RESEARCH NOTE

First Report of Apple Decline Caused by *Botryosphaeria sinensis* in Korea

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ABSTRACT

Apple decline symptoms were frequently observed on cv. Fuji apple orchards located in Gyeonggi, Gyeongbuk, and Gangwon provinces during surveys conducted from May until the end of September 2020. Three fungal strains were isolated from the margins of internal lesions of diseased apple trees, and their morphological characteristics were considered similar to *Botryosphaeria sinensis.* Phylogenetic analysis using internal transcribed spacer (ITS) regions, translation elongation factor 1-alpha *(tef*1*),* beta-tubulin (*tub*2), and the second largest subunit of RNA polymerase II (*rpb*2) gene sequences confirmed the closest relationship of isolates with *B. sinensis* at the species level. According to a pathogenicity test, the appearance of dark-brown discolorations and vascular necrosis on apple branches inoculated with the isolated strain KNUF-20-014 was observed. To the best of our knowledge, this is the first report of *B. sinensis* as the causal agent of apple disease in Korea.

Keywords: Apple decline, *Botryosphaeria sinensis*, cv. Fuji, Phylogeny

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Apple (*Malus domestica*) is one of the most economically important fruits, with more than 31,000 ha cultivation area in South Korea [1]. However, numerous destructive pathogens infect apple trees and significantly reduce the commercial apple production worldwide. Among the fungal diseases, canker, twig dieback, and plant decline frequently occurs in apple orchards [2]. During the apple cultivation period, apple decline symptoms were observed from May until the end of September 2020 in apple (cv. Fuji) orchards located in Gyeonggi, Gyeongbuk, and Gangwon provinces. Mostly, declined trees were young (less than ten years), showing warts on the surface, darkening from the trunk, detachment of epidermis of the stem, embedded pycnidia on the bark, withering of branch, and eventually leading to decline (Fig. 1A-1G, and I). In this present study, isolated fungal strains were described and illustrated as a causal agent of apple decline.

To isolate the causal fungus, the fragment (approximately 2 mm) was taken from the margin of internal lesions, transferred onto potato dextrose agar (PDA; Difco, Detroit, MI, USA), and incubated at 25°C. As a result, the three strains, KNUF-20-014, KNUF-20-072, and KNUF-20-074, were isolated from the diseased apple tree. For molecular identification of strains at the genus and species levels, the total genomic

