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RESEARCH ARTICLE

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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. fusispora*, *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.

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

Fungal species	Isolate No.	Material No.	Host Family	Host species	Regions	Identified by previous study
B. dothidea	MUCC 157	MUMH 10467	Daphniphyllaceae	*Daphniphyllum macropodum*	Aichi	—
	MUCC 221	MUMH 10395	Ericaceae	*Leucothoe fontanesiana*	Aichi	—
	MUCC 245	MUMH 10425	Daphniphyllaceae	*Daphniphyllum macropodum*	Aichi	—
	MUCC 248	MUMH 10429	Lauraceae	*Lindera obtusiloba*	Aichi	—
	MUCC 2521 (MAFF 410826)	—	Rosaceae	*Prunus* sp.	Ibaraki	*B. dothidea*
	MUCC 254	MUMH 10437	Saxifragaceae	*Saxifraga stolonifera*	Aichi	—
	MUCC 2543 (FFPRI 411204)	—	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	MUMH 10273	Araliaceae	*Gamblea innovans*	Aichi	—
B. sinensis	MUCC 2522 (MAFF 410827)	—	Rosaceae	*Prunus* sp.	Ibaraki	*B. dothidea*
	MUCC 2533 (FFPRI 411202)	—	Aucubaceae	*Aucuba japonica*	Tokyo	*Dothiorella* sp.
	MUCC 2537 (FFPRI 411203)	—	Paulowniaceae	*Paulownia tomentosa*	Niigata	*Dothiorella* sp.
B. tenuispora	MUCC 237	MUMH 10420	Ericaceae	*Leucothoe fontanesiana*	Aichi	—
	MUCC 2900	—	Aucubaceae	gall on *Aucuba japonica*	Ibaraki	—
Botryosphaeria sp.	MUCC 2754	—	Fagaceae	*Castanea crenata*	Kumamoto	—
	MUCC 2897	—	Aucubaceae	pupa of *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	—

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 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), 1.9–2.5 mM $MgCl_2$ (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 RPB2 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;

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-α	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])	fRPB2-7cR (Liu et al. [19])	60→58→54

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.

3. Results

3.1 Phylogeny

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) . *Cophinforma 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* (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).

