* |
| *B. dothidea* SC | MUCC 2671 (FFPRI 411230) | Betulaceae | *Alnus japonica* | Tokyo | *G. alnigena* |
| *B. dothidea* SC | MUCC 2672 (FFPRI 411231) | Betulaceae | *Alnus japonica* | Tokyo | *G. alnigena* |
| *B. dothidea* SC | MUCC 2673 (FFPRI 411232) | Sapindaceae | *Acer amoenum* | Shimane | *Guignardia* sp. |
| *B. dothidea* SC | MUCC 2676 | Juglandaceae | *Juglans* sp. | Yamagata | *Guignardia* |

| | | | | | |
|---|---|---|---|---|---|
| | (FFPRI 411233) | | | | *juglandia* |
| *B. dothidea* SC | MUCC 2680 (FFPRI 411234) | Salicaceae | *Populus monilifera* | Yamagata | *Guignardia* sp. |
| *B. dothidea* SC | MUCC 2682 (MAFF 410199) | Juglandaceae | *Juglans* sp. | Yamagata | *Macrophoma juglandis* |
| *B. dothidea* SC | MUCC 2748 | Fagaceae | *Castanea crenata* | Ibaraki | - |
| *B. dothidea* SC | MUCC 2749 | Fagaceae | *Castanea crenata* | Kumamoto | - |
| *B. dothidea* SC | MUCC 2750 | Fagaceae | *Castanea crenata* | Kumamoto | - |
| *B. dothidea* SC | MUCC 2751 | Rosaceae | *Prunus persica* | Ibaraki | - |
| *B. dothidea* SC | MUCC 2755 | Fagaceae | *Castanea crenata* | Kumamoto | - |
| *B. dothidea* SC | MUCC 2756 (K-032) | Fagaceae | *Castanea crenata* | Kumamoto | *B. dothidea* |
| *B. dothidea* SC | MUCC 2900 | Aucubaceae | gall on *Aucuba japonica* | Ibaraki | - |
| *B. qingyuanensis* | MUCC 321 | Araliaceae | *Gamblea innovans* | Aichi | - |

[a)] *Botryosphaeria dothidea* species complex was abbreviated as *B. dothidea* SC.

Table 4–2: List of Japanese *Botryosphaeria* isolates that were observed morphological characteristics used in this study.

| Fungal species | Isolate No. | Material No. | Host Family | Host species | Regions | Identified by previous study |
|---|---|---|---|---|---|---|
| *Botryosphaeria* sp. | MUCC 2754 | - | Fagaceae | *Castanea crenata* | Kumamoto | - |
| | MUCC 2897 | - | Aucubaceae | *Asphondylia aucubae* | Ibaraki | - |
| | MUCC 2898 | - | Aucubaceae | gall on *Aucuba japonica* | Ibaraki | - |
| | MUCC 2899 | - | Aucubaceae | gall on *Aucuba japonica* | Ibaraki | - |
| | MUCC 2901 | - | Aucubaceae | gall on *Aucuba japonica* | Ibaraki | - |
| *B. dothidea* | MUCC 157 | MUMH10467 | Daphniphyllaceae | *Daphniphyllum macropodum* | Aichi | - |
| | MUCC 221 | MUMH10395 | Ericaceae | *Leucothoe fontanesiana* | Aichi | - |
| | MUCC 245 | MUMH10425 | Daphniphyllaceae | *Daphniphyllum macropodum* | Aichi | - |
| | MUCC 248 | MUMH10429 | Lauraceae | *Lindera obtusiloba* | Aichi | - |
| | MUCC 2521 (MAFF410826) | - | Rosaceae | *Prunus* sp. | Ibaraki | *B. dothidea* |
| | MUCC 254 | MUMH10437 | Saxifragaceae | *Saxifraga stolonifera* | Aichi | - |
| | MUCC 2543 (FFPRI411204) | - | Myrtaceae | *Eucalyptus viminalis* | Tokyo | *Dothiorella* sp. |
| | MUCC 2627 | - | Rosaceae | Pyrus pyrifolia | Mie | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| | MUCC 2748 | - | Fagaceae | *Castanea crenata* | Ibaraki | - |
| | MUCC 2749 | - | Fagaceae | *Castanea crenata* | Kumamoto | - |
| | MUCC 2750 | - | Fagaceae | *Castanea crenata* | Kumamoto | - |
| | MUCC 2751 | - | Rosaceae | *Prunus persica* | Ibaraki | - |
| | MUCC 2755 | - | Fagaceae | *Castanea crenata* | Kumamoto | - |
| *B. qingyuanensis* | MUCC 321 | MUMH10273 | Araliaceae | *Gamblea innovans* | Aichi | - |
| *B. sinensis* | MUCC 2522 (MAFF410827) | - | Rosaceae | *Prunus* sp. | Ibaraki | *B. dothidea* |
| | MUCC 2533 (FFPRI411202) | - | Aucubaceae | *Aucuba japonica* | Tokyo | *Dothiorella* sp. |
| | MUCC 2537 (FFPRI411203) | - | Paulowniaceae | *Paulownia tomentosa* | Niigata | *Dothiorella* sp. |
| *B. tenuispora* | MUCC 237 | MUMH10420 | Ericaceae | *Leucothoe fontanesiana* | Aichi | - |
| | MUCC 2900 | - | Aucubaceae | gall on *Aucuba japonica* | Ibaraki | - |

Table 4–3: PCR primer sets and annealing temperatures.

| Region | primer F | primer R | Anealing temperature |
|---|---|---|---|
| ITS | ITS1 (White et al., 1990) | ITS4 (White et al., 1990) | 48 |
| *tef1-α* | EF1-728F (Carbone & Kohn, 1999) | EF1-986R (Carbone & Kohn, 1999) | 52 |
| *tub2* | BT2A (Glass & Donaldson, 1995) | BT2B (Glass & Donaldson, 1995) | 55 |
| *rpb2* | RPB2-5f2 (Liu et al., 1999) | fRPB2-7cR (Liu et al., 1999) | 60→58→54 |

Table 4–4: List of *Botryosphaeria* species used for phylogenetic analysis.

| Species[a] | No. | Host | Country | Accession No. | | | |
|---|---|---|---|---|---|---|---|
| | | | | ITS | tef1-α | tub2 | rpb2 |
| *B. auasmontanum* | CBS 121769$^{T}$ | *Acacia mellifera* | Namibia | EU101303 | EU101348 | - | - |
| *B. corticis* | CBS 119047$^{T}$ | *Vaccinium corymbosum* | USA | DQ299245 | EU017539 | EU673107 | - |
| *B. dothidea* | CBS 115476$^{T}$ | *Prunus* sp. | Switzerland | AY236949 | AY236898 | AY236927 | EU339577 |
| ***B. dothidea* SC** | **MUCC 13 (MAFF 240043)** | ***Quercus serrata*** | **Japan** | **LC593684** | **LC593714** | **LC593742** | **LC593773** |
| ***B. dothidea* SC** | **MUCC 157** | ***Daphniphyllum macropodum*** | **Japan** | **LC585280** | **LC585152** | **LC585176** | **LC585198** |
| ***B. dothidea* SC** | **MUCC 221** | ***Leucothoe fontanesiana*** | **Japan** | **LC585282** | **LC585154** | **LC585178** | **LC585200** |
| ***B. dothidea* SC** | **MUCC 222** | ***Rhododendron* sp.** | **Japan** | **LC593708** | **LC593738** | **LC593766** | **LC593797** |
| ***B. dothidea* SC** | **MUCC 245** | ***Daphniphyllum macropodum*** | **Japan** | **LC585273** | **LC585145** | **LC585169** | **LC585192** |
| ***B. dothidea* SC** | **MUCC 248** | ***Lindera obtusiloba*** | **Japan** | **LC585275** | **LC585147** | **LC585171** | **LC585194** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ***B. dothidea* SC** | **MUCC 254** | ***Saxifraga stolonifera*** | **Japan** | **LC585274** | **LC585146** | **LC585170** | **LC585193** |
| ***B. dothidea* SC** | **MUCC 269** | ***Aronia* sp.** | **Japan** | **LC593685** | **LC593715** | **LC593743** | **LC593774** |
| ***B. dothidea* SC** | **MUCC 272** | ***Daphniphyllum macropodum*** | **Japan** | **LC593692** | **LC593722** | **LC593750** | **LC593781** |
| ***B. dothidea* SC** | **MUCC 322** | ***Euonymus hamiltonianus*** | **Japan** | **LC593686** | **LC593716** | **LC593744** | **LC593775** |
| ***B. dothidea* SC** | **MUCC 2506 (FFPRI 411219)** | ***Prunus yedoensis*** | **Japan** | **LC593690** | **LC593720** | **LC593748** | **LC593779** |
| ***B. dothidea* SC** | **MUCC 2507 (FFPRI 411220)** | ***Castanea crenata*** | **Japan** | **LC593691** | **LC593721** | **LC593749** | **LC593780** |
| ***B. dothidea* SC** | **MUCC 2508 (MAFF 410799)** | ***Betula pendula*** | **Japan** | **LC593689** | **LC593719** | **LC593747** | **LC593778** |
| ***B. dothidea* SC** | **MUCC 2510 (MAFF 410798)** | ***Populus alba*** | **Japan** | **LC593703** | **LC593733** | **LC593761** | **LC593792** |
| ***B. dothidea* SC** | **MUCC 2516 (FFPRI 411221)** | ***Chamaecyparis obtusa*** | **Japan** | **LC593695** | **LC593725** | **LC593753** | **LC593784** |
| ***B. dothidea* SC** | **MUCC 2520 (FFPRI 411222)** | ***Acacia melanoxylon*** | **Japan** | **LC593687** | **LC593717** | **LC593745** | **LC593776** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ***B. dothidea* SC** | **MUCC 2521 (MAFF 410826)** | ***Prunus* sp.** | **Japan** | **LC585270** | **LC585142** | **LC585166** | **LC585189** |
| ***B. dothidea* SC** | **MUCC 2522 (MAFF 410827)** | ***Prunus* sp.** | **Japan** | **LC585277** | **LC585149** | **LC585173** | **LC585195** |
| ***B. dothidea* SC** | **MUCC 2523 (MAFF 410828)** | ***Prunus* sp.** | **Japan** | **LC593693** | **LC593723** | **LC593751** | **LC593782** |
| ***B. dothidea* SC** | **MUCC 2524 (MAFF 410829)** | ***Prunus* sp.** | **Japan** | **LC593711** | **LC593740** | **LC593770** | **-** |
| ***B. dothidea* SC** | **MUCC 2525 (MAFF 410833)** | ***Prunus* sp.** | **Japan** | **LC593704** | **LC593734** | **LC593762** | **LC593793** |
| ***B. dothidea* SC** | **MUCC 2526 (MAFF 410842)** | ***Prunus* sp.** | **Japan** | **LC593697** | **LC593727** | **LC593755** | **LC593786** |
| ***B. dothidea* SC** | **MUCC 2533 (FFPRI 411202)** | ***Aucuba japonica*** | **Japan** | **LC585268** | **LC585140** | **LC585164** | **LC585188** |
| ***B. dothidea* SC** | **MUCC 2536 (FFPRI 411223)** | ***Paulownia tomentosa*** | **Japan** | **LC593696** | **LC593726** | **LC593754** | **LC593785** |
| ***B. dothidea* SC** | **MUCC 2537 (FFPRI 411203)** | ***Paulownia tomentosa*** | **Japan** | **LC585279** | **LC585151** | **LC585175** | **LC585197** |
| ***B. dothidea* SC** | **MUCC 2538** | ***Paulownia*** | **Japan** | **LC593709** | **-** | **LC593767** | **LC593798** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **(FFPRI 411224)** | ***tomentosa*** | | | | | |
| ***B. dothidea* SC** | **MUCC 2539** | ***Paulownia*** | **Japan** | **LC593694** | **LC593724** | **LC593752** | **LC593783** |
| | **(FFPRI 411225)** | ***tomentosa*** | | | | | |
| ***B. dothidea* SC** | **MUCC 2540** | ***Rhus verniciflua*** | **Japan** | **LC593712** | **LC593741** | **LC593771** | **-** |
| | **(FFPRI 411226)** | | | | | | |
| ***B. dothidea* SC** | **MUCC 2541** | ***Castanea*** | **Japan** | **LC593713** | **-** | **LC593772** | **-** |
| | **(MAFF 410141)** | ***crenata*** | | | | | |
| ***B. dothidea* SC** | **MUCC 2542** | ***Castanea*** | **Japan** | **LC593698** | **LC593728** | **LC593756** | **LC593787** |
| | **(FFPRI 411227)** | ***crenata*** | | | | | |
| ***B. dothidea* SC** | **MUCC 2543** | ***Eucalyptus*** | **Japan** | **LC585271** | **LC585143** | **LC585167** | **LC585190** |
| | **(FFPRI 411204)** | ***viminalis*** | | | | | |
| ***B. dothidea* SC** | **MUCC 2544** | ***Eucalyptus*** | **Japan** | **LC593710** | **-** | **LC593768** | **LC593799** |
| | **(FFPRI 411228)** | ***camaldulensis*** | | | | | |
| ***B. dothidea* SC** | **MUCC 2627** | ***Pyrus pyrifolia*** | **Japan** | **LC585284** | **LC585156** | **LC585180** | **LC585202** |
| ***B. dothidea* SC** | **MUCC 2661** | ***Larix kaempferi*** | **Japan** | **LC593688** | **LC593718** | **LC593746** | **LC593777** |
| | **(FFPRI 411229)** | | | | | | |
| ***B. dothidea* SC** | **MUCC 2670** | ***Alnus glutinosa*** | **Japan** | **LC593707** | **LC593737** | **LC593765** | **LC593796** |
| | **(MAFF 410182)** | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ***B. dothidea* SC** | **MUCC 2671 (FFPRI 411230)** | ***Alnus japonica*** | **Japan** | **LC593701** | **LC593731** | **LC593759** | **LC593790** |
| ***B. dothidea* SC** | **MUCC 2672 (FFPRI 411231)** | ***Alnus japonica*** | **Japan** | **LC593705** | **LC593735** | **LC593763** | **LC593794** |
| ***B. dothidea* SC** | **MUCC 2673 (FFPRI 411232)** | ***Acer amoenum*** | **Japan** | **LC593699** | **LC593729** | **LC593757** | **LC593788** |
| ***B. dothidea* SC** | **MUCC 2676 (FFPRI 411233)** | ***Juglans* sp.** | **Japan** | **LC593702** | **LC593732** | **LC593760** | **LC593791** |
| ***B. dothidea* SC** | **MUCC 2680 (FFPRI 411234)** | ***Populus monilifera*** | **Japan** | **LC593700** | **LC593730** | **LC593758** | **LC593789** |
| ***B. dothidea* SC** | **MUCC 2682 (MAFF 410199)** | ***Juglans* sp.** | **Japan** | **LC593706** | **LC593736** | **LC593764** | **LC593795** |
| ***B. dothidea* SC** | **MUCC 2748** | ***Castanea crenata*** | **Japan** | **LC585283** | **LC585155** | **LC585179** | **LC585201** |
| ***B. dothidea* SC** | **MUCC 2749** | ***Castanea crenata*** | **Japan** | **LC585289** | **LC585161** | **LC585185** | **LC585207** |
| ***B. dothidea* SC** | **MUCC 2750** | ***Castanea crenata*** | **Japan** | **LC585269** | **LC585141** | **LC585165** | **-** |
| ***B. dothidea* SC** | **MUCC 2751** | ***Prunus persica*** | **Japan** | **LC585281** | **LC585153** | **LC585177** | **LC585199** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ***B. dothidea* SC** | **MUCC 2755** | ***Castanea crenata*** | **Japan** | **LC585272** | **LC585144** | **LC585168** | **LC585191** |
| ***B. dothidea* SC** | **MUCC 2756 (K-032)** | ***Castanea crenata*** | **Japan** | **-** | **LC593739** | **LC593769** | **LC593800** |
| *B. fabicerciana* | CBS 127193[T] | *Eucalyptus* sp. | China | HQ332197 | HQ332213 | KF779068 | MF410137 |
| *B. fusispora* | MFLUCC 10-0098[T] | *Entada* sp. | Thailand | JX646789 | JX646854 | JX646839 | - |
| *B. guttulata* | CGMCC 3.20094[T] | Dead wood | China | MT327839 | MT331606 | - | - |
| *B. kuwatsukai* | CBS 135219[T] | *Malus domestica* | China | KJ433388 | KJ433410 | - | - |
| *B. minutispermatia* | GZCC 16-0013[T] | Dead wood | China | KX447675 | KX447678 | - | - |
| *B. pseudoramosa* | CGMCC 3.18739[T] | *Eucalyptus* hybrid | China | KX277989 | KX278094 | KX278198 | MF410140 |
| *B. qingyuanensis* | CGMCC 3.18742[T] | *Eucalyptus* hybrid | China | KX278000 | KX278105 | KX278209 | MF410151 |
| ***B. qingyuanensis*** | **MUCC 321** | ***Gamblea innovans*** | **Japan** | **LC585291** | **LC585163** | **LC585187** | **-** |
| *B. ramosa* | CBS 122069[T] | *Eucalyptus camaldulensis* | Australia | EU144055 | EU144070 | KF766132 | - |
| *B. rosaceae* | CGMCC3.18007[T] | *Malus* sp. | China | KX197074 | KX197094 | KX197101 | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *B. scharifii* | CBS 124703[T] | *Mangifera indica* | Iran | JQ772020 | JQ772057 | - | - |
| *B. sinensis* | CGMCC 3.17722[T] | *Populus* sp. | China | KT343255 | - | - | - |
| ***B. tenuispora*** | **MUCC 237[T]** | ***Leucothoe fontanesiana*** | **Japan** | **LC585278** | **LC585150** | **LC585174** | **LC585196** |
| ***B. tenuispora*** | **MUCC 2900** | **gall on *Aucuba japonica*** | **Japan** | **LC585276** | **LC585148** | **LC585172** | **-** |
| *B. wangensis* | CGMCC 3.18744[T] | *Cedrus deodara* | China | KX278002 | KX278107 | KX278211 | MF410153 |
| ***Botryosphaeria* sp.** | **MUCC 2754** | ***Castanea crenata*** | **Japan** | **LC585288** | **LC585160** | **LC585184** | **LC585206** |
| ***Botryosphaeria* sp.** | **MUCC 2897** | **gall on *Aucuba japonica*** | **Japan** | **LC585285** | **LC585157** | **LC585181** | **LC585203** |
| ***Botryosphaeria* sp.** | **MUCC 2898** | **gall on *Aucuba japonica*** | **Japan** | **LC585286** | **LC585158** | **LC585182** | **LC585204** |
| ***Botryosphaeria* sp.** | **MUCC 2899** | **gall on *Aucuba japonica*** | **Japan** | **LC585290** | **LC585162** | **LC585186** | **-** |
| ***Botryosphaeria* sp.** | **MUCC 2901** | **gall on *Aucuba japonica*** | **Japan** | **LC585287** | **LC585159** | **LC585183** | **LC585205** |
| *C. atrovirens* | CBS 124934 | *Pterocarpus angolensis* | South Africa | FJ888473 | FJ888456 | - | - |

