Taxonomical Study of Noteworthy Species of *Botryosphaeria* in Japan

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**ABSTRACT**

The reexamination of the fungal genus *Botryosphaeria* on 12 plant species of 10 families was carried out based on molecular phylogenetic analyses using the regions of translation elongation factor 1–α, β-tubulin, DNA-directed RNA polymerase II subunit, and internal transcribed spacer region of rDNA and morphological characteristics. Japanese isolates 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 fruits of *Aucuba japonica* has been proposed as a new species.

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1. 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*, *B. auasmontanum*, *B. corticis*, *B. fabicerciana*, *B. fusiporta*, *B. guttulata*, *B. kuwatsukai*, *B. minutispermatia*, *B. pseudoramosa*, *B. qingyuanensis*, *B. ramosa*, *B. rosaceae*, *B. scharifii*, *B. sinensis*, and *B. 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) and 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.
2. Materials and methods

2.1 Sample collection and morphological study

Twenty-four 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. These isolates included those from various host plants and insect galls (Table 1). These isolates were cultivated on potato dextrose agar (PDA) medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) or malt agar (Becton Dickinson, Franklin, NJ) at room temperature under room light diffusion. To observe conidiomata and conidia, the isolates were transferred to boiled mulberry agar (BMA [20]). In brief, mulberry leaves were cut into 5-cm squares, boiled for 30–60 s, and dried on a paper towel. These leaves were placed on water agar medium. Mycelial disks 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).

2.2 Molecular and phylogenetic analyses

Genomic DNA was extracted from mycelial disks 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 RP2 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 RP2 0.1 μL), 1.25 μL of 10 × NH4 reaction buffer (Bioline), 1.9–2.5 mM MgCl2 (Bioline; ITS, RPB2, and TEF1-α 2.5 mM and TUB2 1.9 mM), 2.5–5.0 mM each of deoxyribonucleotide triphosphate mixture (Bioline; ITS 2.5 mM and TEF1-α, TUB2, and RP2 5.0 mM), 0.2 μM of each primer, and 5.6% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO), 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 55 °C, 30 s; and extension 72 °C, 1 min).
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 2. 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) 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 spp. collected from GenBank using the software MAFFT version 7 [21].

Maximum likelihood (ML) and Bayesian inference (BI) analyses were used to estimate phylogenetic relationships. ML analyses were performed using raxml HPC-PTHREADS [22]. The strength of the internal branches from the resultant trees was tested by bootstrap analysis [23] using 1000 replications. BI analyses were performed using BEAST version 2.5.1 [24] 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 3). Sequence alignments prepared in this study were deposited in TreeBASE number 26984.

Table 2. PCR primer sets and annealing temperatures.

| Region | Primer F            | Primer R            | Annealing temperature (°C) |
|--------|---------------------|---------------------|---------------------------|
| ITS    | ITS1 (White et al. [16]) | ITS4 (White et al. [16]) | 48                        |
| TEF1-x | EF1-728F (Carbone and Kohn [17]) | EF1-986R (Carbone and Kohn [17]) | 52                        |
| TUB2   | BT2A (Glass and Donaldson [18]) | BT2B (Glass and Donaldson [18]) | 55                        |
| RPB2   | RPB2-5F2 (Liu et al. [19]) | RPB2-7cR (Liu et al. [19]) | 60 to 58 to 54            |

3. Results

3.1 Phylogeny

The ITS + TEF1-x + TUB2 + RPB2 combined data matrix of 41 sequences consisted of 1756 characters (ITS: 536, TEF1-x: 280, RPB2: 576, and TUB2: 364) . Cophiniforma atrovirens (CBS 124934) was selected as the out taxon. The resultant ML tree is shown in Figure 1. The topologies of the generated trees from ML and BI analyses were congruent. As a result of the phylogenetic analysis, Japanese isolates formed five groups with the hitherto known species or newly recognized species. These are B. dothidea (MUC 157, MUC 221, MUC 245, MUC 248, MUC 254, MUC 2521, MUC 2543, MUC 2627, MUC 2748–2751, and MUC 2755), B. tenuispora (MUC 237 and MUC 2900), B. qingyuanensis (MUC 321), B. sinensis (MUC 2522, MUC 2533, and MUC 2537), and Botryosphaeria sp. (MUC 2754, MUC 2897–2899, and MUC 2901).

3.2 Taxonomy

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

Teleomorphic state: It has been reported by Slippers et al.[11].

Anamorphic state on the host plants: Conidiomata solitary, globose, dark brown to dark gray, covered with white to dark green hyphae, 419–490 × 355–437 μm; pycnidial wall composed of depressed or irregular cells in five to eight layers, brown to dark brown, blackish around an ostiole, paler toward the conidiogenous region; paraphyses 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, 1.3–16.3 × 1.5–3.4 μm. Conidia solitary, fusiform to sub fusiform, rounded at the apex, convex to truncate at the base, hyaline, asperate or rarely one-septate, smooth, with granular contents, 15–36 × 3.3–8.4 μm, L/W = 3.45 (min 2.60, max 5.51; n = 101).