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; 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, 1.3–16.3 × 1.5–3.4 µm. Conidia solitary, fusiform to subfusiform, rounded at the apex, convex to truncate at the base, hyaline, aseptate 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 10467, culture MUCC 157); 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			
				ITS	tef1	tub2	rpb2
B. agaves	**CBS 133992**	*Agave* sp.	Thailand	JX646791	JX646856	JX646841	—
B. auasmontanum	**CBS 121769**	*Acacia mellifera*	Namibia	EU101303	EU101348	—	—
B. corticis	**CBS 119047**	*Vaccinium corymbosum*	USA	DQ299245	EU017539	EU673107	—
B. dothidea	**CBS 115476**	*Prunus* sp.	Switzerland	AY236949	AY236898	AY236927	EU339577
B. dothidea	MUCC 157	*Daphniphyllum macropodum*	Japan	LC585280	LC585152	LC585176	LC585198
B. dothidea	MUCC 221	*Leucothoe fontanesiana*	Japan	LC585282	LC585154	LC585178	LC585200
B. dothidea	MUCC 245	*Daphniphyllum macropodum*	Japan	LC585273	LC585145	LC585169	LC585192
B. dothidea	MUCC 248	*Lindera obtusiloba*	Japan	LC585275	LC585147	LC585171	LC585194
B. dothidea	MUCC 2521 (MAFF 410826)	*Prunus* sp.	Japan	LC585270	LC585142	LC585166	LC585189
B. dothidea	MUCC 254	*Saxifraga stolonifera*	Japan	LC585274	LC585146	LC585170	LC585193
B. dothidea	MUCC 2543 (FFPRI 411204)	*Eucalyptus viminalis*	Japan	LC585271	LC585143	LC585167	LC585190
B. dothidea	MUCC 2627	*Pyrus pyrifolia*	Japan	LC585284	LC585156	LC585180	LC585202
B. dothidea	MUCC 2748	*Castanea crenata*	Japan	LC585283	LC585155	LC585179	LC585201
B. dothidea	MUCC 2749	*Castanea crenata*	Japan	LC585289	LC585161	LC585185	LC585207
B. dothidea	MUCC 2750	*Castanea crenata*	Japan	LC585269	LC585141	LC585165	—
B. dothidea	MUCC 2751	*Prunus persica*	Japan	LC585281	LC585153	LC585177	LC585199
B. dothidea	MUCC 2755	*Castanea crenata*	Japan	LC585272	LC585144	LC585168	LC585191
B. fabicerciana	**CBS 127193**	*Eucalyptus* sp.	China	HQ332197	HQ332213	KF779068	MF410137
B. fusispora	**MFLUCC 10-0098**	*Entada* sp.	Thailand	JX646789	JX646854	JX646839	—
B. guttulata	**CGMCC 3.20094**	Dead wood	China	MT327839	MT331606	—	—
B. kuwatsukai	**CBS 135219**	*Malus domestica*	China	KJ433388	KJ433410	—	—
B. minutispermatia	**GZCC 16-0013**	Dead wood	China	KX447675	KX447678	—	—
B. pseudoramosa	**CGMCC 3.18739**	*Eucalyptus* hybrid	China	KX277989	KX278094	KX278198	MF410140
B. qingyuanensis	**CGMCC 3.18742**	*Eucalyptus* hybrid	China	KX278000	KX278105	KX278209	MF410151
B. qingyuanensis	MUCC 321	*Gamblea innovans*	Japan	LC585291	LC585163	LC585187	—
B. ramosa	**CBS 122069**	*Eucalyptus camaldulensis*	Australia	EU144055	EU144070	KF766132	—
B. rosaceae	**CGMCC 3.18007**	*Malus* sp.	China	KX197074	KX197094	KX197101	—
B. scharifii	**CBS 124703**	*Mangifera indica*	Iran	JQ772020	JQ772057	—	—
B. sinensis	**CGMCC 3.17722**	*Populus* sp.	China	KT343255	—	—	—
B. sinensis	MUCC 2522 (MAFF 410827)	*Prunus* sp.	Japan	LC585277	LC585149	LC585173	LC585195
B. sinensis	MUCC 2533 (FFPRI 411202)	*Aucuba japonica*	Japan	LC585268	LC585140	LC585164	LC585188
B. sinensis	MUCC 2537 (FFPRI 411203)	*Paulownia tomentosa*	Japan	LC585279	LC585151	LC585175	LC585197
B. tenuispora	**MUCC 237**	*Leucothoe fontanesiana*	Japan	LC585278	LC585150	LC585174	LC585196
B. tenuispora	MUCC 2900	gall on *Aucuba japonica*	Japan	LC585276	LC585148	LC585172	–
B. wangensis	**CGMCC 3.18744**	*Cedrus deodara*	China	KX278002	KX278107	KX278211	MF410153
Botryosphaeria sp.	MUCC 2754	*Castanea crenata*	Japan	LC585288	LC585160	LC585184	LC585206
Botryosphaeria sp.	MUCC 2897	pupa of *Asphondylia aucubae*	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	—	—

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 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 *Castanea crenata*, Japan, Ibaraki, Tsukuba, 4 Sep 2017, by A. Sasaki (culture MUCC 2748 = NP-004); on *C. 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).

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.