[a)] *Botryosphaeria dothidea* species complex was abbreviated as *B. dothidea* SC.

[T)] Ex-type, ex-neotype, and ex-epitype isolates are indicated.

Japanese isolates sexamied in this study are indicated in bold.

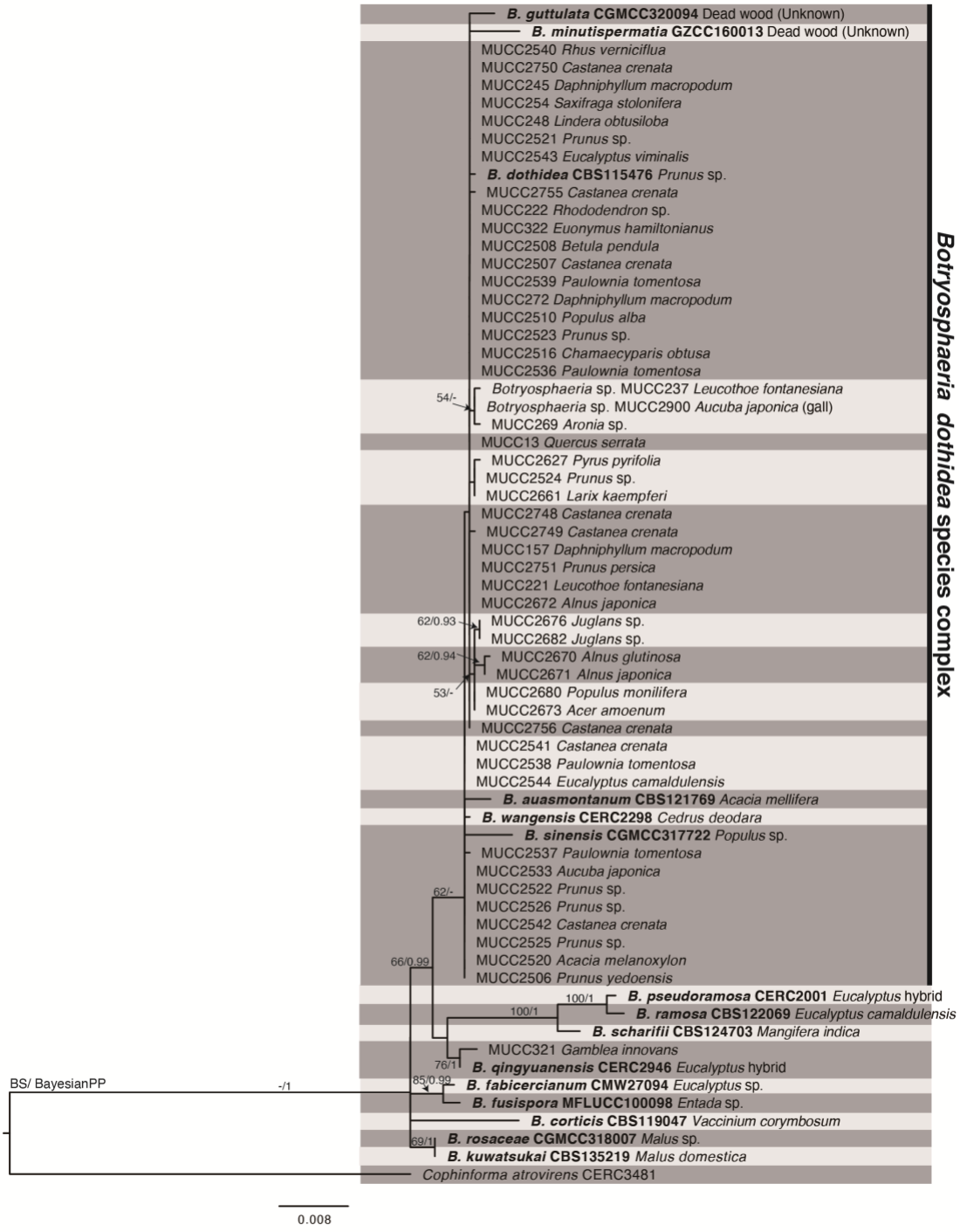


Fig. 4–1: Phylogenetic tree of *Botryosphaeria* spp. constructed by ML using the combined ITS, RPB2, TEF1-α, and TUB2 gene region datasets. ML bootstrap values and Bayesian PPs are given near the branches (BS/PP). Ex-type strains are in boldface.

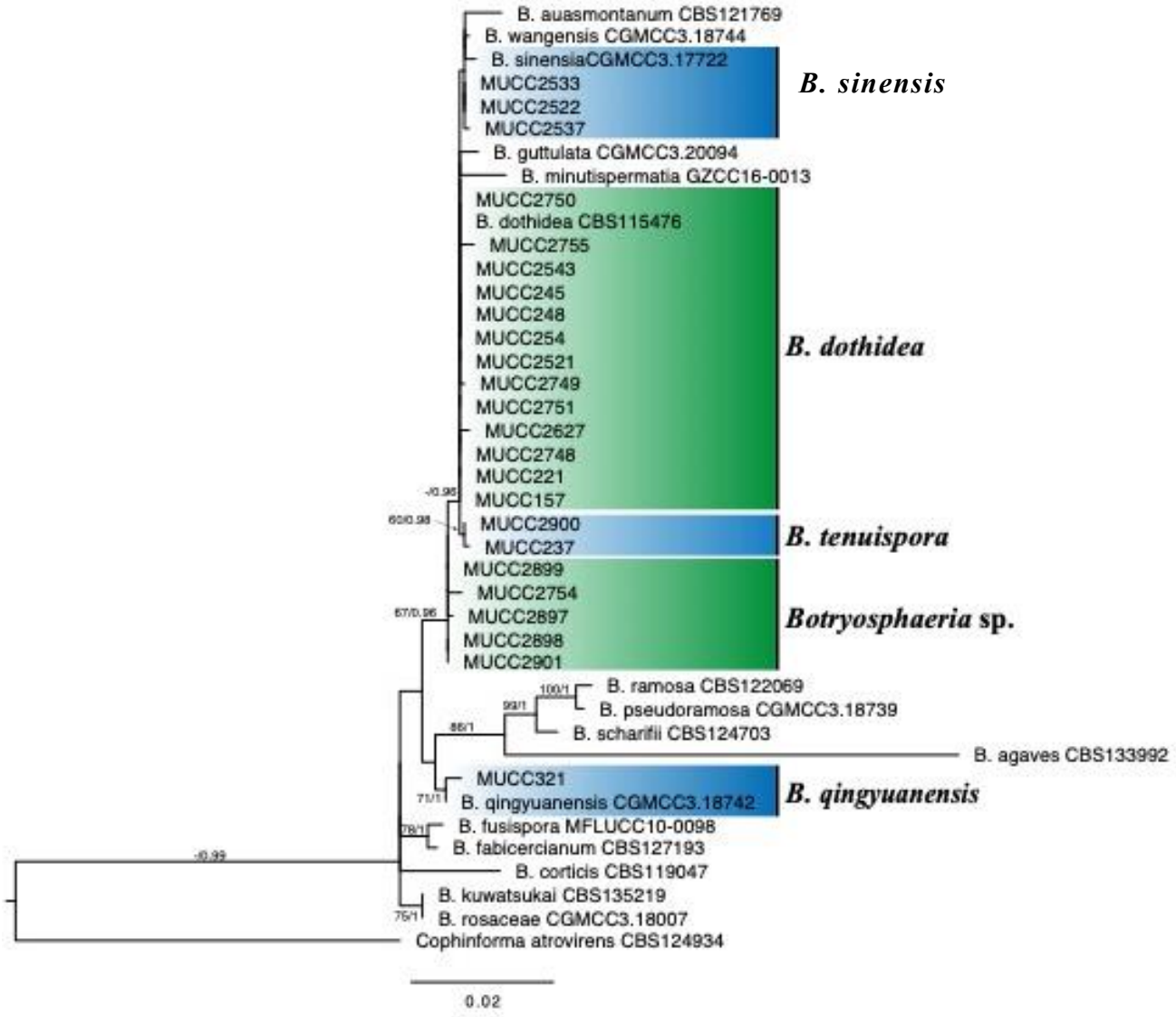


Fig. 4–2: Phylogenetic tree of *Botryosphaeria* spp. constructed by ML using the combined ITS, RPB2, TEF1-α, and TUB2 gene region datasets. ML bootstrap values and Bayesian PPs are given near the branches (BS/PP). Ex-type strains are in boldface.

MUCC 221); on *Daphniphyllum macropodum*, ibid, 18 Jul 2006, by I. Araki & K. Motohashi (MUMH 10425, culture MUCC 245); on *Lindera obtusiloba*, ibid, 18 Jul 2006, by I. Araki & K. Motohashi (MUMH 10429, culture MUCC 248); on *Saxifraga stolonifera*, ibid, 18 Jul 2006, by I. Araki & K. Motohashi (MUMH 10437, culture MUCC 254); on *Prunus* sp., **Japan**, Ibaraki, Tsukuba, May 1993, by T. Yamada (culture MUCC 2521 = MAFF 410826); on *Eucalyptus viminalis*, **Japan**, Tokyo, Koto, 2 Jul 1986, by unknown (culture MUCC 2543 = FFPRI 411204); on *Pyrus pyrifolia*, **Japan**, Mie, Tsu, 9 Aug 2018, by Y. Hattori (culture MUCC 2627); on *C. crenata*, **Japan**, Ibaraki, Tsukuba, 4 Sep 2017, by A. Sasaki (culture MUCC 2748 = NP-004); on *Castanea crenata*, **Japan**, Kumamoto, Uki, 8 Sep 2017, by Y. Fukunaga (culture MUCC 2749 = K-001); on *Castanea crenata*, ibid, 8 Sep 2017, by Y. Fukunaga (culture MUCC 2750 = K-004); on *Prunus persica*, **Japan**, Ibaraki, Tsukuba, 27 Jan 2017, by H. Nakamura (culture MUCC 2751; and on *Castanea crenata*, **Japan**, Kumamoto, Uki, 8 Sep 2017, by Y. Fukunaga (culture MUCC 2755 = K-027).

**Note:** Thirteen Japanese isolates were identified as *B. dothidea* based on phylogenetic analysis and morphological characteristics. The morphology of conidia varied, with an L/W ratio of 3.4 to 5.6 (Table 4–5). All isolates grew well and formed conidiomata and conidia on the BMA medium.

***Botryosphaeria qingyuanensis*** G.Q. Li & S.F. Chen, Persoonia 40: 83, 2008.

**Teleomorphic state:** It has not been reported.

**Anamorphic state on the host plants:** Symptoms brown to reddish-brown, small at the edge of the leaf, later enlarged and coalescent, expanded toward the whole leaf. Conidiomata amphigenous, epidermal, merged, solitary, scattered, black to dark brown, ellipsoid, 105–146.5 × 87–132 µm; pycnidial wall composed of depressed or irregular cells in three to five layers, brown to dark brown, blackish around an ostiole, paler toward the conidiogenous region; paraphyzes hyaline, rounded at the apex, septate. Conidiophores reduced to conidiogenous cells. Conidiogenous cells hyaline, cylindrical,

phialidic conidiogenesis, or holoblastic conidiogenesis after percurrent proliferation at the tip, smooth, 6.3–12.8 × 1.4–2.5 µm (n = 10). Conidia solitary, fusiform to ellipsoid, obtuse at both ends, hyaline, aseptate, smooth, with granular contents, 17–24 × 3.6–6.5 µm, L/W = 4.16 (min 2.96, max 6.12; n = 25).

**Cultural characteristics on PDA:** Colonies were gray to dark gray with dense aerial mycelia, reaching 90 mm at 7 days after inoculation.

**Host:** *Eucalyptus* hybrid [21], *Gamblea innovans* (this study).

Materials examined: on *Gamblea innovans*, **Japan**, Aichi, Nagoya, 14 Nov 2005, by I. Araki & K. Motohashi (MUMH 10273, culture MUCC 321).

**Note:** From a phylogenetic analysis, MUCC 321 formed the same clade as *B. qingyuanensis* (CGMCC 3.18742). The width of the conidia of MUCC 321 was slightly narrower than that of *B. qingyuanensis* [MUCC 321: 17.5–24 × 3.6–6.5 vs. CGMCC 3.18742: (15) 19.5–24.5 (28.5) × (5) 6–6.5 (7.5); Li et al. 2018]. *B. qingyuanensis* was isolated from the twigs of a *Eucalyptus* tree in China and known only from the type locality. MUCC 321 was isolated from the leaf spots on *G. innovans*. This study was the first report of the new habitat and host plant from Japan.