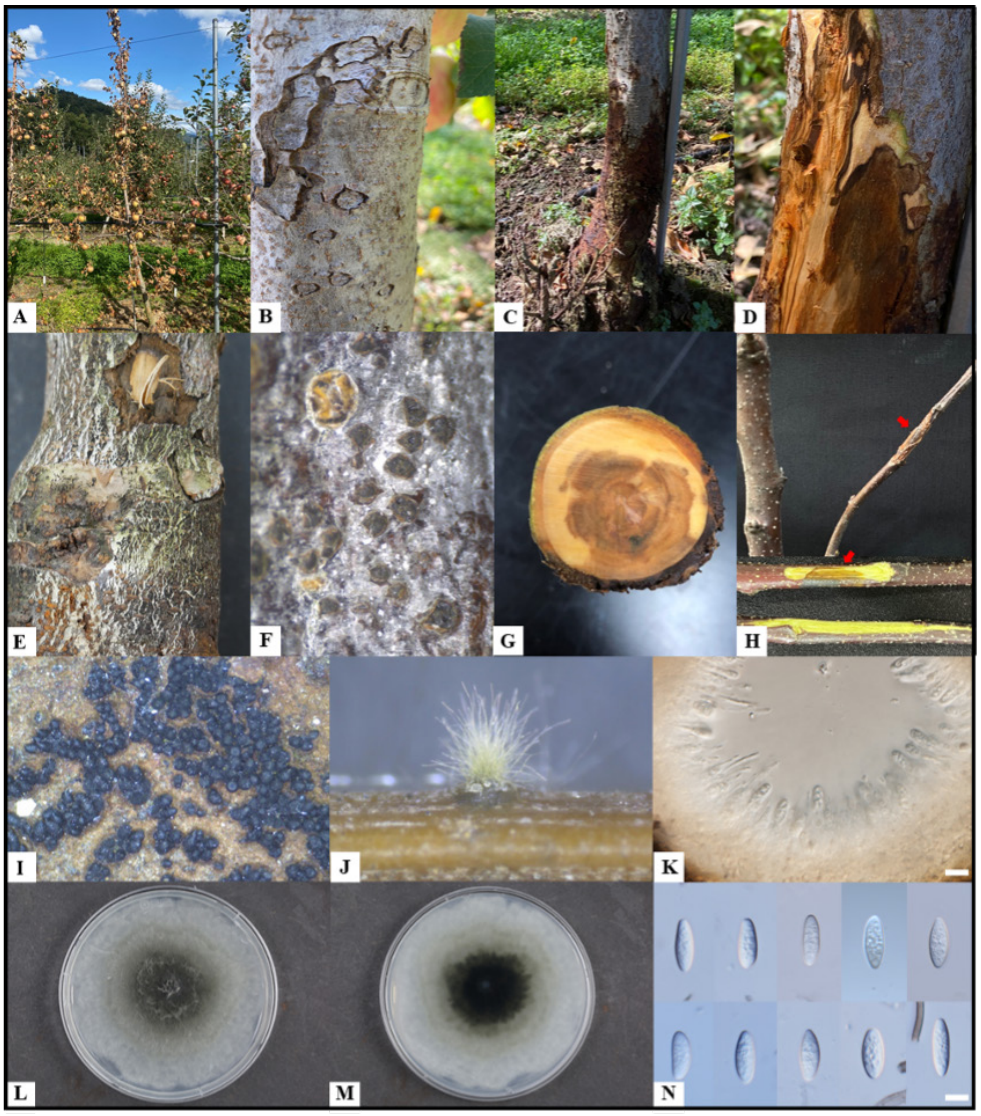

Fig. 1. Natural apple decline symptoms caused by *Botryosphaeria sinensis* and description of the KNUF-20-014. A, declined apple tree; B, warts on a diseased tree; C and D, blackened trunk and observed symptoms inside; E and F, black dots on a diseased trunk; G, observed necrosis on the internal of a diseased tree; H, pathogenicity test result shows canker and necrosis on the inoculated branch of cv. Fuji and its internal symptom (red arrows indicate inoculated zones); I and J, pycnidia on a diseased tree and on pine needle agar (PNA) medium; K, cross-sections through pycnidium; L and M, colony on malt extract agar after incubation at 28℃ for five days (L, front; M, reverse); N, conidia (scale bar=10 μm).

DNA was extracted from the isolates using a HiGene Genomic DNA prep kit (BIOFACT, Daejeon, Korea) according to the manufacturer's instructions. Molecular identification of the strains was conducted using the nucleotide sequences of internal transcribed spacer (ITS) regions and translation elongation factor 1-alpha (*tef*1), beta-tubulin (*tub*2), and second largest subunit of RNA polymerase II (*rpb*2) genes. The ITS regions, *tef*1, *tub*2, and *rpb*2 genes were amplified using the primer sets ITS1/ITS4 [3], EF1-668F/EF1-1251R [4],

Bt2a/Bt2b [5], and RPB2-5F2/fRPB2-7cR [6], respectively. Amplified products of the polymerase chain reaction (PCR) were purified with EXOSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced by Macrogen Co. Ltd. (Daejeon, Korea). Amplification of the ITS, *tef*1, *tub*2, and *rpb*2 loci of strains KNUF-20-014, KNUF-20-072, and KNUF-20-074 yielded fragments of 526, 512, 413, and 1,020 bp; 520, 500, 422, and 1,005 bp; and 497, 496, 423, and 1,029 bp, respectively. The comparative sequence analyses of the molecular markers of the three strains revealed their similarity of 100%, indicating their affiliation to the same species. A BLAST search of the NCBI database showed that the sequences obtained from ITS, *tef*1, *tub*2, and *rpb*2 loci of the three strains exhibited highest similarities of 99.8% (517 bp out of 518 bp), 100% (267 bp out of 267 bp), 100% (363 bp out of 363 bp), and 100% (577 bp out of 577) with that of *Botryosphaeria sinensis* MUCC 2533 (accession numbers LC585268, LC585140, LC585164, LC585188, respectively). To confirm the closest relationship between the three strains and *B. sinensis* at the species level, a phylogenetic analysis was conducted using concatenated sequences of the ITS *regions*, *tef*1, *tub*2, and *rpb*2 genes. The sequences of allied species were retrieved from the NCBI database (Table 1). A phylogenetic tree was constructed with maximum-likelihood method using the program MEGA7 [7]. The evolutionary distances were calculated using the Kimura two-parameter model [8] and the topology of the tree was evaluated using bootstrap analysis based on 1,000 replicates. In the phylogenetic tree, the isolated strains occupied a position within the genus *Botryosphaeria* and clustered together with *B. sinensis*, indicating their closest relationship at the species level (Fig. 2).

Table 1. List of species used in phylogenetic analyses with the GenBank accession numbers.