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 10395, culture MUCC 221); on Leucothoe catesbaei, ibid, June 19, 2006, by I. Araki & K. Motohashi (MUMH 10395, culture MUCC 221); on Daphniphyllum macropodum, ibid, 18 Jul 2006, by...
### Table 3. List of Botryosphaeria species used for phylogenetic analysis.

| Fungal species | Isolates No. | Host | Country | Accession numbers |
|----------------|--------------|------|---------|-------------------|
| B. agaves      | CBS 133992   | Agave sp. | Thailand | JX646791, JX646856, JX646841 |
| B. austromontanum | CBS 121769 | Accacia melleifera | Namibia | EU010303, EU010348 |
| B. corticis     | CBS 119047   | Vaccinium corymbosum | USA | DQ299245, EU017539, EU367107 |
| B. dothidea     | CBS 115476   | Prunus sp. | Switzerland | AY236049, AY236089, AY236097, EU343977 |
| B. dothidea     | MUC 157      | Daphniphyllum macropodum | Japan | LC582800, LC583152, LC583176, LC583198 |
| B. dothidea     | MUC 221      | Leucothoe fontanesiana | Japan | LC582822, LC583154, LC583178, LC583200 |
| B. dothidea     | MUC 245      | Daphniphyllum macropodum | Japan | LC582733, LC583145, LC583169, LC583192 |
| B. dothidea     | MUC 248      | Linderia obuslora | Japan | LC582753, LC583147, LC583171, LC583194 |
| B. dothidea     | MUC 2521     | Prunus sp. | Japan | LC582720, LC583146, LC583166, LC583189 |

(MAFF 410826)

| B. dothidea     | MUC 254      | Saxifraga stolonifera | Japan | LC582724, LC583146, LC583170, LC583193 |
| B. dothidea     | MUC 2543     | Eucalyptus viminalis | Japan | LC582721, LC583143, LC583167, LC583190 |

(FFPRI 411204)

| B. dothidea     | MUC 2627     | Pyrus pyrifolia | Japan | LC582824, LC583156, LC583180, LC583202 |
| B. dothidea     | MUC 2748     | Castanea crenata | Japan | LC582823, LC583155, LC583179, LC583201 |
| B. dothidea     | MUC 2749     | Castanea crenata | Japan | LC582829, LC583161, LC583185, LC583207 |
| B. dothidea     | MUC 2750     | Prunus persica | Japan | LC582629, LC583141, LC583165, LC583189 |
| B. dothidea     | MUC 2751     | Prunus persica | Japan | LC582821, LC583157, LC583177, LC583199 |
| B. dothidea     | MUC 2755     | Castanea crenata | Japan | LC582722, LC583144, LC583168, LC583191 |
| B. dothidea     | MUC 2756     | Castanea crenata | Japan | LC582722, LC583144, LC583168, LC583191 |

| B. fabriceriana | CBS 127193   | Eucalyptus sp. | China | HSQ32197, HSQ32213, FF79068, MF140137 |
| B. fusispora    | MFLUCC 10-0098 | Entada sp. | Thailand | JX646789, JX646854, JX646839 |
| B. gattulata    | CGMCC 3.20094 | Dead wood | China | MT327839, MT313606 |
| B. kuwatsukii   | CBS 135219   | Malus domestica | Korea | KAI33388, KAI33410 |
| B. minitisspermatia | GZCC 16-0013 | Dead wood | China | XX447657, XX447678 |
| B. pseudomorosa | CGMCC 3.18739 | Eucalyptus hybrid | China | KX277989, KX278094, KX278198, MF101400 |
| B. qingyuanensis | CGMCC 3.18742 | Eucalyptus hybrid | China | KX278000, KX278015, KX278209, MF101511 |
| B. ramosa       | CBS 122069   | Gymelia innovans | Japan | LC582911, LC583163, LC583187 |
| B. rosaeae      | CGMCC 3.18007 | Mutus sp. | Australia | EU114405, EU114407, KF766132 |
| B. scharifii     | CBS 124703   | Mangifera indica | Iran | IQ772020, IQ772057 |
| B. sinensis     | CGMCC 3.17722 | Populus sp. | China | KT343255 |
| B. sinens      | MUC 2522     | Prunus sp. | Japan | LC582727, LC583149, LC583173, LC583195 |

