RESEARCH ARTICLE

Morphology and Phylogeny of *Cytospora erumpens*, Unreported Species Isolated from Apple Trunk in Korea

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ABSTRACT

A fungal strain was isolated from a darkened apple tree trunk and designated as KNUF-21-C5. The cultural and morphological characteristics of the isolated strain were evaluated using potato dextrose and malt extract agar (MEA). After 7 days, the colonies on MEA were circular, dense, rough, lacking aerial mycelia, olivaceous-yellow at the center with white margins, and reached 90 mm in diameter. Morphologically, the conidiophores were straight and hyaline, and the conidiogenous cells were enteroblastic, phialidic, hyaline, and cylindrical. The conidia were unicellular, elongated-allantoid, and measured 5.1–6.6 × 1.3–1.6 µm in diameter. These characteristics were consistent with those of the *Cytospora* species. To identify the species, molecular analysis was performed using sequences of the internal transcribed spacer regions, large subunit of 28S rRNA, actin, translation elongation factor 1-alpha, and RNA polymerase II subunit gene, it showed 98.0–100% similarity to *C. erumpens* CFCC 53163. The KNUF-21-C5 strain was clustered with *C. erumpens* CFCC 53163 in phylogenetic trees, and the conidial size was similar to the type strain of *C. erumpens* (5.1–6.6 × 1.3–1.6 µm vs. 5.6–6.7 × 1.3–1.7 µm). Based on fungal characteristics and phylogenetic analysis, KNUF-21-C5 was identified as *C. erumpens*. The pathogenicity of KNUF-21-C5 in apples was confirmed by inoculation of apple twigs. This is the first record of *C. erumpens* associated with Cytospora canker on apples in Korea.

Keywords: Apple, Cytospora canker, *Cytospora erumpens*, Morphology, Phylogenetic analysis

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INTRODUCTION

Apple (*Malus* × *domestica* Borkh.) is one of the most economically significant fruits cultivated all around the world [1]. In Korea, apples are highly valuable fruits grown on 33,313 ha, with a production of 460,088 tons in 2024 [2]. The production and quality of apples can be affected by various fungal

pathogens, and some species, including *Diplodia*, *Neofusicoccum*, *Botryosphaeria*, and *Cytospora*, cause canker and dieback symptoms in apple trees and twigs [3,4]. Among these, Cytospora canker (also called Valsa canker), caused by *Cytospora* species, was first reported on apples in Japan in 1903 and is one of the most destructive diseases that cause significant economic losses in East Asia [5]. The genus *Cytospora* (Diaporthales) was established by Ehrenberg in 1818, and more than 100 species have been described as phytopathogens, endophytes, and saprobes [6]. *Cytospora* is associated with several sexual genera such as *Valsa*, *Leucocytospora*, *Lecuostoma*, and *Valsella* [7]. In 2005, these sexual genera were synonymized under *Valsa* by Adams et al., and they were classified as the oldest name, *Cytospora* following the application of the "one fungus-one name" principle [7]. Cytospora canker disease can occur when the host is weakened by abiotic stress, such as during droughts, by colonizing the bark of host trees through wounds and other mechanical injuries, and extensively invading into phloem and xylem [6,8]. The canker begins with water-soaked symptoms on trunks or branches, and the epidermis turns reddish brown [9]. The lesions may later dry, forming sunken, dark brown cankers that can cause the death of the entire tree. Fruiting bodies are formed on infected hosts and yellow-orange to red spore tendrils can exude from fruiting bodies under moist conditions [7]. The fruiting bodies contained single or labyrinthine locules with hyaline conidiophores and allantoid conidia. During an apple disease survey in 2021, a sample of a declined apple tree was collected from an apple orchard in Gyeonggi Province, Korea. In this study, a *Cytospora* species was isolated from apple trunks and identified based on its cultural and morphological characteristics, along with a phylogenetic analysis.