***Botryosphaeria sinensis*** Y.P. Zhou & Y. Zhang ter, Phytotaxa 245: 45, 2016.

**Teleomorphic state:** It has been reported.

**Anamorphic state formed on BMA:** Conidiomata formed within 7 days, solitary or aggregate, globose to subglobose, dark brown to dark gray, covered with white to dark green hyphae, 304–382 × 316–400 µm; pycnidial wall composed of depressed or irregular cells in three to five layers, brown to dark brown, blackish around the ostiole, paler toward the conidiogenous region; paraphyzes hyaline, rounded at the apex, septate. Conidiophores reduced to conidiogenous cells. Conidiogenous cells hyaline, cylindrical, smooth, phialidic conidiogenesis with periclinal thickening, or holoblastic conidiogenesis after percurrent proliferation at the tip 1.6–2.6 × 6.8–11.2 µm (n = 3). Conidia solitary, fusiform, or irregularly fusiform, rounded at the apex, convex to

Table 4–5: Morphological characteristics of the genus *Botryosphaeria*.

| Species | Conidia | | | Isolate | Literature |
|---|---|---|---|---|---|
| | Conidial bodies (µm) | Average | L/W | | |
| *B. auasmontanum*$^{T}$ | (8.1–)8.8–11.3(–13) × (2.5–)2.9–3.9(–5) | 10.1 × 3.4 | 3.0 | - | Slippers et al. (2014) |
| ***Botryosphaeria* sp.** | **14.6–29 × 3.2–6.2** | **22.8 × 5.2** | **4.4** | **MUCC 2899** | **this study** |
| *B. dothidea*$^{T}$ | (20–)23–27(–30) × 4–5(–6) | 26.2 × 5.4 | 4.9 | - | Slippers et al. (2004) |
| ***B. dothidea*** | **22–30 × 3.9–6.3** | **25.8 × 5.2** | **4.9** | **MUCC 157** | **this study** |
| ***B. dothidea*** | **21–29 × 3.8–6** | **25.0 × 5.1** | **4.8** | **MUCC 221** | **this study** |
| ***B. dothidea*** | **20–30 × 5.3–7** | **25.1 × 6.1** | **4.1** | **MUCC 245** | **this study** |
| ***B. dothidea*** | **18.5–30 × 3.8–8.8** | **25.2 × 5.2** | **4.8** | **MUCC 248** | **this study** |
| ***B. dothidea*** | **16–24 × 3.3–7.3** | **19.7 × 5.7** | **3.4** | **MUCC 2521 (MAFF 410826)** | **this study** |
| ***B. dothidea*** | **17–30 × 4.2–6.2** | **25.6 × 5.1** | **4.9** | **MUCC 254** | **this study** |
| ***B. dothidea*** | **19–26 × 4.8–6** | **22.9 × 5.4** | **4.2** | **MUCC 2543 (FFPRI411204)** | **this study** |
| ***B. dothidea*** | **22.5–29.4 × 4.2–6.6** | **26.1 × 5.4** | **4.8** | **MUCC 2627** | **this study** |
| ***B. dothidea*** | **15–26 × 4–7.4** | **22.0 × 5.5** | **4.0** | **MUCC 2748** | **this study** |
| ***B. dothidea*** | **25–33 × 3.8–6.3** | **28.7 × 5.1** | **5.6** | **MUCC 2749** | **this study** |

| | | | | | |
|---|---|---|---|---|---|
| ***B. dothidea*** | **17–36 × 3.8–6.9** | **26.7 × 5.2** | **5.1** | **MUCC 2750** | **this study** |
| ***B. dothidea*** | **22.5–31 × 4.4–6.9** | **26.1 × 5.4** | **4.8** | **MUCC 2751** | **this study** |
| ***B. dothidea*** | **21–35 × 4–6.7** | **26.8 × 5.1** | **5.2** | **MUCC 2755** | **this study** |
| *B. guttulata*[T] | (17.1–)18.5–19.3(– 20.3) × (4.1–)4.4–4.9(– 5.2) | 18.9 × 4.7 | 4.0 | - | Chen et al. (2020) |
| *B. minutispermatia*[T] | 8–14 × 3–4 | 13.0 × 3.5 | 3.7 | - | Ariyawansa et al. (2016) |
| *B. qingyuanensis*[T] | (15–)19.5–24.5(–28.5) × (5–)6–6.5(–7.5) | 22.0 × 6.2 | 3.5 | - | Li et al. (2018) |
| *B. qingyuanensis* | 17–24 × 3.6–6.5 | 21.4 × 5.2 | 4.1 | MUCC 321 | this study |
| *B. sinensis*[T] | (15–)19–29 × 5–7 | 24.3 × 5.9 | 4.1 | - | Zhou et al. (2016) |
| *B. sinensis* | 16–23 × 4.8–6 | 20.4 × 5.4 | 3.7 | MUCC 2522 (MAFF410827) | this study |
| *B. sinensis* | 14–27 × 3–5.8 | 24.0 × 4.6 | 5.2 | MUCC 2537 (FFPRI411203) | this study |
| ***B. tenuispora*[T]** | **23–32 × 4–6.7** | **27.3 × 5.1** | **5.4** | **MUCC 237** | **this study** |
| *B. wangensis*[T] | (20.5–)22–26(–29) × (4.5–)5.5–6.5(–7.5) | 23.8 × 6.0 | 3.9 | - | Li et al. (2018) |

[T] Ex-type, ex-neotype, and ex-epitype strains are indicated. Japanese isolates examined in this study are indicated in bold.

truncate at the base, hyaline, aseptate or ralely one-two septate, smooth with granular contents, 16–23 × 4.8–6 μm, L/W = 3.72 (min 3.27, max 4.04; n = 5).

**Cultural characteristics on PDA:** Colonies were gray to dark gray with dense aerial mycelia, reaching 90 mm at 7 days after inoculation.

**Host:** *Juglans regia*, *Morus* sp., *Populus* sp. [22], *Paulownia tomentosa, Prunus* sp. (this study).

**Materials examined:** on *Prunus* sp., **Japan**, Ibaraki, Tsukuba, May 1993, by T. Yamada (culture MAFF 410827 = MUCC 2522); on *Aucuba japonica*, **Japan**, Tokyo, Minato, 12 Feb 1980, by T. Konayashi (culture MUCC 2533 = FFPRI 411202); and on *Paulownia tomentosa*, **Japan**, Niigata, Uonuma, 10 Jul 1978, by H. Hayashi (culture MUCC 2537 = FFPRI 411203).

**Note:** From the results of the molecular and phylogenetic analyses, three examined isolates were located in the same clade composed of ex-type isolates of *B. auasmontanum* (CBS 121769), *B. sinensis* (CGMCC 3.17722), and *B. wangensis* (CGMCC 3.18744). This clade was not supported statistically on the ML tree but the Bayesian tree. *B. sinensis*, *B. wangensis*, MUCC 2522, MUCC 2533, and MUCC 2537 formed an inner clade supported moderately with a PP value (0.91). The conidia size of MUCC 2522 (16–23 × 4.8–6) was somewhat smaller than *B. sinensis* [(15) 19–29 × 5–7] and *B. wangensis* [(20.5) 22–26 (29) × (4.5) 5.5–6.5 (7.5); Zhou et al. 2016, Li et al. 2018[20,21]]. The ITS and *tef1-α* sequences of MUCC 2522 were identical to *B. sinensis*. Also, the conidia of MUCC 2537 (14–27 × 3–5.8) were narrower than that of *B. sinensis* and *B. wangensis* (Table 4–5). Only a few mutations were observed in the *tef1-α* regions of MUCC 2537 compared to *B. sinensis*.

***Botryosphaeria tenuispora*** Y. Hattori & C. Nakashima, sp. nov. [MB837514], Fig. 4–3.

**Etymology:** Name derived from the shape of the slender conidia.

**Teleomorphic state:** Unknown.

**Anamorphic state formed on the host:** Leaf spots brown to yellowish-brown, small at

the edge, later enlarged and coalescent, expanded toward the whole of a leaf. Conidiomata epidermal, merged, solitary, scattered, black to dark brown, ellipsoid, 446.68 × 476.03 μm; pycnidial wall composed of depressed or irregular cells in five to eight layers, brown to dark brown, blackish around the ostiole, paler toward the conidiogenous region; paraphyzes hyaline, rounded at the apex, septate. Conidiophores reduced to conidiogenous cells. Conidiogenous cells hyaline, cylindrical, phialidic conidiogenesis with periclinal thickening, or holoblastic conidiogenesis after percurrent proliferation at the tip, smooth, 8.8–26.5 × 1.9–4.4 μm. Conidia fusiform to cylindro-clavate, rounded at the apex, convex to truncate at the base with fine frill, hyaline, aseptate, smooth, with granular contents, 23–32 × 4–6.7 μm, L/W = 5.40 (min 3.51, max 6.85; n = 115).

**Cultural characteristics on PDA:** Colonies were gray to dark gray with dense aerial mycelia, reaching 90 mm at 7 days after inoculation. On BMA: Conidiomata formed within 7 days, solitary or aggregate, dark brown to dark gray, covered with white, yellowish-green, to dark green hyphae, 287–635 ×266–597 μm (MUCC 2900).

**Holotypus:** on *Leucothoe catesbaei*, **Japan**, Aichi, Nagoya, 18 Jul 2006, by I. Araki (MUMH 10420, ex-type culture MUCC 237).

**Host:** *Aucuba japonica*, *Leucothoe. catesbaei* (this study).

**Materials examined:** on *Leucothoe catesbaei*, **Japan**, Aichi, Nagoya, 18 Jul 2006, by I. Araki (MUMH 10420, culture MUCC 237); from the fruit gall induced by *Asphondylia aucubae* on *Aucuba japonica*, **Japan**, Ibaraki, Tsukuba, May 23, 2019, by N. Uechi (culture MUCC 2900 = 18-2).

**Note:** On the resultant tree of molecular and phylogenetic analyses, this species formed a single clade. The clade composed of the two examined isolates was moderately supported by the statistical values of ML and BI (ML BS: 68, BI PP: 0.98). This species is phylogenetically closely related to *B. auasmontanum*, *B. dothidea*, *B. minutispermatia*, *B. sinensis*, and *B. wangensis*. However, the L/W ratio of *B. tenuispora* was bigger than that of the hitherto known species in the same clade (Table 4–5). Moreover, the size of

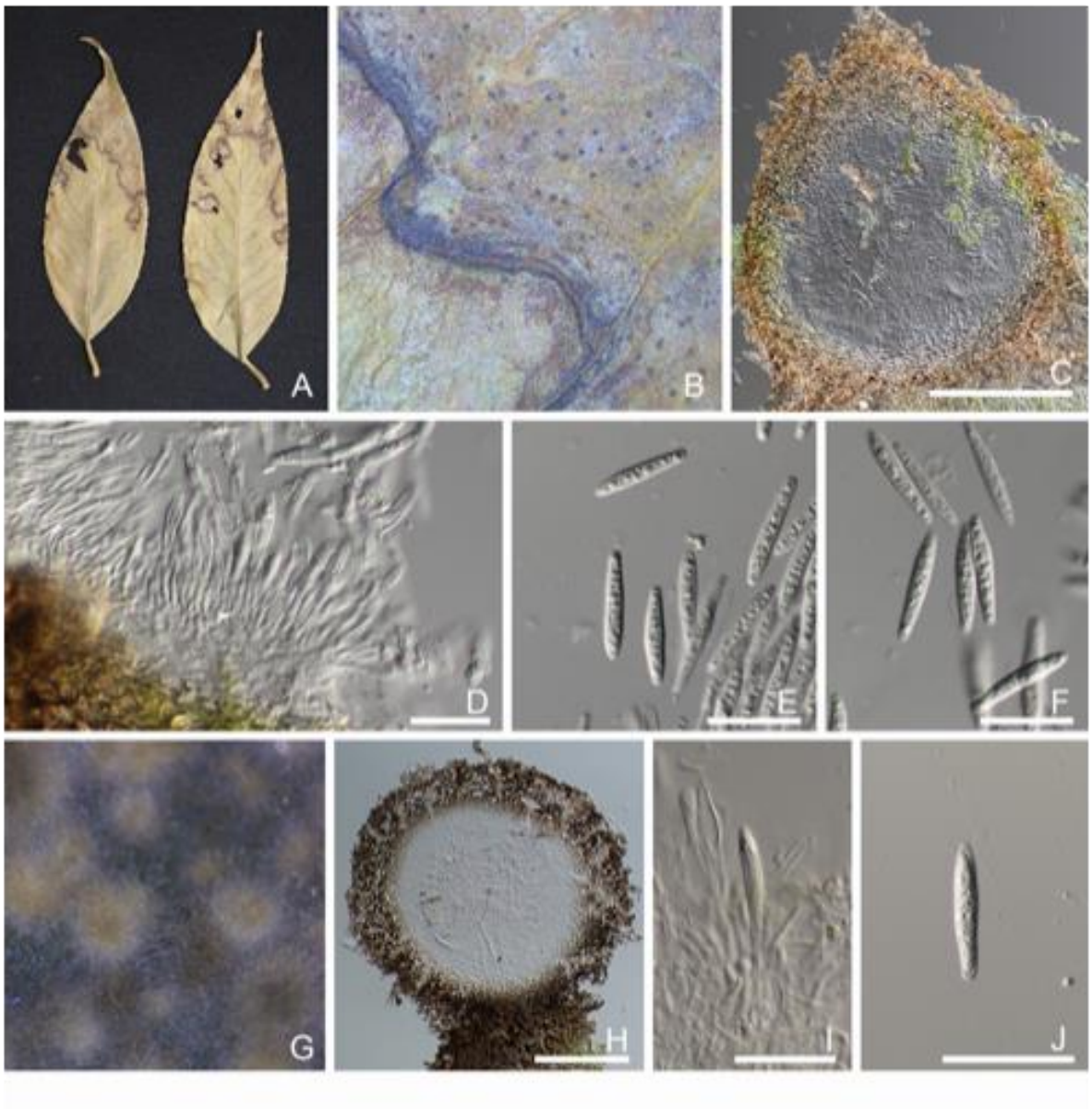


Fig. 4–3: Morphological features of *Botryosphaeria tenuispora* [**A**–**F**: MUMH 10420 (MUCC 237) and **G**–**I**: MUCC 2900]. (**A**) Specimen MUMH 10420. (**B**) Symptoms with pycnidia forming on the leaf of *Leucothoe fontanesiana*. (**C**) Vertical section of pycnidium in the leaf tissue. (**D**) Conidia and conidiophores. (**E** and **F**) Conidia. (**G**) Conidiomata formation on BMA after 7 days. (**H**) Conidiomata. (**I**) Conidium and conidiophores. (**J**) Conidium. Scale bars, 200 μm (**C** and **H**) and 25 μm (**D**–**F** and **I**–**J**).

the conidia of *B. tenuispora* (23–32 × 4–6.7 µm) was larger than that of *B. auasmontanum* [(8.1) 8.8–11.3 (13) × (2.5) 2.9–3.9 (5) µm] and *B. minutispermatia* (8–14 × 3–4; [23,24]).

***Botryosphaeria* sp.**, Fig. 4–4.

**Teleomorphic state:** Unknown.

**Anamorphic state formed on the host:** Conidiomata formed on BMA within 7 days, solitary or aggregate, dark brown to dark gray, covered with white to dark green hyphae, globose to ellipsoid, 252–712 × 208–422 µm; pycnidial wall composed of depressed or irregular cells in five to eight layers, brown to dark brown, blackish around the ostiole, paler toward the conidiogenous region; paraphyzes hyaline, rounded at the apex, septate. Conidiophores reduced to conidiogenous cells. Conidiogenous cells hyaline, cylindrical, smooth, phialidic conidiogenesis with periclinal thickening, or holoblastic conidiogenesis after percurrent proliferation at the tip, 8.5–16.8 × 1.3–3.1 µm. Conidia fusiform to cylindro-clavate, rounded at the apex, convex to truncate at the base with fine frill, hyaline, aseptate, smooth, with granular contents, 14.6–29 × 3.2–6.2 µm, L/W = 4.38 (min 3.39, max 5.88; n = 100).

**Cultural characteristics on PDA:** Colonies were gray to dark gray with dense aerial mycelia, reaching 90 mm at 7 days after inoculation.