(MAFF 410827)

| B. sinensis     | MUC 2533     | Aucuba japonica | Japan | LC582729, LC583151, LC583175, LC583197 |
| B. sinensis     | MUC 2537     | Paulownia tormentosa | Japan | LC582729, LC583151, LC583175, LC583197 |
| B. tenuispora   | MUC 237      | Leucothoe fontanesiana | Japan | LC582728, LC583150, LC583174, LC583196 |
| B. tenuispora   | MUC 2900     | gall on Aucuba japonica | Japan | LC582726, LC583148, LC583172 |
| B. wangenii     | CGMCC 3.18744 | Cedrus deodara | China | KX278002, KX278107, KX278211, MF101533 |
| Botryosphaeria sp. | MUC 2754 | Castanea crenata | Japan | LC582888, LC583160, LC583184, LC583206 |
| Botryosphaeria sp. | MUC 2897 | pupa of Asphondylia auctae | Japan | LC582821, LC583157, LC583177, LC583203 |
| Botryosphaeria sp. | MUC 2898 | gall on Aucuba japonica | Japan | LC582826, LC583158, LC583182, LC583204 |
| Botryosphaeria sp. | MUC 2899 | gall on Aucuba japonica | Japan | LC582820, LC583156, LC583186 |
| Botryosphaeria sp. | MUC 2901 | gall on Aucuba japonica | Japan | LC582827, LC583159, LC583183, LC583205 |

| C. atrovires | CBS 124934 | Pierocarpus angolensis | South Africa | FB88473, FB88456, —, — |

Ex-type strains are in boldface.

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 248); on I. Araki & K. Motohashi (MUMH 10425, culture MUCC 254), an isolate of B. dothidea, was identified as B. dothidea based on their phylogenetic analyses and morphological characteristics. The morphology of conidia, varied, with an L/W ratio of 3.4–5.6 (Table 4). All isolates grew well and formed conidiomata and conidia on the BMA medium.

**Botryosphaeria qingyuanensis** G.Q. Li & S.F. Chen, Persoonia 40: 88, 2003.

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 amphiogenous, epidermal, merged, solitary, scattered, black to dark brown, ellipsoidal, 105–146.5 × 87–132 μm; pycnidial wall composed of depressed or irregular cells in three to five layers, Kumamoto, Uki, 8 Sep 2017, by Y. Fukunaga (culture MUCC 2755 = K-027).

Thirteen Japanese isolates were identified as *B. dothidea* based on their phylogenetic analyses and morphological characteristics. The morphology of conidia varied, with an L/W ratio of 3.4–5.6 (Table 4). All isolates grew well and formed conidiomata and conidia on the BMA medium.
Figure 1. Phylogenetic tree of *Botryosphaeria* spp. constructed by ML using the combined ITS, RPB2, TEF1-α, and TUB2 gene sequence datasets. ML bootstrap values and Bayesian PPs are given near the branches (BS/PP). Ex-type strains are in boldface.

Table 4. Morphological characteristics of the genus *Botryosphaeria*.

| Species                  | Conidial bodies (μm) | Average | L/W | Isolate | Literature          |
|--------------------------|----------------------|---------|-----|---------|---------------------|
| *B. australis montanum*  | (8.1–8.8–11.3–13) × 2.5–2.9–3.9–5 | 10.1 × 3.4 | 3.0 | —       | Slippers et al. [25] |
| *B. dothisidea*          | (20–23–27–30) × 4–5–6 | 26.2 × 5.4 | 4.9 | MUCC 157 | This study          |
| *B. dothisidea*          | (20–23–27–30) × 4–5–6 | 25.8 × 5.2 | 4.9 | MUCC 157 | This study          |
| *B. dothisidea*          | 21–29 × 3.6–6        | 25.0 × 5.1 | 4.8 | MUCC 221 | This study          |
| *B. dothisidea*          | 20–30 × 5.3–7        | 25.1 × 6.1 | 4.1 | MUCC 245 | This study          |
| *B. dothisidea*          | 18.5–30 × 3.8–8.8    | 25.2 × 5.2 | 4.8 | MUCC 248 | This study          |
| *B. dothisidea*          | 16–24 × 3.3–7.3      | 19.7 × 5.7 | 3.4 | MUCC 2521| This study          |
| *B. dothisidea*          | 17–30 × 4.2–6.2      | 25.6 × 5.1 | 4.9 | MUCC 254 | This study          |
| *B. dothisidea*          | 19–26 × 4.8–6        | 22.9 × 5.4 | 4.2 | MUCC 2543| This study          |
| *B. dothisidea*          | 22.5–29.4 × 4–6.6    | 26.1 × 5.4 | 4.8 | MUCC 2627| This study          |
| *B. dothisidea*          | 22.5–29.4 × 4–6.6    | 22.0 × 5.5 | 4.0 | MUCC 2748| This study          |
| *B. dothisidea*          | 25–33 × 3.8–6.3      | 28.7 × 5.1 | 5.6 | MUCC 2749| This study          |
| *B. dothisidea*          | 17–36 × 3.8–6.9      | 26.7 × 5.2 | 5.1 | MUCC 2750| This study          |
| *B. dothisidea*          | 22.5–31 × 4.4–6.9    | 26.1 × 5.4 | 4.8 | MUCC 2751| This study          |
| *B. dothisidea*          | 21–35 × 4–6.7        | 26.8 × 5.1 | 5.2 | MUCC 2755| This study          |
| *B. guttulata*           | (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. [13]    |
| *B. minutispermatica*    | 8–14 × 3–4           | 13.0 × 3.5 | 3.7 | —       | Ariyawansa et al. [26]|
| *B. qingyuanensis*       | (15–19.5–24.5–28.5) × 5–6–6.5–7.5 | 22.0 × 6.2 | 3.5 | —       | Li et al. [27]      |
| *B. qingyuanensis*       | 17–24 × 3.6–6.5      | 21.4 × 5.2 | 4.1 | MUCC 321 | This study          |
| *B. sinensis*            | (15–19–29–5–7)      | 24.3 × 5.9 | 4.1 | —       | Zhou et al. [28]    |
| *B. sinensis*            | 16–23 × 4.8–6        | 20.4 × 5.4 | 3.7 | MUCC 2522| This study          |
| *B. sinensis*            | 14–27 × 3–5.8        | 24.0 × 4.6 | 5.2 | MUCC 2537| This study          |
| *B. tenuispora*          | 23–32 × 4–6.7        | 27.3 × 5.1 | 5.4 | MUCC 237 | This study          |
| *B. wangensis*           | (20.5–22–26–29) × 4.5–5.5–6.5–7.5 | 23.8 × 6.0 | 3.9 | —       | Li et al. [27]      |
| *Botryosphaeria* sp.     | 14.6–29 × 3.2–6.2    | 22.8 × 5.2 | 4.4 | MUCC 2899| This study          |