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,

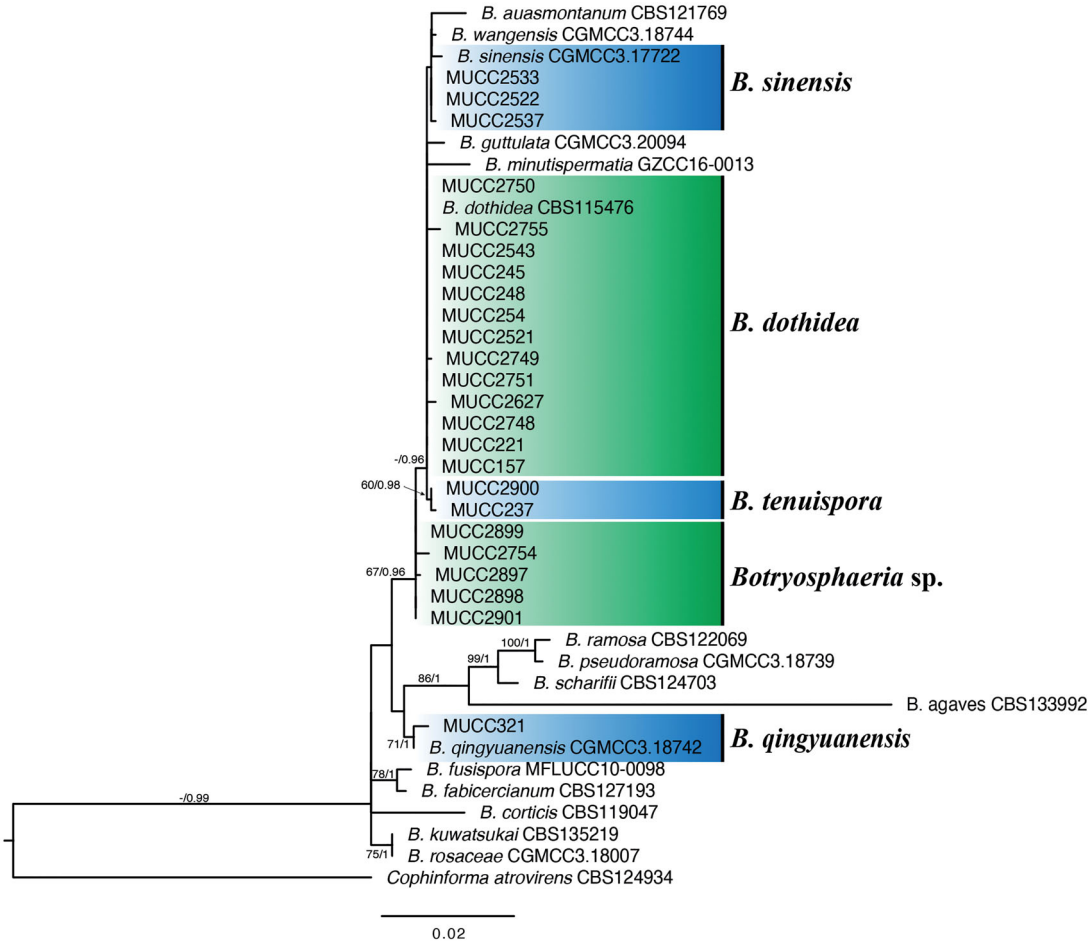

Figure 1. Phylogenetic tree of *Botryosphaeria* spp. constructed by ML using the combined ITS, RPB2, TEF1-α, and TUB2 gene sequence datasets. ML bootstrap values and Bayesian PPs are given near the branches (BS/PP). Ex-type strains are in boldface.

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

	Conidia				
Species	Conidial bodies (μm)	Average	L/W	Isolate	Literature
B. auasmontanum	**(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. dothidea	**(20–)23–27(–30) × 4–5(–6)**	**26.2 × 5.4**	**4.9**	—	Slippers et al. [25]
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 (FFPRI 411204)	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	**(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. minutispermatia	**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 (MAFF 410827)	This study
B. sinensis	14–27 × 3–5.8	24.0 × 4.6	5.2	MUCC 2537 (FFPRI 411203)	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.