**Host:** *Aucuba japonica, Castanea crenata*.

**Materials examined:** from a pupa of *Asphondylia aucubae*, **Japan**, Ibaraki, Tsukuba, April 29, 2019, by N. Uechi (culture MUCC 2897 = 2); fruit galls induced by *Ashpondylia aucubae* on *Aucuba japonica*, ibid, May 23, 2019, by N. Uechi (culture MUCC 2898 = 17-2); fruit galls induced by *Ashpondylia aucubae* on *Aucuba japonica*, ibid, May 23, 2019, by N. Uechi (culture MUCC 2899 = 21-1); fruit galls induced by *Ashpondylia aucubae* on *Aucuba japonica*, ibid, May 23, 2019, by N. Uechi (MUCC 2901 = 23-1); and on *C. crenata*, **Japan**, Kumamoto, Uki, 8 Sep 2017, by Y. Fukunaga

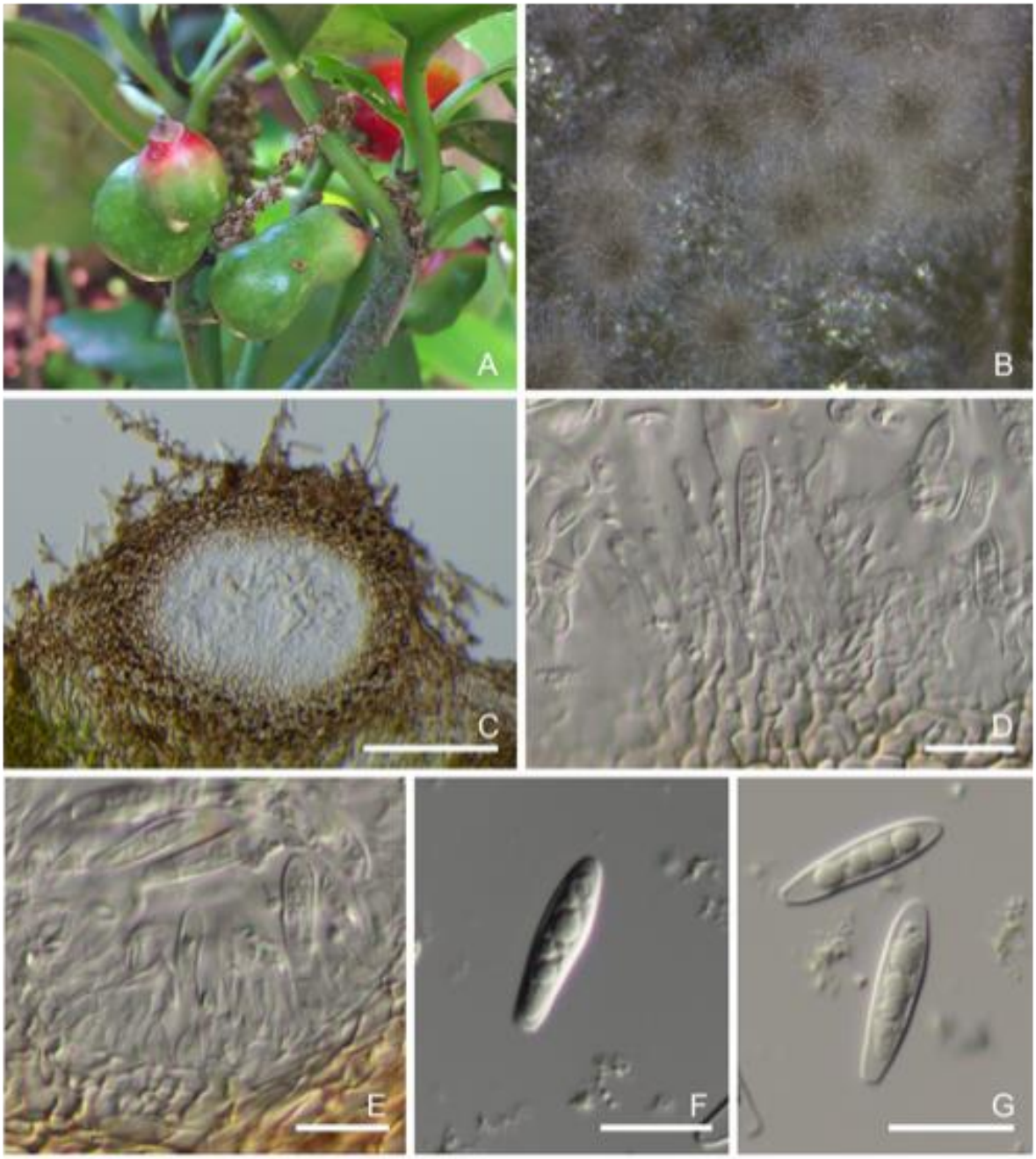


Fig. 4–4: Morphological features of *Botryosphaeria* sp. (**A** and **B**: MUCC 2899). (**A**) fruit galls by *Asphondylia aucabae* on *Aucuba japonica*. (**B**) Conidiomata formation on the BMA after 7 d. (**C**) Vertical section of pycnidium in the leaf tissue. (**D**–**E**) Conidia and conidiophores. (**F**–**G**) Conidia. Scale bars, 200 μm (**C**) and 25 μm (**D**–**G**).

(culture MUCC 2754 = K-018).

**Note:** All isolates, except MUCC 2754, were obtained from the insect (*Asphondylia aucubae*) galls and pupa, which were induced on fruit of *A. japonica*. In contrast, MUCC 2754 was isolated from diseased chestnuts. The relationship among these isolates is unclear. On the Bayesian tree, these isolates were recognized as an independent clade but were supported with a somewhat weak PP value (0.79). This suggested that it should be treated as a species.

## Discussion

In this study, isolates of the genus *Botryosphaeria* in Japan were reexamined for their taxonomical position based on molecular phylogeny and morphology. As a result, these isolates were divided into five clades: *B. dothidea*, *B. qingyuanensis*, *B. sinensis*, *B. tenuispora*, and *Botryosphaeria* spp. *Botryosphaeria qingyuanensis* and *B. sinensis* have been newly added to the Japanese mycoflora. *Botryosphaeria tenuispora* was described as a new species based on its phylogenetic position and morphological characteristics of the conidia. Although *B. dothidea* is known as a polyxenic species, it was confirmed that plural *Botryosphaeria* sp. were sharing one host plant species, *B. dothidea* and *B. sinensis* infected and established the habitat on *Prunus* sp., *B. dothidea* and *Botryosphaeria* sp. were from *C. crenata*, *B. tenuispora* and *Botryosphaeria* sp. were from *A. japonica*, and *B. dothidea* and *B. tenuispora* were from *Leucothoe fontanesiana*. In contrast, the current taxonomic position of the hitherto known Japanese species, such as *B. laricina* that causes the shoot blight of genus *Larix* [25,26] and *B. yedoensis* that inhabits *Prunus* spp. [27], are still unclear. More detailed studies based on phylogeny and morphology are required. *Botryosphaeria* spp. are often isolated from the insect gall. The relationships between gall midges and host plants have been discussed. *Asphondylia* species on *Acacia* and *B. dothidea* [8] and *Asphondylia prosopidis* on *Prosopis* tree and *B. dothidea* have been studied [9]. In Italy and Poland, *B. dothidea* isolated from the *Asphondylia* gall on

Lamiaceae had identical sequences. In contrast, the fungus isolated from the gall collected in the Southern Hemisphere showed mutations in those sequences [10]. In this study, the isolates from galls and pupa on the fruit of *A. japonica* affected by *A. aucubae* and one isolate from *C. crenata* formed a single clade on the BI tree with a weak PP value (0.79; *Botryosphaeria* spp. on Fig. 4–2). The morphological characteristics of conidia and the ecological niche of the isolates suggested that it should be treated as a new species.

In this study, three strains of *Botryosphaeria* that were isolated from the galls and twigs of *A. japonica*, a native plant in East Asian countries, were recognized (Fig. 4–2). The species diversity of *Botryosphaeria* on *Aucuba* and its origin is interesting. The insect gall on *Aucuba* is formed by monophagous gall midge, *A. aucubae* [28]. This indicates that the monophagous midge does not act as a vector of *Botryosphaeria* from plants belonging to different plant genera. In contrast, as described above, *B. dothidea* has often been reported to be related to the gall, and its dispersal has been discussed [8,9]. In Japan, the warty stem blight of *A. japonica* by *Botryosphaeria* sp. has been reported [29]. However, its taxonomical position in the current species criteria based on phylogeny is unknown. Furthermore, MUCC 2533 isolated from the branch of *A. japonica* was identified as *B. sinensis*. In the future, it is necessary to clarify the relationship among *Botryosphaeria* sp. related to diseases, galls, *Asphondylia* species, and host plants. These studies would contribute to revealing the interspecific interaction, such as the cospeciation and expansion of niches, of fungi.

*Botryosphaeria dothidea* is distributed worldwide and has many hosts. According to the U.S. Department of Agriculture fungal host database, *B. dothidea* has been recorded to infest 403 plant species [30]. In this study, 49 Japanese isolates were identified as *B. dothidea* species complex (Fig. 4–2). In recent years, some new species, *B. sinensis* [22], *B. minutispermatia* [24], *B. wangensis* [21], and *B. guttulata* [13] have been described as closely related species of *B. dothidea*. These species are distinguished from other closely related species by their phylogenetic positions and morphological characteristics. However, the phenotypic characteristics of the conidia of *B. dothidea* have been reported

to be various and unstable [10]. In this study, the morphology of the conidia of 13 isolates of *B. dothidea* was examined. The combined characteristic phylogeny and morphology is useful and stable for the recognition of the species.

*B. tenuispora*, proposed as a new species in this study, is closely related to *B. dothidea*. It formed an independent clade as an inner clade of *B. dothidea* (Fig. 4–2) and had a typically higher L/W ratio than the hitherto known species (Table 4–5). This taxon is recognized using the combined data of ITS+ TEF1-α+TUB2 regions, which are regions that are currently used regions for the molecular recognition of *Botryosphaeria* sp. [4,21]. In phylogenetic analyses, including those of Japanese strains, statistical support values for clades of *B. dothidea* and the hitherto known species closely related to *B. dothidea* were generally low. It is necessary to find stable phenotypic characters in morphology and the additional loci to analyze the phylogenetic relationships. Moreover, as the reports of the new species of the genus *Botryosphaeria* are eccentrically located in East Asia, a more global taxonomic, ecological, and phylogenetic survey of this genus is required in the future.

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## Chapter 5

# Taxonomical re-examination of genus *Neofusicoccum* in Japan


## ABSTRACT

*Neofusicoccum* is a genus of plant pathogenic fungi associated with various woody plants. Since *Neofusicoccum* has very similar morphological characteristics to the genus Botryospaheria, molecular phylogenetic analysis is essential to determine its taxonomic position. In Japan, a comprehensive taxonomic study of the genus Neofusicoccum has not been conducted. To elucidate the species diversity in Japan, we reexamined Japanese isolates of *Neofusicoccum* based on their morphology and molecular phylogenetic relationships, using the internal transcribed spacer (ITS) regions rpb2, tef1-α, and tub2. The Japanese isolates were divided into five clades recognized as the species. These species were *N. parvum*, other *Neofusicoccum* spp., and three new species proposed in this study, *N. hyperici*, *N. miyakoense*, and *N. okinawaense*. Furthermore, Physalospora laricina, which causes shoot blight of larch (*Larix* spp.), was transferred to the genus Neofusicoccum, and we propose its epitype and ex-epitype isolate.



**Keyword:** *Botryosphaeriaceae*, epitypification, *Larix*, new species, phylogeny,

# 1. Introduction

*Neofusicoccum* (Botryosphaeriaceae) is a genus of plant pathogenic fungi associated with various woody plants that causes shoot blight, canker, and dieback of fruit and forest trees (Phillips et al., 2008). The genus was established by Crous et al. (2006), based on the type species *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A. J. L. Phillips, and is closely related to the genus *Fusicoccum*. *Neofusicoccum* is characterized by fusiform to ellipsoid aseptate conidia and "dichomera-like" synanamorphs, which septate and turn brown with age, to form globose to pyriform conidia (Barber et al., 2005; Phillips et al., 2005; Crous et al., 2006). The sexual morphs of Botryosphaeriaceae are similar in morphology, such that these structures are difficult to distinguish among the genera *Botryosphaeria, Fusicoccum,* and *Neofusicoccum*. These genera have been historically confused. The genus *Fusicoccum,* which had been regarded as an asexual morph of the genus *Botryosphaeria,* is known to be polyphyletic and synonymous with various other genera (Phillips et al., 2002; Crous et al. 2006). Therefore, molecular phylogenetic analysis is essential to discriminate between *Fusicoccum* and other *Fusicocum*-like genera (Marin-Felix et al., 2017). Taxonomic studies of the genus *Neofusicoccum* and its related genera have been conducted based on worldwide multi-locus phylogenetic analyses (Dissanayake et al., 2016; Yang et al., 2017; Marin-Felix et al., 2017; Zhang et al., 2017). New generic criteria based on molecular phylogeny have resulted in an increased number of species, due to reclassification of species in the hitherto known Botryosphaeriaceae. According to the fungal names database Mycobank (https://www.mycobank.org, accessed 30 Sep 2020), forty-six species of *Neofusicoccum* have been described.

In Japan, several diseases caused by *Neofusicoccum* species have been reported: black leaf blight of *Cymbidium* spp. caused by *N. parvum* (Suzukiet al., 2018); canker of *Platanus* × *acerifolia* caused by *N. parvum* (Motohashi et al., 2016); stem end rot of *Mangifera indica* by *N. parvum* (Takushi et al., 2017) and *Neofusicoccum* sp. (Hara et al.,

2016); fruit rot of chestnut caused by *Neofusicoccum* sp. (Sasaki et al., 2019). Etiological studies have been conducted in response to severe damage of important crops, including identifying the causal fungi; however, a comprehensive study on the diversity of Japanese *Neofusicoccum* species from various hosts has not yet been conducted. Moreover, our molecular phylogenetic analysis of Botryosphaeriaceae showed that many isolates previously identified as *Botryosphaeria* spp. belong to the *Neofusicoccum* clade (Hattori et al., 2019). In many Japanese isolates of the genus *Neofusicoccum*, the taxonomic positioning is unclear. The purpose of this study was to elucidate the species diversity of the genus *Neofusicoccum* in Japan. Therefore, we examined the morphological characteristics and molecular phylogenetic relationships of isolates preserved in culture collections as species of *Botryosphaeria, Fusicoccum,* and *Guignardia*, in addition to the isolates obtained during this study.