Ex-type strains are in boldface.
brown to dark brown, blackish around an ostiole, paler toward the conidiogenous region; paraphyses 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 [27], Gamblea innovans (this study).

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

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]. Botryosphaeria qingyuanensis was isolated from the twigs of an 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.

Figure 2. 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, 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).
Botryosphaeria sinensis Y.P. Zhou & Y. Zhang, Phytotaxa 245: 45, 2016.

Teleomorphic state: It has been reported [28].

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; pycnidal 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; paraphyses 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 truncate at the base, hyaline, aseptate or rarely 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. [28], 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

Figure 3. 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).
Paulownia tomentosa, Japan, Niigata, Uonuma, 10 Jul 1978, by H. Hayashi (culture MUCC 2537 = FFPI 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. wangenensis* (CGMCC 3.18744). This clade was statistically supported only in Bayesian trees. *Botryosphaeria sinensis, B. wangenensis, MUCC 2522, MUCC 2533*, and *MUCC 2537* formed an inner clade supported moderately with a PP value of 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. wangenensis* [(20.5) 22–26 (29) × (4.5) 5.5–6.5 (7.5); Zhou et al. and Li et al. [24,27]. 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. wangenensis* (Table 4). 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], Figure 2.

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; paraphyses 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, 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).

Holotype: 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 *Ashpondyli a aucaubae* 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. minutispermata, B. sinensis*, and *B. wangenensis*. However, the L/W ratio of *B. tenuispora* was bigger than that of the hitherto known species in the same clade (Table 4). Moreover, the size of 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. minutispermata* (8–14 × 3–4; [25,26]).

**Botryosphaeria sp.** Figure 3.

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; paraphyses 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 *Asphondyli a aucaubae*, Japan, Ibaraki, Tsukuba, April 29, 2019, by N. Uechi (culture MUCC 2897 = 2); fruit galls induced by *Ashpondyli a aucaubae* on *Aucuba japonica*, ibid, May 23, 2019, by N. Uechi (culture MUCC 2898 = 17-2); fruit galls induced by *Asphondyli a aucaubae* on *Aucuba japonica*, ibid, May 23, 2019, by N. Uechi (culture MUCC 2899 = 21-1); fruit galls induced by *Asphondyli a aucaubae* on *Aucuba japonica*, ibid, May 23, 2019, by N. Uechi (MUCC
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.

4. 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 [29,30] and B. yedoensis that inhabits Prunus spp. [31], 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 Figure 1). 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 (Figure 1). 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 [32]. 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 [33]. 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 [34]. In this study, 13 Japanese isolates from nine plant species in seven families were identified as B. dothidea. In recent years, some new species, B. sinensis [28], B. minutispermatia [26], B. wangensis [27], 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.

Botryosphaeria 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 (Figure 1) and had a typically higher L/W ratio than the hitherto known species (Table 4). 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. [4,27]. 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.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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