Fungal species	Isolates No.	Host	Country	Accession number			
				ITS	*tef*1	*tub*2	*rpb*2
Botryosphaeria dothidea	MUCC 245	*Daphniphyllum macropodum*	Japan	LC585273	LC585145	LC585169	LC585192
Botryosphaeria dothidea	MUCC 2521	*Prunus* sp.	Japan	LC585270	LC585142	LC585166	LC585189
Botryosphaeria dothidea	MUCC 2627	*Pyrus pyrifolia*	Japan	LC585284	LC585156	LC585180	LC585202
Botryosphaeria fabicerciana	CBS 127193	*Eucalyptus* sp.	China	HQ332197	HQ332213	KF779068	MF410137
Botryosphaeria fusispora	CERC 2274	*Eucalyptus* hybrid	China	KX277969	KX278074	KX278179	MF410118
Botryosphaeria qingyuanensis	CGMCC 3.18743	*Eucalyptus* hybrid	China	KX278001	KX278106	KX278210	MF410152
Botryosphaeria sinensis	MUCC 2522	*Prunus* sp.	Japan	LC585277	LC585149	LC585173	LC585195
Botryosphaeria sinensis	MUCC 2533	*Aucuba japonica*	Japan	LC585268	LC585140	LC585164	LC585188
Botryosphaeria sinensis	**KNUF-20-076**	***Malus domestica***	**Korea**	**MW644757**	**LC611450**	**LC611453**	**LC638625**
Botryosphaeria sinensis	**KNUF-20-014**	***Malus domestica***	**Korea**	**MW644755**	**LC611448**	**LC611451**	**LC638623**
Botryosphaeria sinensis	**KNUF-20-072**	***Malus domestica***	**Korea**	**MW644756**	**LC611449**	**LC611452**	**LC638624**
Botryosphaeria wangensis	CGMCC 3.18745	*Cedrus deodara*	China	KX278003	KX278108	KX278212	MF410154
Botryosphaeria wangensis	CGMCC 3.18744	*Cedrus deodara*	China	KX278002	KX278107	KX278211	MF410153
Neofusicoccum parvum	ATCC 58191	*Populus nigra*	New Zealand	AY236943	AY236888	AY236917	EU821963

The fungal strains isolated in this study and their accession numbers are indicated in bold.
ITS, internal transcribed spacer; *tef*1, translation elongation factor-1; *tub*2, beta-tubulin; *rpb*2, RNA polymerase II gene.

The representative isolate, KNUF-20-014, was cultured on pine needle agar (PNA) and malt extract agar (MEA) to check the cultural and morphological characteristics. On MEA the colonies grew up to 80 mm in

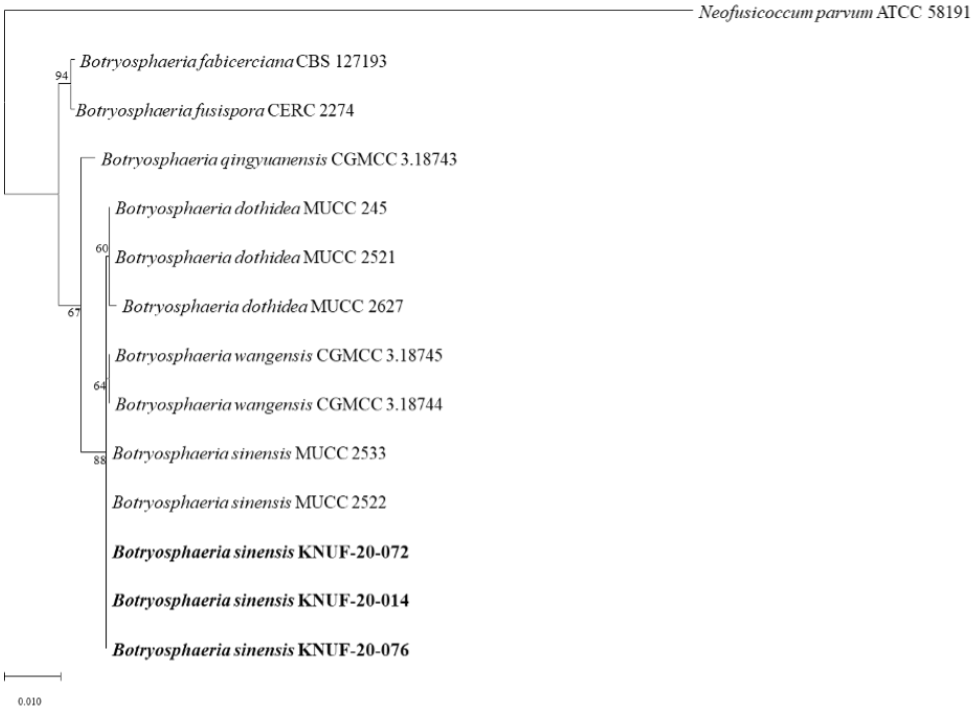

Fig. 2. Maximum-likelihood phylogenetic tree, based on internal transcribed spacer (ITS) regions, translation elongation factor-1 (*tef*1*)*, beta-tubulin *(tub*2*)*, and RNA polymerase II *(rpb*2*)* gene sequences, showing the phylogenetic position of strains KNUF-20-076, KNUF-20-014, and KNUF-20-072, among related strains of the genus *Botryosphaeria*. Bootstrap values greater than 60% (percentage of 1,000 replications) are shown at branching points. The tree was rooted using *Neofusicoccum parvum* ATCC 58191 as an outgroup. Bar, 0.01 substitutions per nucleotide position.