## 2. Materials and methods

### *2.1. Fungal isolation*

Mango (*Mangifera indica*) and Coffee (*Coffea* sp.) leaves or branches showing symptoms of leaf or shoot blight were collected from Miyako-jima Island, Okinawa, Japan. Isolates of *Neofusicoccum* spp. were obtained using the single conidia isolate method described by Nakashima et al. (2016). The isolates were cultivated on a potato dextrose agar (PDA) medium (Nissui Pharmaceutical Co., Ltd., Japan) and transferred to a pine needle agar (PNA) medium to observe conidiomata and conidia (Smith et al. 1996). For accurate identification, the twelve isolates and two specimens that had been preserved as *Botryosphaeria*, *Fusicoccum*, or *Guinardia* species at the Laboratory of Forest Pathology, Forestry and Forest Products Research Institute (FFPRI, TFM: FPH) (Tsukuba, Ibaraki, Japan) and the culture collection of the Laboratory of Phytopathology, Mie University (TSU-MUCC) (Tsu, Mie, Japan) (Table 1) were revitalized. Herbarium specimens were borrowed from the Iwate University Museum (IUM) (Iwate, Japan) for

Table 5–1. List of Japanese *Neofusicoccum* isolates and specimens used in this study.

| Fungal species | MUCC No. | Original No. | Material No. | Host Family | Host species | Regions | Identified by previous study | Reference |
|---|---|---|---|---|---|---|---|---|
| *N. hyperici* | 241 | – | MUMH 10423 | Hypericaceae | *Hypericum patulum* | Aichi | *Fusicoccum* sp. | this study |
| *N. hyperici* | 2509 | B1-2 (MAFF 410797) | – | Hypericaceae | *Hypericum galioides* | Tokyo | *Botryosphaeria* sp. | this study |
| *N. hyperici* | 650 | – | MUMH 10606 | Stachyuraceae | *Stachyurus praecox* | Aichi | *Fusicoccum* sp. | this study |
| *N. laricinum* | 2660 | GC74 (MAFF 410183) | – | Pinaceae | *Larix kaempferi* | Hokkaido | *Botryosphaeria laricina* | Motohashi et al. (2009) |
| *N. laricinum* | 2662 | GC59 (FFPRI 411215) | TFM: FPH-4038 | Pinaceae | *Larix decidua* | Ibaraki | *Guignardia laricina* | this study |
| *N. laricinum* | 2663 | GC38 (FFPRI 411216) | – | Pinaceae | *Larix decidua* | Iwate | *Guignardia laricina* | this study |
| *N. laricinum* | 2666 | GC14 (FFPRI 411217) | – | Pinaceae | *Larix kaempferi* | Aomori | *Guignardia laricina* | this study |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *N. laricinum* | 2669 | GC82 (FFPRI 411218) | – | Pinaceae | *Larix kaempferi* | Iwate | *Guignardia laricina* | this study |
| *N. miyakoense* | 2585 | – | MUMH 11936 | Coffeeae | *Coffea* sp. | Okinawa, Miyako-jima island | – | this study |
| *N. miyakoense* | 2586 | – | MUMH 11937 | Anacardiaceae | *Mangifera indica* | Okinawa, Miyako-jima island | – | this study |
| *N. okinawaense* | 789 | MAFF 240624 | MUMH 10839 | Lythraceae | *Lagerstroemia speciosa* | Okinawa, Main island | *Fusicoccum* sp. | Nakashima (2008) |
| *N. parvum* | 2511 | B1-10 (FFPRI 411214) | TFM: FPH-5589 | Tamaricaceae | *Tamarix tenuissima* | Niigata | *Botryosphaeria* sp. | this study |
| *N. parvum* | 392 | – | MUMH 10368 | Ericaceae | *Rhododendron hybrids* | Aichi | *Fusicoccum* sp. | this study |

the morphological studies. All isolates were cultured at room temperature (22 °C). All dried cultures and specimens for microscopic studies were kept in the mycological herbarium at Mie University (TSU-MUMH), and these established isolates have been maintained in the culture collection of TSU-MUMH and TSU-MUCC.

### *2.2. Molecular and phylogenetic analysis*

Genomic DNA was extracted from mycelial disks after culturing for seven days on PDA plates using a DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Targeted sequences of the rDNA ITS, rpb2, tef1-α, and tub2 gene-coding regions were amplified with a T100 Thermal Cycler (Bio-Rad, Tokyo, Japan) by polymerase chain reaction (PCR). The total volume of the PCR mixture was 12.5 μL. It consisted of 1 to 10 ng genomic DNA, 0.05 μL of 0.25 unit Taq DNA polymerase (Bioline, London, UK; tef1-α and rpb2: 0.1 μL), 1.25 μL of 10 × NH4 reaction buffer (Bioline), 1.9 to 2.5 mM MgCl2 (Bioline; ITS, rpb2, and tef1-α: 2.5 mM, tub2: 1.9 mM), 2.5 to 5.0 mM each of deoxyribonucleotide triphosphate mixture (Bioline; ITS: 2.5 mM, tef1-α, tub2, rpb2: 5.0 mM), 0.2 μM of each primer, 5.6% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA; added only for tef1-α amplification), and sterilized distilled water up to 12.5 μL. The rDNA ITS was amplified with the primers V9G (de Hoog & Gerrits van den Ende, 1998) and ITS4 (White et al., 1990); rpb2 with the primers RPB2-5f2 and fRPB2-7cR (Liu et al., 1999); tef1-α with the primers EF1-728F, and EF1-986R (Carbone & Kohn, 1999); and tub2 with the primers BT2A and BT2B (Glass & Donaldson, 1995). The PCR conditions for ITS, tef1-α, and tub2 were: initial denaturation at 94 °C for 5 min; 40 cycles of amplification (denaturation at 94 °C for 45 s; annealing at 48 °C (tef1-α: 52 °C, tub2: 55 °C) for 30 s; and extension at 72 °C for 90 s); and final extension at 72 °C for 7 min. The PCR conditions for rpb2 were: initial denaturation at 95 °C for 5 min; touch-down amplification (5 cycles of 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 120 s; 5 cycles of 95 °C for 45 s, 58 °C for 45 s, and 72 °C for 120 s; and 30 cycles of 95 °C for 45 s, 54 °C for 45 s, and 72 °C for 120 s), and final

elongation at 72 °C for 8 min. The amplicon was sequenced in both directions using the respective PCR primers and the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 3730xl DNA analyzer installed at the Mie University Advanced Science Research Promotion Center (Tsu, Mie, Japan). The sequences were assembled and aligned with 46 sequences of Neofusicoccum species recollected from GenBank, and aligned using MAFFT software version 7 (Katoh et al., 2019). Maximum likelihood (ML) and Bayesian inference (BI) analyses were used to estimate phylogenetic relationships. ML analyses were performed using raxml HPC-PTHREADS software (Stamatakis, 2006). The strength of the internal branches from the resultant trees was tested by bootstrap (BS) analysis (Felsenstein, 1985) using 1,000 replications. BI analyses were performed using BEAST version 2.5.1 (Bouckaert et al., 2019) to estimate the posterior probabilities (PPs) of tree topologies based on metropolis-coupled Markov chain Monte Carlo (MCMC) searches, which used the MCMC algorithm of four chains in parallel from a random tree topology. The MCMC analysis lasted 10,000,000 generations. Trees were sampled and saved every 1,000 generations. The first 25% of saved trees were discarded as burn-in, and the PPs were determined from the remaining trees. Representative sequences for all taxa were uploaded to GenBank (Table 2). The sequence alignments prepared in this study were deposited in TreeBASE number S27074.

## 3. Results

### *3.1. Phylogeny*

A total of 13 isolates were obtained from the samples in this study (Table 1). The ITS + rpb2 + tef1-α + tub2 combined dataset consisted of 60 sequences with a total of 1,829 characters (ITS: 551, rpb2: 593, tef1-α: 301, tub2: 384), including alignment gaps. The out-group taxon was *Botryosphaeria dothidea* (CBS 100564). The two trees estimated using ML and BI had the same species grouping. As a result of the phylogenetic

Table 5–2. List of *Neofusicoccum* species used for phylogenetic analysis.

| Fungal species | Isolates No. | Host | Country | Accession numbers | | | |
|---|---|---|---|---|---|---|---|
| | | | | ITS | tef1 | tub2 | rpb2 |
| *Botryosphaeria dothidea* | CBS100564 | *Paeonia* sp. | Netherlands | KX464085 | KX464555 | KX464781 | KX463951 |
| *Neofusicoccum algeriense* | CBS 137504$^{T}$ | *Vitis vinifera* | Algeria | KJ657702 | KJ657721 | – | – |
| *N. andinum* | CBS 117453$^{T}$ | *Eucalyptus* sp. | Venezuela | AY693976 | AY693977 | KX464923 | KX464002 |
| *N. arbuti* | CBS 116131$^{T}$ | *Arbutus menziesii* | USA | AY819720 | KF531792 | KF531793 | KX464003 |
| *N. australe* | CBS 139662$^{T}$ | *Acacia* sp. | Australia | AY339262 | AY339270 | AY339254 | EU339573 |
| *N. batangarum* | CBS 124924$^{T}$ | *Terminalia catappa* | Cameroon | FJ900607 | FJ900653 | FJ900634 | FJ900615 |
| *N. brasiliense* | CMM 1285$^{T}$ | *Mangifera indica* | Brazil | JX513628 | JX513608 | KC794030 | – |
| *N. buxi* | CBS 116.75$^{T}$ | *Buxus sempervirens* | France | KX464165 | KX464678 | – | KX464010 |
| *N. cordaticola* | CBS 123634$^{T}$ | *Syzygium cordatum* | South Africa | EU821898 | EU821868 | EU821838 | EU821928 |
| *N. corticosae* | CBS 120081$^{T}$ | *Eucalyptus corticosa* | Australia | MN161920 | KX464682 | KX464958 | KX464013 |
| *N. cryptoaustrale* | CBS 122813$^{T}$ | *Eucalyptus* sp. | South Africa | FJ752742 | FJ752713 | FJ752756 | KX464014 |
| *N. eucalypticola* | CBS 115679$^{T}$ | – | – | AY615141 | AY615133 | AY615125 | – |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *N. eucalyptorum* | CBS 115791[T] | *Eucalyptus grandis* | South Africa | AF283686 | AY236891 | AY236920 | – |
| *N. grevilleae* | CBS 129518[T] | *Grevillea aurea* | Australia | JF951137 | – | – | – |
| *N. hellenicum* | CERC 1947[T] | *Pistacia vera* | Greece | KP217053 | KP217061 | KP217069 | – |
| *N. hongkongense* | CERC 2973[T] | *Araucaria cunninghamii* | China | KX278052 | KX278157 | KX278261 | KX278283 |
| ***N. hyperici*** | **MUCC 241[T]** | ***Hypericum patulum*** | **Japan** | **LC589125** | **LC589137** | **LC589147** | **LC589160** |
| ***N. hyperici*** | **MUCC 2509** | ***Hypericum galioides*** | **Japan** | **LC589126** | **LC589138** | **LC589148** | **LC589161** |
| ***N. hyperici*** | **MUCC 650** | ***Stachyurus praecox*** | **Japan** | **LC589124** | **LC589136** | **–** | **LC589159** |
| *N. illicii* | CGMCC 3.18310[T] | *Illicium verum* | China | KY350149 | – | KY350155 | – |
| *N. italicum* | MFLUCC 15-0900[T] | *Vitis vinifera* | Italy | KY856755 | KY856754 | – | – |
| *N. kwambonambiense* | CBS 123639[T] | *Syzygium cordatum* | South Africa | EU821900 | EU821870 | EU821840 | EU821930 |
| ***N. laricinum*** | **MUCC 2660** | ***Larix kaempferi*** | **Japan** | **LC589131** | **LC589142** | **LC589153** | **LC589166** |
| ***N. laricinum*** | **MUCC 2662[T]** | ***Larix decidua*** | **Japan** | **LC589129** | **LC589140** | **LC589151** | **LC589164** |
| ***N. laricinum*** | **MUCC 2663** | ***Larix decidua*** | **Japan** | **LC589130** | **LC589141** | **LC589152** | **LC589165** |
| ***N. laricinum*** | **MUCC 2666** | ***Larix kaempferi*** | **Japan** | **LC589132** | **LC589143** | **LC589154** | **LC589167** |
| ***N. laricinum*** | **MUCC 2669** | ***Larix kaempferi*** | **Japan** | **LC589128** | **LC589139** | **LC589150** | **LC589163** |
| *N. lumnitzerae* | CMW 41469[T] | *Lumnitzera racemosa* | South Africa | KP860881 | KP860724 | KP860801 | KU587925 |
| *N. luteum* | CBS 562.92[T] | *Actinidia deliciosa* | New Zealand | KX464170 | KX464690 | KX464968 | KX464020 |
| *N. macroclavatum* | CBS 118223[T] | *Eucalyptus globulus* | Australia | DQ093196 | DQ093217 | DQ093206 | KX464022 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *N. mangiferae* | CBS 118531$^{T}$ | *Mangifera indica* | Australia | AY615185 | DQ093221 | AY615172 | – |
| *N. mangroviorum* | CMW 41365$^{T}$ | *Avicennia marina* | South Africa | KP860859 | KP860702 | KP860779 | KU587905 |
| *N. mediterraneum* | CBS 121718$^{T}$ | *Eucalyptus* sp. | Greece | MH863145 | – | – | KX464024 |
| *N. microconidium* | CERC3497$^{T}$ | *Eucalyptus urophylla* × *Eucalyptus grandis* | China | KX278053 | KX278158 | KX278262 | MF410203 |
| ***N. miyakoense*** | **MUCC 2585$^{T}$** | ***Coffea* sp.** | **Japan** | **–** | **LC589146** | **LC589157** | **LC589170** |
| ***N. miyakoense*** | **MUCC 2586** | ***Mangifera indica*** | **Japan** | **LC589133** | **LC589144** | **LC589155** | **LC589168** |
| *N. nonquaestitum* | CBS 126655$^{T}$ | *Umbellularia californica* | USA | GU251163 | GU251295 | GU251823 | KX464025 |
| *N. occulatum* | CBS 128008$^{T}$ | *Eucalyptus grandis* hybrid | Australia | EU301030 | EU339509 | EU339472 | EU339558 |
| ***N. okinawaense*** | **MUCC 789$^{T}$** | ***Lagerstroemia speciosa*** | **Japan** | **LC589134** | **LC589145** | **LC589156** | **LC589169** |
| *N. pandanicola* | MFLUCC 17-2270$^{T}$ | *Pandanus* sp. | China | MH275072 | MH412778 | – | – |
| *N. parvum* | CBS 138823$^{T}$ | *Populus nigra* | New Zealand | AY236943 | AY236888 | AY236917 | EU821963 |
| ***N. parvum*** | **MUCC 2511** | ***Tamarix tenuissima*** | **Japan** | **LC589127** | **–** | **LC589149** | **LC589162** |
| ***N. parvum*** | **MUCC 392** | ***Rhododendron hybrids*** | **Japan** | **LC589123** | **LC589135** | **–** | **LC589158** |
| ***N. pennatisporum*** | **MUCC 510$^{T}$** | ***Allocasuarina fraseriana*** | **Australia** | **EF591925** | **EF591976** | **EF591959** | **–** |
| *N. pistaciae* | CBS 595.76$^{T}$ | *Pistacia vera* | Greece | KX464163 | KX464676 | KX464953 | KX464008 |
| *N. pistaciarum* | CBS 113083$^{T}$ | *Pistacia vera* | USA | KX464186 | KX464712 | KX464998 | KX464027 |
| *N. pistaciicola* | CBS 113089$^{T}$ | *Pistacia vera* | USA | KX464199 | KX464727 | KX465014 | KX464033 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *N. protearum* | CBS 114176[T] | *Leucadendron salignum* x *L. laureolum* cv. Silvan Red | South Africa | AF452539 | KX464720 | KX465006 | KX464029 |
| *N. pruni* | CBS 121112[T] | *Prunus salicina* | South Africa | EF445349 | EF445391 | KX465016 | KX464034 |
| *N. ribis* | CBS 115475[T] | *Ribes* sp. | USA | AY236935 | AY236877 | AY236906 | EU339554 |
| *N. sinense* | CGMCC 3.18315[T] | Unknown woody plant | China | KY350148 | KY817755 | KY350154 | – |
| *N. sinoeucalypti* | CGMCC 3.18752[T] | *Eucalyptus urophylla* × *Eucalyptus grandis* | China | KX278061 | KX278166 | KX278270 | KX278290 |
| *N. stellenboschiana* | CBS 110864[T] | *Vitis vinifera* | South Africa | AY343407 | AY343348 | KX465047 | KX464042 |
| *N. terminaliae* | CBS 125263[T] | *Terminalia sericea* | South Africa | GQ471802 | GQ471780 | KX465052 | KX464045 |
| *N. umdonicola* | CBS 123645[T] | *Syzygium cordatum* | South Africa | EU821904 | EU821874 | EU821844 | EU821934 |
| *N. ursorum* | CBS 122811[T] | *Eucalyptus* sp. | South Africa | FJ752746 | FJ752709 | KX465056 | KX464047 |
| *N. variabile* | CBS 143480[T] | *Mimusops caffra* | South Africa | MH558608 | – | MH569153 | – |
| *N. versiforme* | CBS 118101[T] | *Eucalyptus camaldulensis* | Australia | KF766154 | KX464757 | KF766128 | KX464041 |
| *N. viticlavatum* | CBS 112878[T] | *Vitis vinifera* | South Africa | AY343381 | AY343342 | KX465058 | KX464048 |
| *N. vitifusiforme* | CBS 110887[T] | *Vitis vinifera* | South Africa | AY343383 | AY343343 | KX465061 | KX464049 |

[T)] Ex-type, ex-neotype, and ex-epitype isolates are indicated. Japanese isolates examined in this study are indicated in bold.