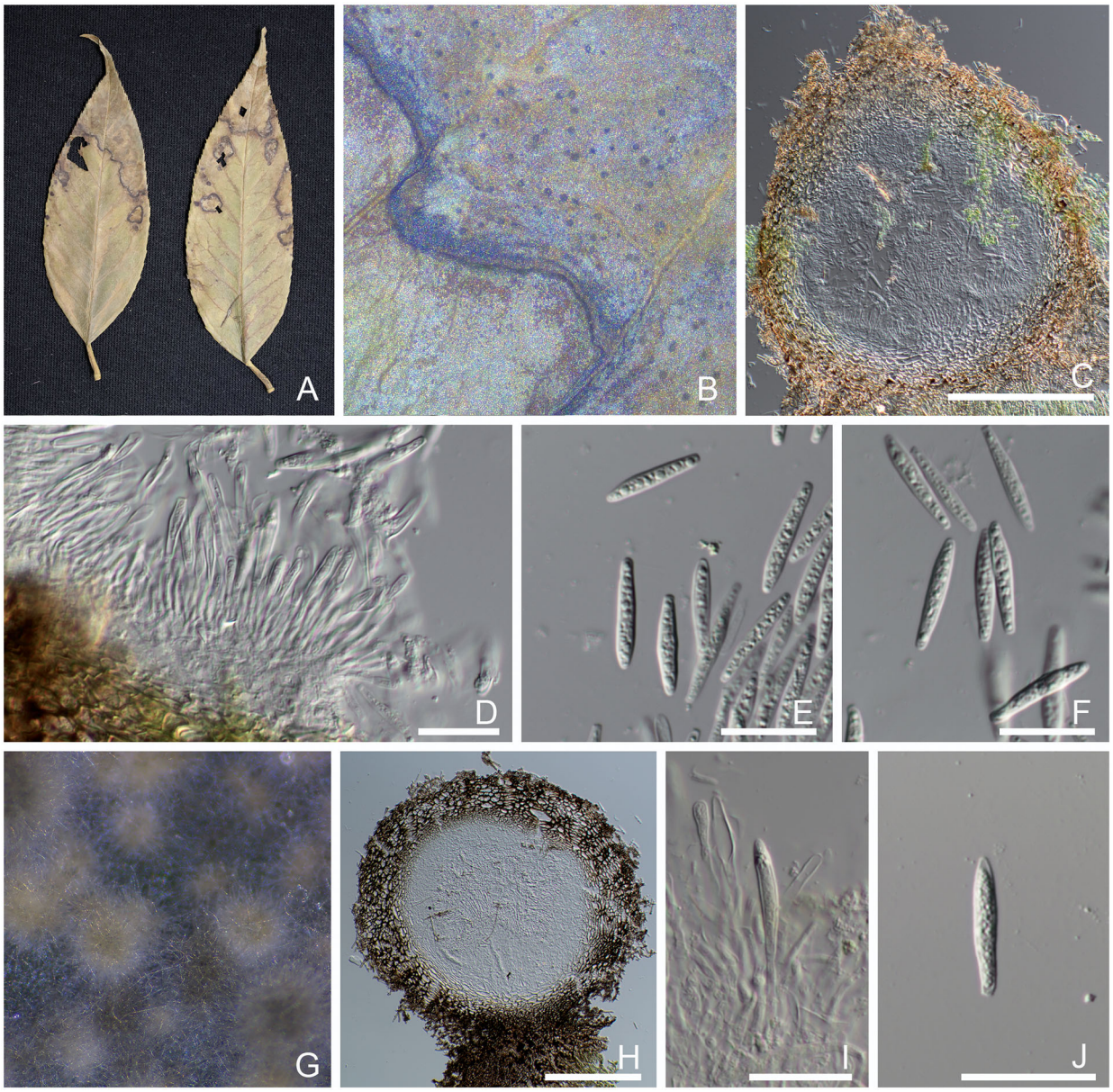

Figure 2. Morphological features of *Botryosphaeria tenuispora* [A–F: MUMH 10420 (MUCC 237) and G–I: MUCC 2900]. (A) Specimen MUMH 10420. (B) Symptoms with pycnidia forming on the leaf of *Leucothoe fontanesiana*. (C) Vertical section of pycnidium in the leaf tissue. (D) Conidia and conidiophores. (E, 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).

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 [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 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.