MATERIAL AND METHODS

Sample collection and isolation of fungal strain

Apple tree samples (cv. Gamhong) showing decline and darkening symptoms were collected from an apple orchard in Paju-si, Gyeonggi Province, Korea. To isolate the causal agent, the bark from the darkened trunk was removed using a scalpel. Then, the pieces of dark-brown tissue on the trunk were transferred onto potato dextrose agar (PDA; Difco, Detroit, MI, USA), and incubated at 25°C in the dark. After a few days, the margins of the colonies were sub-cultured on fresh PDA. The isolated strain was designated as KNUF-21-C5 and was maintained in 20% glycerol at –80°C for further study. The isolated strain has been deposited in the Korean Agricultural Culture Collection (KACC410961).

Cultural and morphological characteristics

The strain KNUF-21-C5 was cultured on PDA and malt extract agar (MEA; Difco, Detroit, MI, USA) for 7 days at 25°C to observe the cultural and morphological characteristics. Mycelial plugs were extracted from colonies grown on PDA using a 4 mm cork borer. The conidiophores, conidiogenous cells, and conidia in the pycnidia were observed and measured using an optical microscope (BX-50; Olympus, Tokyo,

Japan). Fungal growth was quantified by measuring the diameter of colonies using Vernier calipers (Mitutoyo, Kawasaki, Japan).

Genomic DNA extraction and amplification

Total genomic DNA was extracted from the strain grown on PDA using the HiGene™ Genomic DNA Prep Kit (BIOFACT, Daejeon, Korea) following the manufacturer's protocol. The internal transcribed spacer (ITS) regions, large subunit of 28S rRNA (LSU), actin (*ACT*), translation elongation factor 1-alpha (*TEF1*), and RNA polymerase II subunit (*RPB2*) genes of the strain were amplified using primer pairs of ITS1F/ITS4, LROR/LR7, ACT-512F/ACT-783R, EF1-688F/EF1-1251R, and RPB2-5F2/fRPB2-7cR, respectively [10–17]. The PCR products were analyzed by gel electrophoresis and stained with ethidium bromide. The purification of amplified products was conducted using ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced by Solgent (Daejeon, Korea). The obtained sequences of the ITS regions, LSU, *ACT*, *TEF1*, and *RPB*2 were submitted to the National Center for Biotechnology Information (NCBI) (Table 1).

Table 1. GenBank accession numbers of *Cytospora* species used for phylogenetic analysis

Species	Strain number	Isolation source	Origin	GenBank Accession number				
				ITS	LSU	*ACT*	*TEF*1	*RPB*2
Cytospora albodisca	CFCC 53161[T]	*Platycladus orientalis*	China	MW418406	MW418418	MW422899	MW422921	MW422909
C. albodisca	CFCC 54373	*Platycladus orientalis*	China	MW418407	MW418419	MW422900	MW422922	MW422910
C. ceratospermopsis	CFCC 89626[T]	*Juglans regia*	China	KR045647	KR045726	KU711011	KU710934	KU710978
C. ceratospermopsis	CFCC 89627	*Juglans regia*	China	KR045648	KR045727	KU711012	KU710935	KU710979
C. erumpens	CFCC 53163	*Prunus padus*	China	MK673059	MK673089	MK673029	MK672948	MK673000
C. erumpens	**KNUF-21-C5**	***Malus domestica***	**Korea**	**PQ821439**	**PQ821445**	**PQ827002**	**PQ827004**	**PQ827003**
C. gigaspora	CFCC 50014	*Juniperus procumbens*	China	KR045630	KR045710	KU710999	KU710922	KU710959
C. gigaspora	CFCC 89634[T]	*Salix psammophila*	China	KF765671	KF765687	KU711000	KU710923	KU710960
C. leucostoma	CFCC 53140	*Prunus sibirica*	China	MN854445	MN854656	MN850760	MN850753	MN850746
C. leucostoma	CFCC 53141	*Prunus sibirica*	China	MN854446	MN854657	MN850761	MN850754	MN850747
C. mali	ARI-15-US	*Malus domestica*	Korea	PP974558	PP976972	LC830507	LC830505	LC830509
C. mali	ARI-23-GW	*Malus domestica*	Korea	PP974557	PP976971	LC830506	LC830504	LC830508
C. mali	CFCC 50030	*Malus pumila*	China	MH933643	MH933677	MH933550	MH933524	MH933608
C. mali	CFCC 50031	*Crataegus* sp.	China	KR045636	KR045716	KU711004	KU710927	KU710965
C. mali-spectabilis	CFCC 53181[T]	*Malus spectabilis*	China	MK673066	MK673096	MK673036	MK672953	MK673006
C. nivea	CFCC 89641	*Elaeagnus angustifolia*	China	KF765683	KF765699	KU711006	KU710929	KU710967
C. olivacea	CFCC 53176[T]	*Sorbus tianschanica*	China	MK673068	MK673098	MK673038	MK672955	MK673008
Diaporthe eres	CBS 145040	*Lactuca satia*	Netherlands	MK442579	MK442521	MK442634	MK442693	MK442663