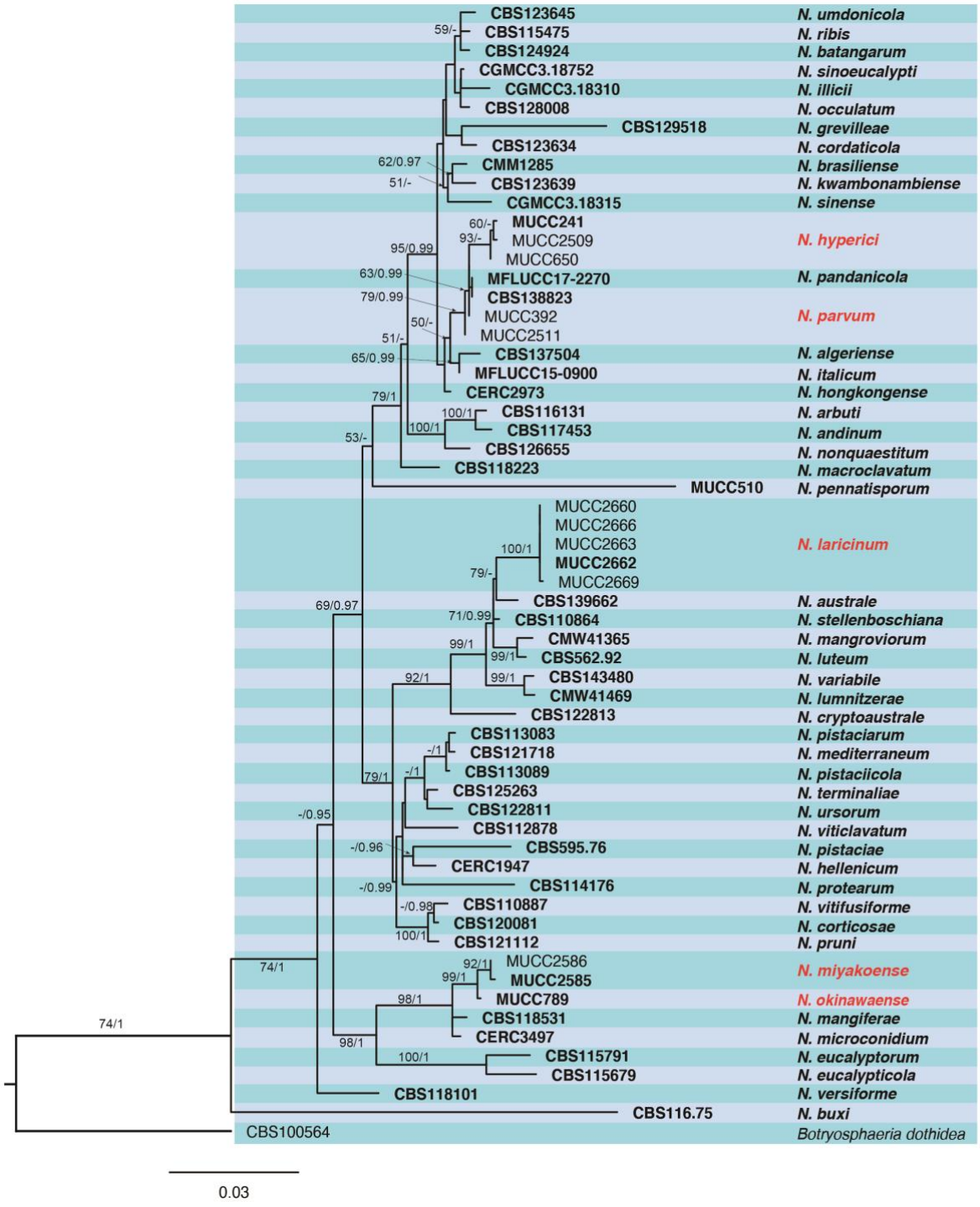


Fig. 5–1 – Phylogenetic tree of *Neofusicoccum* spp. Tree constructed by ML using combined ITS, rpb2, tef1-α, and tub2 gene region datasets. ML bootstrap values and Bayesian PPs are given near the branches (ML/PP). Ex-type strains are in bold. Ex-type strains are in bold. Species identified in this study are shown in red.

analysis, the 13 Japanese *Neofusicoccum* isolates in this study were divided into five clades. The first clade was composed of isolates from *Hypericum patulum* (MUCC 241), *H. galioides* (MUCC 2509), and *Stachyurus praecox* (MUCC 650). The second clade was composed of an ex-type isolate of *N. parvum* (CBS 138823), isolates from *Rhododendron hybrids* (MUCC 392), and *Tamarix tenuissima* (MUCC 2511). The third clade was composed of isolates from *Larix decidua* (MUCC 2662; MUCC 2663) and *L. kaempferi* (MUCC 2660; MUCC 2666; MUCC 2669). The fourth clade was from *Coffea* sp. (MUCC 2585) and *Mangifera indica* (MUCC 2586). The fifth clade was from *Lagerstroemia speciosa* (MUCC 789) (Fig. 1).

### *3.2. Taxonomy*

***Neofusicoccum hyperici*** Y. Hattori & C. Nakash., sp. nov., Fig. 5–2.

MycoBank no.: MB 837717.

**Etymology:** Named after the genus name of the host plant (*Hypericum patulum*) from which the type strain was obtained.

**Sexual morph:** Unknown.

**Asexual structures formed on the host and PNA:** Leaf spots yellowish-brown to brown, small at the edge, later enlarged and coalescent, expanded toward the whole of a leaf. Conidiomata pycnidial, epidermal, merged, superficial on PNA, solitary, globose, dark brown to black, ellipsoid, unilocular, with a central ostiole, 63–110 × 46–89 μm; pycnidial wall composed of depressed or irregular cells in three to four layers, dark brown to black. Conidiophores reduced to conidiogenous cells; conidiogenous cells discrete, hyaline, cylindrical to ampulliform, determinate, with periclinal thickening, rarely proliferating percurrently, 4.4–7 × 1.6–2.3 μm. Paraphyses not seen. Conidia holoblastic sporulation for first conidia, phialidic sporulation for following conidia, hyaline, smooth, aseptate, various in shape, cylindrical to fusiform, reniform, straight or slightly curved, granulate, subtruncate to bluntly rounded at the base, acute to rounded at the apex, 15–18 × 5.3–6.4 μm (av. = 15.96 × 6.00 μm, n = 50; L/W = 2.66).

**Cultural characteristics on PDA:** colony is white to dark gray, with floccose and dense aerial mycelia, and reaching 90 mm at 14 d after inoculation at room temperature (22 ℃). Holotypus: on *Hypericum patulum*, JAPAN, Aichi, Nagoya, 18 Jul 2006, by I. Araki (MUMH 10423, ex-type culture MUCC 241).

**Host:** *Hypericum galioides*, *Hypericum patulum, Stachyurus praecox*.

**Other materials examined:** on *Hypericum galioides*; JAPAN, Tokyo, Hachioji, 4 Jul 1963, by T. Kobayashi (culture MUCC 2509 = MAFF 410797); on *Stachyurus praecox*, JAPAN, Aichi, Nagoya, 17 Jul 2007, by I. Araki (MUMH 10606, culture MUCC 650).

**Note:** In molecular phylogenetic analysis, two strains isolated from the genus *Hypericum* (MUCC 241; MUCC 2509) and one strain from the genus *Stachyrus* (MUCC 650) formed a clade strongly supported by a 93% ML-BS value. In addition, MUCC 241 and MUCC 2509 formed an internal clade within the clade, but no difference in morphological characteristics was found among the three isolates. *Neofusicoccum hyperici* is phylogenetically closely related to *N. parvum* (CBS 138823) and *N. pandanicola* (MFLUCC 17-2270). However, the conidia size of *N. pandanicola* (15–26 × 8–12 μm) (Tibpromma et al., 2018) and *N. parvum* (11–)14–18(–23) × (7–)8–10(–11) μm (Pennycook & Samuels, 1985) were larger than *N. hyperici* (15–18 × 5.3–6.4 μm). *Neofusicoccum hyperici* showed almost no difference in morphological characteristics between the host plants and the PNA.

***Neofusicoccum laricinum*** (Sawada) Y. Hattori & C. Nakash., comb. nov., Fig. 5–3.

MycoBank no.: MB 837720.

≡ *Physalospora laricina* Sawada Bull. Gov. For. Exp. Stn. Tokyo 46: 126, 1950.

= *Guignaridia laricina* (Sawada) W. Yamam. & Kaz. Itô, Science Reports of the Hyogo University of Agriculture 5: 9, 1961.

= *Botryosphaeria laricina* (Sawada) Shang, Acta Mycologia Sinica 6: 249, 1987.

**Sexual morph:** *Physalospora laricina* (fide Sawada, 1950): Caulicolous. Diseased twigs defoliated from the mddle to the tip, with exudate resin. Fruit bodies lined, erumpent.

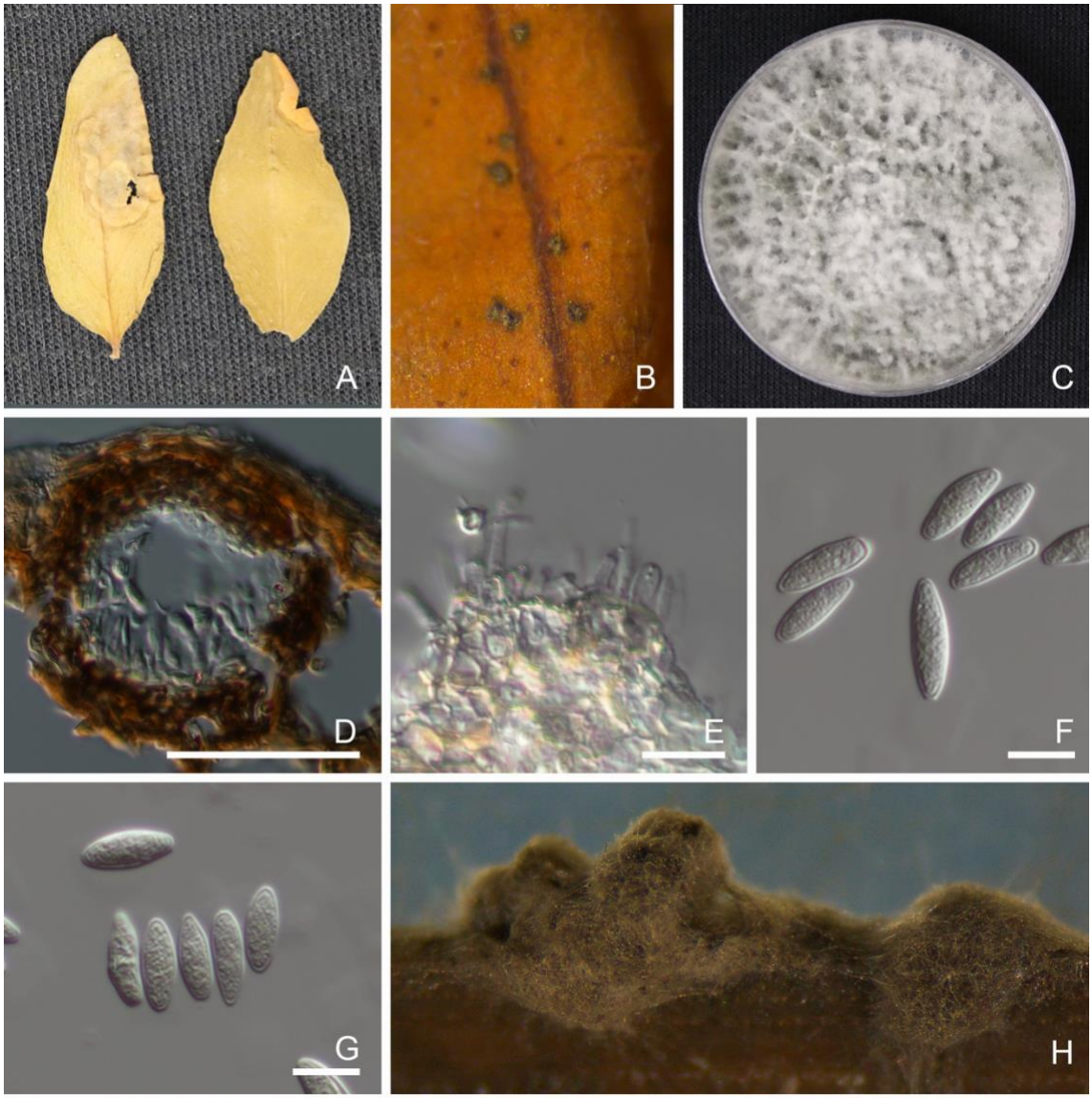


Fig. 5–2 – Morphological features of *Neofusicoccum hyperici* (MUMH 10423 and MUCC 241). A: Specimen MUMH 10420. B: Symptoms with pycnidia forming on *Hypericum patulum* leaf. C: Colony on PDA (MUCC 241). D: Vertical section of pycnidium in leaf tissue. E: Conidia and conidiophores. F: Conidia. *Bars*: D 50 µm; E 20 µm; F 10 µm.

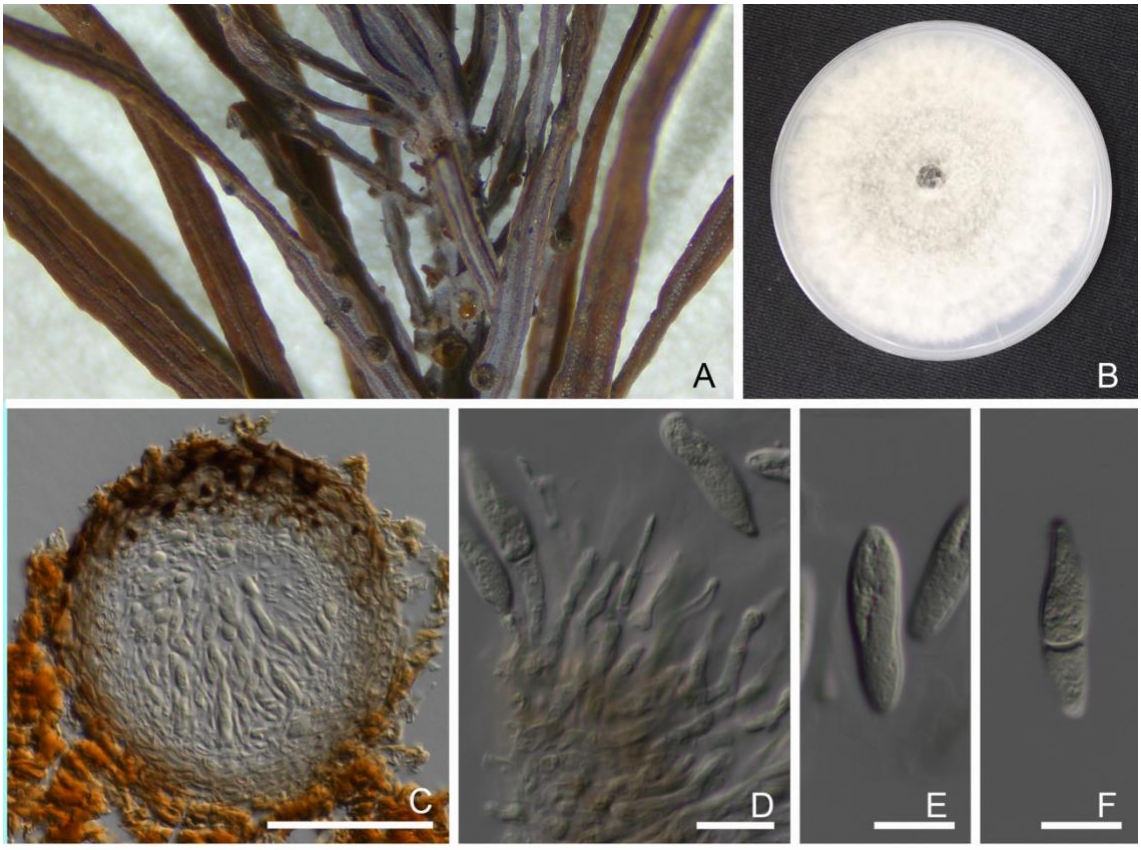


Fig. 5–3 – Morphological features of *Neofusicoccum laricinum* (FPH-4038 and MUCC 2662). A: Specimen FPH-4038; symptoms with pycnidia forming on *Larix decidua* leaf. B: Colony on PDA (MUCC 2662). C: Vertical section of pycnidium in leaf tissue. D: Conidia and conidiophores. E, F: Conidia. *Bars*: D 50 µm; E, F 20 µm.