three days at 28°C, showing gray aerial mycelium, white and gray surface with an olivaceous black reverse (Fig. 1L and 1M), the pycnidia were produced on PNA after 21 days (Fig. 1J and 1K), conidia were hyaline, aseptate, obtuse apex, fusiform, smooth with granular contents, and 19.7-28.9×4.9-7.3 μm (av. 23.4×6.0 μm, l/w 4.1, n=50) (Fig. 1N). These cultural and morphological characteristics were similar to those of the previously described *B. sinensis* [9], but strain KNUF-20-014 was readily distinguishable from *B. dothidea* by its conidia size. The average conidia length of the strain (23.4 μm) was distinctly shorter than that of *B. dothidea* (26.2 μm), while the width of its conidia (6.0 μm) was longer than that of *B. dothidea* (5.4 μm) [10]. The conidial length:width ratio of strain KNUF-20-014 (4.1) was the same as the reported value for *B. sinensis* [9], but clearly different from that of *B. dothidea* (4.9) [10].

Currently, there are controversies regarding the classification of a few species of the genus *Botryosphaeria* [11,12]. Based on phylogenetic analysis using the three combined ITS, *tef*1, and *tub*2 sequences, *B. sinensis* [9], *B. minutispermatia* [13], *B. quercus* [14], *B. qinlingensis* [15], and *B. wangensis* [16] were reclassified as a later synonym of *B. dothidea by* Zhang et al. [12]. At the same time, Hattori et al. [11] reexamined the genus *Botryosphaeria* based on phylogenetic analyses using the four molecular markers, namely ITS, *tef*1,

*tub*2, and *rpb*2, and showed that *B. sinensis* and *B. dothidea* represent separate species. Simultaneously, the conidia size was highlighted as a key morphological characteristic for the differentiating species in the genus *Botryosphaeria*. Typically, an increase in the number of genes used in multi locus sequence analysis (MLSA) resulted in more accurate identification of isolated strains, therefore, we conducted MLSA using the four molecular markers in our study. According to the phylogenetic analysis, cultural and morphological characteristics, KNUF-20-014 was identified to be the same with *B. sinensis* at the species level but differed from *B. dothidea*. These data are in good agreement with the results of the reexamination of the genus *Botryosphaeria* conducted by Hattori et al. [11].

A pathogenicity test of strain KNUF-20-014 was conducted with healthy branches cv. Fuji. The branches were inoculated by placing a mycelial plug (4-5 mm) from the five-day-old colony on fresh wound sites made with a sterilized needle, while PDA plugs were placed into similar wounds on the three branches as mock inoculation. The inoculation points were wrapped in parafilm to maintain the moisture for three days at 25℃ in a growth chamber. After four weeks, all inoculated branches showed dark-brown discolorations and vascular necrosis, whereas no symptoms were observed in the mock inoculated branches (Fig. 1H). Pathogenicity test was conducted three times, and the pathogen was re-isolated from the inoculated branches.

Botryosphaeria species are known as plant saprobes, pathogens, and endophytes, with global distribution, on a wide variety of mainly woody hosts [13,17]. Diseases caused by pathogens belonging to the genus *Botryosphaeria* have resulted in significant losses in various economically important agricultural crops. Apple black rot caused by *B. obtusa* has resulted in fruit loss of 25-50% in the USA [18] and 20% annual losses of vineyard productivity from *B. stevensii* infection were reported in France [19]. Among them, *B. dothidea* is known to be a serious pathogen mainly of woody plants that were reported in 66 countries and confirmed in more than 24 host genera [20]. In Korea, *B. dothidea* has been previously reported to cause canker and warts on infected apple branches, mainly occurring on the cv. Hongro and rarely observed on the cv. Fuji [21,22]. However, in this study, *B. sinensis* was mainly isolated from the cv. Fuji, which could be related to host specificity.

B. sinensis was recorded on the twigs of *Populus* sp., *Morus alba*, *Juglans regia* in China [9], *Paulownia tomentosa* and *Prunus* sp. in Japan [11], and recently identified on *Mangifera indica* in Australia [23]. Our results increase the awareness of *Botryosphaeria* distribution, thereby improving our understanding of apple decline associated with *B. sinensis* and can be used for developing the control methods to prevent economic losses.

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