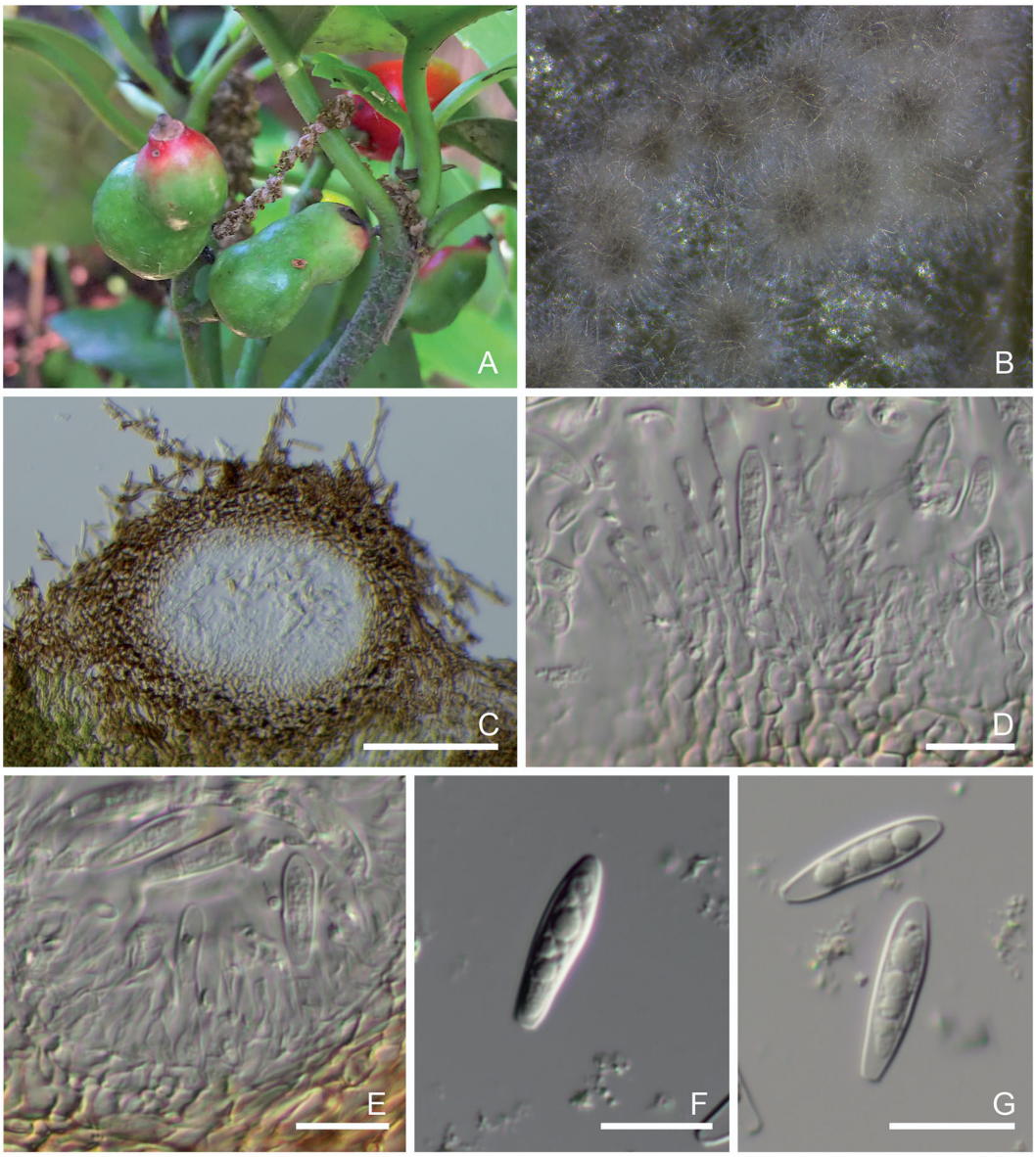

Figure 3. Morphological features of *Botryoshaeria* sp. (A and B: MUCC 2899). (A) fruit galls by *Asphondylia aucabae* on *Aucuba japonica*. (B) Conidiomata formation on the BMA after 7 d. (C) Vertical section of pycnidium in the leaf tissue. (D–E) Conidia and conidiophores. (F–G) Conidia. Scale bars, 200 µm (C) and 25 µm (D–G).

Botryosphaeria sinensis Y.P. Zhou & Y. Zhang ter, 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; 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 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. [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

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 statistically supported only in Bayesian trees. *Botryosphaeria sinensis*, *B. wangensis*, 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. wangensis* [(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. wangensis* (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; 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 cylindroclavate, 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). 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. minutispermatia* (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; 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 cylindroclavate, 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 (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.

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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