Ascomata epidermal, blackish, globose, 368 µm in diameter; ostiole erumpent, 60 µm in diameter; paraphyses developed, intricate, 3 µm in width. Ascus clavate, rounded at the apex, stipitate at the base, hyaline, 114–135 × 22–26 µm. Ascospores ellipsoid, smooth, hyaline, 24–27 × 13 µm.

**Asexual structures formed on the host:** Conidiomata pycnidial, epidermal, merged, solitary, globose, dark brown, subglobose, unilocular, with a central ostiole, 204–246 × 207–212 µm; pycnidial wall composed of depressed or irregular cells in three to four

layers, brown to dark brown. Conidiophores reduced to conidiogenous cells; conidiogenous cells discrete, hyaline, cylindrical to ampulliform, determinate, with periclinal thickening, or proliferating percurrently, 9–23 × 2.4–5 μm. Paraphyses not seen. Conidia holoblastic sporulation for first conidia, phialidic sporulation for following conidia, hyaline, smooth, aseptate, slightly colored and septate with age, ellipsoid to fusiform, granulate, subtruncate to bluntly rounded at the base, rounded to subacute at the apex, with a short frill at the both ends, 23–38 × 7–12 μm (av. = 29.85 × 8.50 μm, n = 33; L/W = 3.57).

**Cultural characteristics on PDA:** colony is white to gray, with dense aerial mycelia, reaching 90 mm at 14 d after inoculation at room temperature.

Syntypus: on *Larix kaempferi*, JAPAN, Aomori, Sanbongi, 27 Sep 1949, by K. Sawada (IUM-FS515), on *Larix kaempferi*, ibid, 27 Sep 1949, by K. Sawada (IUM-FS516); on *L. kaempferi*, ibid, 27 Sep 1949, by K. Sawada (IUM-FS517); on *L. kaempferi*, JAPAN, Aomori, Yokohama, 3 Oct 1949, by K. Sawada & S. Murai (IUM-FS518); on *L. kaempferi*, ibid, 3 Oct 1949, by K. Sawada & S. Murai (IUM-FS519); on *L. kaempferi*, ibid, 3 Oct 1949, by K. Sawada & S. Murai (IUM-FS520).

**Epitype for anamorphic state (designated here):** on *Larix decidua*, JAPAN, Ibaraki, Mito, 14 Jun 1973, by H. Kondo (TFM: FPH-4038, ex-epitype culture FFPRI 411215 = MUCC 2662).

**Host:** *Larix kaempferi* (Sawada, 1950)*, L. decidua*

**Other materials examined:** on *Larix kaempferi*, JAPAN, Hokkaido, Tomakomai, 27 Jul 1971, by S. Yokota (culture MUCC 2660 = MAFF 410183); on *L. decidua*, JAPAN, Ibaraki, Mito, 14 Jun 1973; on *L. decidua*, JAPAN, Iwate, Morioka, 18 Aug 1965, by Y. Yokosawa (culture MUCC 2663 = FFPRI 411216); on *Larix kaempferi*, JAPAN, Aomori, Kamikita, 9 Jun 1961, by T. Uozumi (culture MUCC 2666 = FFPRI 411217); on *Larix kaempferi*, JAPAN, Iwate, Iwate, 3 Jul 1986, by T. Shoji (culture MUCC 2669 = FFPRI 411218).

**Note:** *Physalospora laricina* has been recognized as an important pathogenic fungus

causing larch shoot blight disease in Japan and has been extensively studied for its ecology and pathogenicity (Uozumi, 1961; Sato et al., 1963), but it was identified only on the basis of its morphological characteristics. In the observation of syntype specimens of P. laricina (IUM-FS515, IUM-FS516, IUM-FS517, IUM-FS518, IUM-FS519, IUM-FS520), only the sexual morph was observed; there is no mention of the asexual morph in the description by Sawada (1950). However, many studies of this disease and its causal fungus have been conducted in Japan because *Larix* species are very important for silviculture in northern Japan; from these studies, the asexual morphs of *P. laricina* and *Macrophoma* sp. have been reported. Inoculation tests on the sexual and asexual morphs have confirmed isogenicity among the isolates of both states (Uozumi, 1961; Sato et al., 1963). As mentioned above, we examined several isolates of present species. However, we did not observe ascomata or conidiomata on artificial media, although Uozumi (1961) reported their formation on such media. The morphological characteristics of the asexual morph of herbarium specimen TFM: FPH-4038 (origin of isolate MUCC 2662) are identical to *Macrophoma* sp. recorded by Uozumi (1961), and under the current criteria belong to the genus *Neofusicoccum*. Moreover, the examined isolates formed a single clade in the phylogenetic tree; therefore, we suggest that these isolates should be treated as an independent species. This species was transferred to the genus *Guignardia* by Yamamoto (1961) and later transferred to *Botryosphaeria* by Shang (1987). Based on the results of molecular analysis in this study, we propose to transfer the genus to *Neofusicoccum* and designate the epitype FPH-4038 (ex-epitype culture MUCC 2662).

***Neofusicoccum miyakoense*** Y. Hattori & C. Nakash., sp. nov., Fig. 5–4.

MycoBank no.: MB 837718.

**Etymology:** Name refers to Miyako-jima, the Island in Japan where this fungus was collected.

**Sexual morph:** Unknown.

**Asexual structures formed on the host:** Caulicolous or foliicolous. Lesions turned to

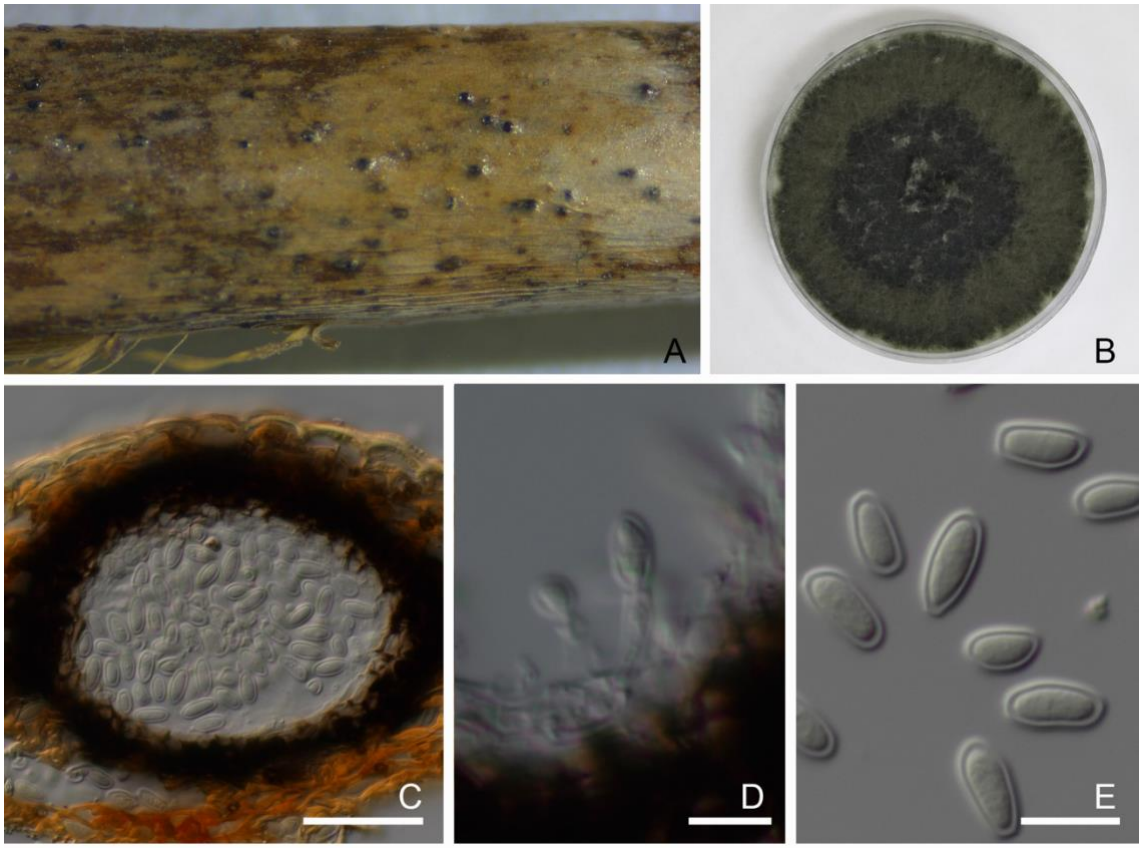


Fig. 5–4 – Morphological features of *Neofusicoccum miyakoense* (MUMH 11936 and MUCC 2585). A: Specimen MUMH 11936; symptoms with pycnidia forming on *Coffea* sp. branch. B: Colony on PDA (MUCC 2585). C: Vertical section of pycnidium in the leaf tissue. D: Conidia and conidiophores. E: Conidia. *Bars*: D 50 µm; E, F 10 µm.

pale brown to brown. Conidiomata pycnidial, scattered, visible as small dots on the lesion, epidermal, immersed, solitary, globose to subglobose, suppressed, dark brown to black, ellipsoid, unilocular, with a central ostiole, 118–185 × 102–132 µm; pycnidial wall composed of depressed or irregular cells in three to four layers, dark brown to black. Conidiophores reduced to conidiogenous cells; conidiogenous cells discrete, hyaline, cylindrical to ampulliform, determinate, with periclinal thickening, 4.2–9.3 × 1.5–2.8 µm. Paraphyses not seen. Conidia holoblastic sporulation for first conidia, phialidic

sporulation for following conidia, hyaline to slightly colored, smooth, with granular contents, aseptate, ellipsoid, rounded at both ends, or truncated at the base, rounded at the apex, 10–14.6 × 5–7.5 μm (av. = 12.11 × 6.18 μm, n = 100; L/W = 1.96).

Cultural characteristics on PDA: colony is dark gray to olivaceous gray, with floccose aerial mycelia, dense at the center, reaching 90 mm at 14 d after inoculation at room temperature.

**Holotypus:** on *Coffea* sp., JAPAN, Okinawa, Miyako-jima, 28 Jul 2018, by Y. Hattori & C. Nakashima (MUMH 11936, ex-type culture MUCC 2585).

**Host:** *Coffea* sp., *Mangifera indica*

**Other Material examined:** on *Mangifera indica*, JAPAN, Okinawa, Miyako-jima, 28 Jul 2018, by Y. Hattori & C. Nakashima (MUMH 11937, culture MUCC 2586).

**Note:** As a result of phylogenetic analysis, an independent clade was formed with strongly supported statistical values (ML-BS: 92%, and BI-PP: 1). Although this species is phylogenetically closely related to *N. mangiferae* (CBS118531) and *N. microconidium* (CERC 3497), the average size of conidia of *N. mangiferae* (13.6 × 5.4 μm) (Phillips et al., 2013) and *N. microconidium* (12.3 × 5 μm) (Li, Liu, Li, Liu, & Chen, 2018) are longer and narrower than that of *N. miyakoense* (12.11 × 6.18 μm). Furthermore, the shape of *M. miyakoense* conidia is ellipsoidal, while that of *N. microconidium* is narrower and fusiform, and that of *N. mangiferae* is ellipsoid to nearly fusiform. See also *N. okinawaense*. I did not observe ascomata or conidiomata on the PNA.

***Neofusicoccum okinawaense*** Y. Hattori & C. Nakash., sp. nov., Fig. 5–5.

MycoBank no.: MB 837719.

**Etymology:** Name refers to Okinawa, the Island in Japan where this fungus was collected.

**Sexual morph:** Unknown.

**Asexual structures formed formed on the host:** Leaf spots grayish-white to brown, small at the edge, later enlarged and coalescent, expanded toward the whole of a leaf. Conidiomata pycnidial, epidermal, submerged, solitary, globose to subglobose, dark

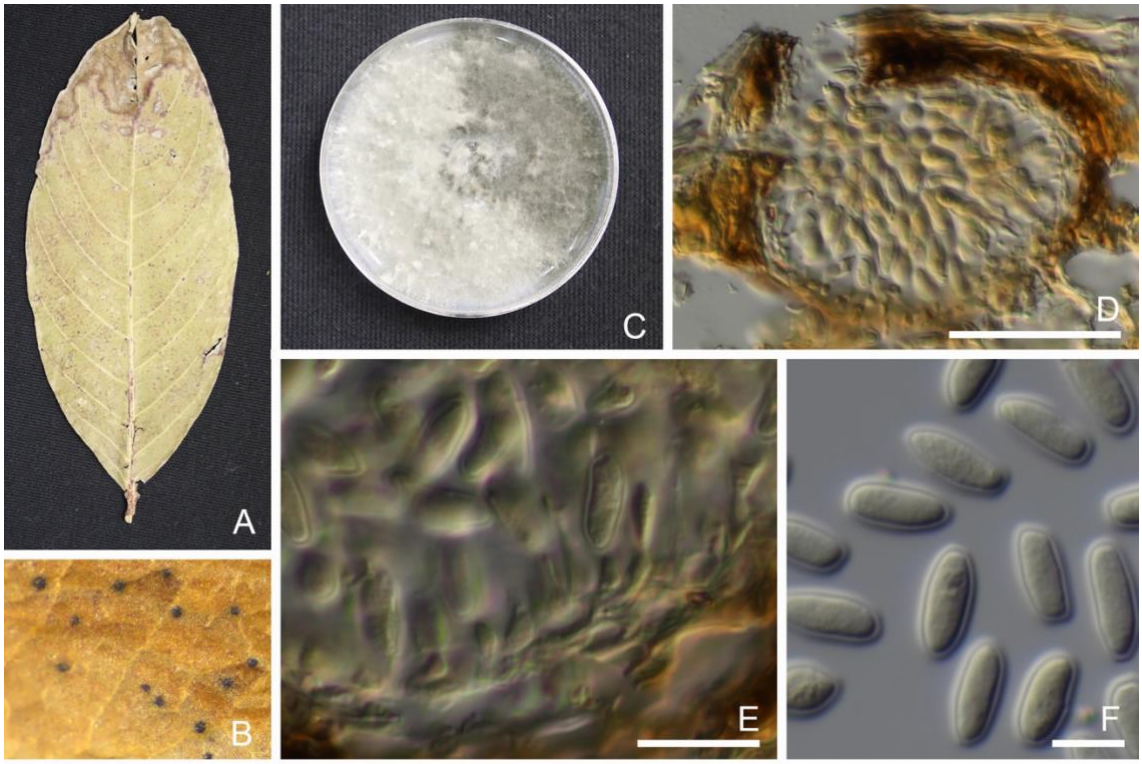


Fig. 5–5 – Morphological features of *Neofusicoccum okinawaense* (MUMH 10839 and MUCC 789). A: Specimen MUMH 10839. B: Symptoms with pycnidia forming on *Lagerstroemia speciosa.* C: Colony on PDA (MUCC 789). D: Vertical section of pycnidium in the leaf tissue. E: Conidia and conidiophores. F: Conidia. *Bars*: D 50 µm; E, F 10 µm.

brown to black, ellipsoid, unilocular, with a central ostiole, 149–164 × 120–149 µm; pycnidial wall composed of depressed or irregular cells in three to four layers, dark brown to black. Conidiophores reduced to conidiogenous cells; conidiogenous cells discrete, hyaline, cylindrical, with periclinal thickening, 4.7–8.7 × 2.7–3.8 µm. Paraphyses not seen. Conidia holoblastic sporulation for first conidia, phialidic sporulation for following conidia, hyaline to pale yellowish-brown, smooth with granular contents, unicellular, aseptate, ellipsoid to wider fusiform, rounded at both ends, or subtruncate at the base,

11.8–15 × 5.2–6.7 μm (av. = 13.60 × 5.99 μm, n = 18; L/W = 2.28).

**Cultural characteristics on PDA:** colony is white to gray, covered with floccose aerial mycelia, reaching 90 mm at 14 d after inoculation at room temperature.

**Holotypus:** on *Lagerstroemia speciosa*, JAPAN, Okinawa, Nakagami, 19 Nov 2007, by C. Nakashima (MUMH 10839, ex-type culture MUCC 789 = MAFF 240624).

**Host:** *Lagerstroemia speciosa*

**Note:** As a result of phylogenetic analysis, MUCC 789 formed a clade with *N. miyakoense* (MUCC 2585, MUCC 2586). The clade was strongly supported by ML-BS (99%) and BI-PP (1). Although this species is phylogenetically closely related to *N. miyakoense,* later species formed an independent clade composed of two isolates obtained from different host plants. Furthermore, these two species were included within a basal clade of the genus *Neofusicoccum*, with *N. mangiferae* in the genus *Mangifera* from Australia and *N. microconidium* in *Eucalyptus* from China. The host plants for Japanese *Neofusicoccum* species were three foreign species introduced for cultivation in recent years, *Coffea* sp. and *Mangifera indica* for *N. miyakoense*, and *Lagerstroemia speciosa* for *N. okinawasense*. Based on the genetic distance among the species, they might be strongly related to the common host plant genera *Mangifera* and *Eucalyptus* for speciation and host jumping. The average conidial size and L/W ratio of *N. okinawaense* (13.60 × 5.99 μm, n = 7; L/W = 2.28) is larger than *N. miyakoense* (12.11 × 6.18 μm, n = 100; L/W = 1.96). I did not observe ascomata or conidiomata on the PNA.

***Neofusicoccum parvum*** (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, Studies in Mycology 55: 248.

**Description and illustration:** see Pennycook & Samuels, 1985.

**Host in Japan:** *Cymbidium* spp. (Suzuki et al., 2018)*, Mangifera indica* (Takushi et al., 2017), *Platanus* × *acerifolia* (Motohashi et al., 2016), *Rhododendron hybrids, Taminarix tenuissima* (this study).

**Materials examined:** on *Rhododendron* hybrids, JAPAN, Aichi, Nagoya, 9 May 2006,

by Unknown collector (MUMH 10368, culture MUCC 392); on *Taminarix tenuissima*, JAPAN, Niigata, Iwafune, 22 Jul 1983, by T. Kobayashi (TFM: FPH-5589, culture MUCC 2511 = FFPRI 411214).

**Note:** According to the USDA fungal host database (https://nt.ars-grin.gov/fungaldatabases/fungushost/FungusHost.cfm) up to Oct 6, 2020, there were no reports of *N. parvum* on genus *Taminarix*, suggesting that *Taminarix tenuissima* is a new host.

## Discussion:

In this study, the taxonomical positions of 13 Japanese isolates of the genus *Neofusicoccum* were examined based on their morphology and molecular phylogeny. As a result, these isolates were divided into five clades. Based on their phylogenetic position and morphological characteristics, four of them were described as new species: *N. hypelici*, *N. laricinum*, *N. miyakoense*, and *N. okinawaense*. *Neofusicoccum hyperici* was closely related to *N. parvum* and *N. pandanicola*, however it was morphologically distinct. This species is characterized by small conidia and an independent phylogenetic position. The size and L/W ratio of conidia are useful characteristics for differentiation and delimitation among *Neofusicoccum* species (Crous et al., 2006; Marin-Felix et al., 2017; Yang et al., 2017; Li et al., 2018). These criteria were supported in the present study. However, species limitations by other phenotypic characteristics, such as the host range and geological distribution, remain unclear.

*Neofusicoccum parvum* is distributed worldwide and is known to have an extremely broad host range as a pathogen or endophyte (Slippers & Wingfield, 2007). For example, this species has been reported in the following plants: grapevine (Úrbez-Torres & Gubler, 2009; Chacón et al., 2020), walnut (Yu et al., 2015), and mango (Slippers et al., 2005; Takushi et al., 2017). Additionally, plural species or strains can be found on the same leaf (Slippers & Wingfield, 2007). These studies suggest that extensive, global sampling will

be necessary to fully understand the host associations and distribution of Botryosphaeriaceae, including the genus *Neofusicoccum* (Jami et al., 2014). In this study, two isolates of *N. parvum* were identified from two plants, including a newly recorded host, *Tamarix tenuissima*. The expansion of the known host range following expanded sampling appears to be a common pattern of recent studies on the Botryosphaeriaceae, and is drastically changing the perceptions of host association (Jami et al., 2014). Future efforts are still needed to collect and isolate the genus *Neofusicoccum* from various host plants in Japan and around the world.

The current species delimitation by morphology and phylogenetic relationships has resulted in an explosive increase in the number of species of *Neofusicoccum*. In this study, we examined isolates of *N. laricinum* originating from both sexual and asexual morphs, and collected from various places and dates in Japan. Sequence analyses showed that only a few nucleotides differed among isolates on the same host genus (2/551 nucleotides in ITS). Furthermore, all regions other than ITS were the same. Conversely, sequence analyses of *N. parvum* showed 15 nucleotide differences in the ITS region among the isolates in different genera. Furthermore, the isolates of *N. hyperici* showed two nucleotide differences in the tef1-a region among the different host genera. These results support the previous studies. The homology of the sequences used in this study was not suitable for identifying the species level. The phylogenetic analyses based on a combination of the four regions (ITS, tef1-a, tub2 and rpb2) indicated that the species form independent phylogenetic clades supported by high BS values (Li et al., 2018). The multiple species concepts that comprise morphological features, host range, and geographical distribution, as well as phylogenetic relationships, are considered important to species recognition.

In this study, the new combination *N. laricinum* was transferred from the genus *Physalospora* to the genus *Neofusicoccum*, due to its morphological characteristics and molecular phylogenetic position. This study reveals, for the first time, the molecular phylogenetic position of *N. laricinum*. Similar taxonomic problems exist among many

Japanese tree diseases and their pathogens, such as blight disease of coniferous trees (Kobayashi, 1957; 1962) and dieback in *Paulownia* (Itô & Kobayashi, 1951).

*Neofusicoccum miyakoense* collected from Miyako-jima Island, and *N. okinawaense* collected from the main island of Okinawa, are proposed as new species in this study. Although the two species have similar morphological characteristics, such as ellipsoidal conidia, they have been recognized as separate clades in the molecular phylogenetic tree. Both Miyako-jima Island and the main island of Okinawa belong to the Ryukyu Islands in the Okinawa Prefecture, Japan, but they have different geographical backgrounds. While the Ryukyu Islands are oceanic Islands formed by volcanic activity, Miyako-jima Island is located about 300 km from the main island of Okinawa and is known to have a unique ecosystem based on its geological history of having been once submerged. Several new species of tree pathogens have been reported on Miyako-jima Island in previous studies (Kobayashi & Kawabe, 1992, Nonaka et al., 2013), and these geographic isolations and differences might be involved in the unique evolution of the species.

In conclusion, the results of re-examination of the genus *Neofusicoccum* based on morphological characteristics and phylogenetic analysis revealed a high degree of diversity in this genus in Japan. However, the strains reexamined in this study are part of the genus *Neofusicoccum* collected in Japan, and many isolates may still be treated as other genera. Therefore, further study is required to understand the full diversity of the genus *Neofusicoccum* in Japan.

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# Chapter 6

## General discussion and conclusion

In this study, I re-examined the taxonomy of Japanese Botryosphaeriales by analyzing their morphological characteristics, culture characteristics, molecular phylogenetic relationships, and pathogenicities. These analyses confirmed the high diversity of tree-parasitic Botryosphaeriales in Japan, which comprise four species in the genus *Botryosphaeria*, including one new species and one new combination; eight species in the genus *Lasiodiplodia*, including four new species and two new combinations; five species in the genus *Neofusicoccum*, including three new species and one new combination; and five species in the genus *Phyllosticta*, including two new species and one new combination. Located in the Far East, Japan has more than 2,500 endemic species of vascular plants (Kato & Ebihara 2011). On the other hand, Japan is known to have many species in common with Eurasia, which has repeatedly been connected and separated throughout its geographical history, and with North America, which was connected by Beringia during the last ice age (Murata 1977). Therefore, it is suggested that plant parasitic fungi, which are closely related to the host plants, also have various origins. In particular, many unknown isolates were found in the Ogasawara Islands and the Ryukyu Islands in this study. The unique geography of these regions, which are further isolated from the Japanese archipelago, may have caused the speciation of unique Botryosphaeriaceae species. These interesting results might be a good example for understanding the speciation of plant pathogen, which includes the endemic and monoxenic species, and the cosmopolitan and polyxenic species.

In recent years, many new species of Botryosphaeriales have been described, particularly in the genera *Botryosphaeria* (Dissanayake et al. 2016; Chen et al. 2020) and

*Lasiodiplodia* (Dou et al. 2017; Jayasiri et al. 2019), and several unknown lineages were also identified in this study. Some of the species identified in this study appeared to have different host plant taxa, leaf L/W ratios, pathogenicities, and optimum growth temperatures, which will be useful for characterizing them biologically. However, the phylogenetic analyses of the fungi in this order were found to be inadequate as, for example, known species in the genus *Botryosphaeria* formed a species complex despite meeting the current criteria for species determination. Therefore, in the future, it will be necessary to search for gene regions that have a distinct discriminatory sensitivity for each genus and species complex within a genus.

Fungi in Botryosphaeriales are discussed only in terms of their importance as plant pathogens in Japan. However, it has recently been shown that some species are endophytes or saprophytes, the role of which in ecosystems remains unclear (Phillips et al. 2019). In addition, although most of the strains used in this study were pathogenic isolates, the formation of a new clade of Japanese isolates from insects suggests the existence of diverse life cycles in this order that are not visible to humans. Elucidating the original ecological niche and ecological role of Botryosphaeriaceales fungi will lead to an understanding of the acquisition and switching of invasive and endogenous behaviors of these fungi. In addition, predicted climate change and the migration of fungi due to human activities may lead to more disease outbreaks caused by Botryosphaeriales. An accurate understanding of these fungi is essential for the development of more precise plant disease control strategies and quarantine systems. Therefore, it is necessary to collect Botryosphaeriales species from a various sources and environments, not only plants, and to clarify their morphological characteristics, pathogenicity, host range and specificities, and distributional details to better understand Botryosphaeriales.

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# Summary

Fungi in the order Botryosphaeriales include pathogens that parasitize the leaves, fruits, and branches of plants and cause various diseases. These fungi are important plant pathogens globally, as they cause fruit rot, leaf blight, and branch blight in useful trees, such as fruit trees and trees that are used for urban greening and afforestation. In Japan, many tree diseases have been reported to be associated with Botryosphaeriales, but most of the Japanese species in this order have been described based solely on their morphological characteristics, with their molecular phylogenetic positions remaining unclear, highlighting the urgent need to review the Japanese Botryosphaeriales based on new criteria that reflect molecular phylogenetic relationships. Therefore, in this study, I conducted a taxonomic study of Japanese Botryosphaeriales fungi based on their morphological characteristics, culture characteristics, molecular phylogenetic relationships, and pathogenicities.

The taxonomy of the species in the genus *Phyllosticta*,which are parasitic on conifer (Cupressaceae) trees in Japan, was conducted based on current criteria, including morphological characteristics, culture characteristics, phylogenetic relationships, and pathogenicities. Phylogenetic analyses revealed that this genus included several clades comprising plant pathogens that were isolated from a specific host as well as clades comprising endophytic species isolated from various conifer species. Each clade was recognized as a species based on the morphological characteristics and other features. Five species of *Phyllosticta* sensu stricto were recognized, two of which were newly recorded in the Japanese mycoflora. Furthermore, a new combination of *P. pilospora* and its epitype and an epitype and ex-type strain for *P. cryptomeriae* were proposed.

To clarify the species diversity of *Lasiodiplodia* in Japan, I examined 30 Japanese isolates based on their morphological characteristics, culture characteristics, and phylogenetic relationships. Phylogenetic analyses using a matrix composed of ITS, *tef1*-

*α*, *tub2*, and *rpb2* sequences revealed that these isolates were divided into 11 clades that corresponded to *L. theobromae*, *L. brasiliensis, L. swieteniae*, *L. pseudotheobromae*, and *Lasiodiplodia* spp. These species were also identified based on their morphological characteristics and the optimum growth temperature of each isolate, two of which were new additions to the Japanese mycoflora. In addition, four other species (*L. latispora, L. parvispora, L. ryukyuensis*, and *L. yaguchii*) were proposed.

Reexamination of Japanese species in the genus *Botryosphaeria* isolated from 12 plant species belonging to ten families was carried out based on their morphological characteristics and molecular and phylogenetic analyses using the *tef1-α*, *tub2*, and *rpb2* protein-coding regions and the ITS region of rDNA. These Japanese isolates were divided into five clades that were identified as *B. dothidea*, *B. qingyuanensis*, *B. sinensis*, and *Botryosphaeria* sp. Among these, *B. qingyuanensis* and *B. sinensis* have recently been added to the Japanese mycoflora and are not host-specific. In addition, *B. tenuispora* isolated from *Leucothoe fontanesiana* and insect galls on the leaves of *Aucuba japonica* was proposed as a new species.

Reexamination of Japanese species in the genus *Neofusicoccum* was carried out based on their morphological characteristics and molecular phylogenetic relationships using the ITS, *rpb2, tef1-α*, and *tub2* regions. These Japanese isolates were divided into five clades, which were recognized as the species *N. parvum*, other *Neofusicoccum* spp., and three new species proposed in this study (*N. hyperici*, *N. miyakoense*, and *N. okinawaense*). In addition, *Physalospora laricina,* which causes shoot blight in larch, was transferred to the genus *Neofusicoccum* and its epitype and ex-epitype isolates were proposed.

These findings confirm the high diversity of tree-parasitic Botryosphaeriales in Japan. Some of the species identified in this study appeared to have different host plant taxa, leaf L/W ratios, pathogenicities, and optimum growth temperatures, which will be useful for characterizing them biologically. However, the phylogenetic analysis of the fungi in this order was found to be inadequate despite it meeting the current criteria for species determination. Therefore, there is a need to clarify the morphological characteristics, molecular phylogenetic relationships, pathogenicities, host ranges and specificities, and distributional details of Botryosphaeriales in the future to gain a better understanding of

their roles as plant pathogens, endophytes, and saprophytes in ecosystems.

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# List of publications

1. **Yukako Hattori**, Keiichi Motohashi, Kazuaki Tanaka, Chiharu Nakashima (2020) Taxonomical re-examination of the genus *Phyllosticta* – parasitic fungi on Cupressaceae trees in Japan. Forest Pathology, 50, e12630.

2. **Yukako Hattori**, Yuho Ando, Chiharu Nakashima (2021) Phylogenetic study of the tree parasitic *Botryosphaeria* isolates in Japan. Japanese Journal of Mycology, 62 (Accepted on 3rd Dec. 2020).

3. **Yukako Hattori**, Yuho Ando, Atsuko Sasaki, Nami Uechi, Chiharu Nakashima. Taxonomical study of noteworthy species of *Botryosphaeria* in Japan. Mycobiology. (Accepted on 11th Feb. 2021).

The contents of Chapters 3 and 5 were submitted as follows.

1. Yukako Hattori, Lynn Nakano, Chiharu Nakashima. Species Diversity of Genus *Lasiodiplodia* in Japan. Fungal Systematics and Evolution. (Submitted on 18th Aug. 2020)
2. Yukako Hattori, Yuho Ando, Chiharu Nakashima. Taxonomical re-examination of the genus *Neofusicoccum* in Japan. Mycoscience. (Submitted on 26th Oct. 2020)