ITS: internal transcribed spacer regions; LSU: large subunit of 28S rRNA; *ACT*: actin; *TEF1*: translation elongation factor 1-alpha; *RPB2*: RNA polymerase II subunit.
[T] type strain.
The strain identified in this study is indicated in bold.

Molecular phylogenetic analysis

Phylogenetic analysis was performed using sequences obtained from the NCBI database (Table 1). The ambiguous regions were removed from the alignments, and the evolutionary distance matrices were calculated using the maximum-likelihood (ML) method with ClustalX and the Tamura-Nei model [18]. Phylogenetic trees were constructed using the ML method in MEGA 11.0, with bootstrap values derived from 1,000 replications [19].

Pathogenicity test

To test Koch's postulates, a pathogenicity test was conducted by inoculating apple twigs with the KNUF-21-C5 strain according to the inoculation method described previously [7]. The twigs were washed with tap water, sterilized with 70% ethanol for 4 min, and dried for 2 min. The bark of the twigs was removed using a disinfected scalpel and inoculated with a 4 mm diameter mycelial plug from a 5-day-old colony grown on PDA. The inoculated twigs were incubated in a moist chamber at 25°C for 2 weeks. After incubation, small pieces from the inoculation sites were transferred onto PDA medium to re-isolate the inoculated strain.

RESULTS AND DISCUSSION

Cytospora erumpens Norph., Bulgakov, T.C. Wen & K.D. Hyde, Mycosphere 8: 64 (2017) [MB#552604]

Cultural and morphological characteristics of KNUF-21-C5

The colonies of the KNUF-21-C5 strain on PDA were flat, circular, irregular, lacking aerial mycelia, yellow to cream at the center, white toward the edges, and reached 90 mm after 3 days. On MEA, the colonies exhibited a circular form, dense mycelia, and a rough, irregular, subhyaline texture with white margins. The center of the colonies was yellow to olivaceous-yellow, reaching a diameter of 90 mm after 7 days. Colonies were flat and lacked aerial mycelia. The reverse side of the colonies also displayed bright khaki at the center and white margins (Fig. 1A–D). Pycnidia, exudating dark orange to brownish masses of conidia, were observed in the colonies (Fig. 1E). Conidiophores were straight, cylindrical, unbranched, or occasionally branched at the base and were reduced to conidiogenous cells. Conidiogenous cells formed within the pycnidial wall, appearing hyaline, blastic, enteroblastic, phialidic, and cylindrical with smooth walls (Fig. 1F). Conidia were unicellular, hyaline, allantoid to subcylindrical, slightly curved, and smooth-walled, and typically measured 5.1–6.6 × 1.3–1.6 μm (avg. 5.6 × 1.4 μm, $n = 50$) (Fig. 1G). The culture and morphological characteristics of KNUF-21-C5 were similar to those previously reported for *C. erumpens* [20].

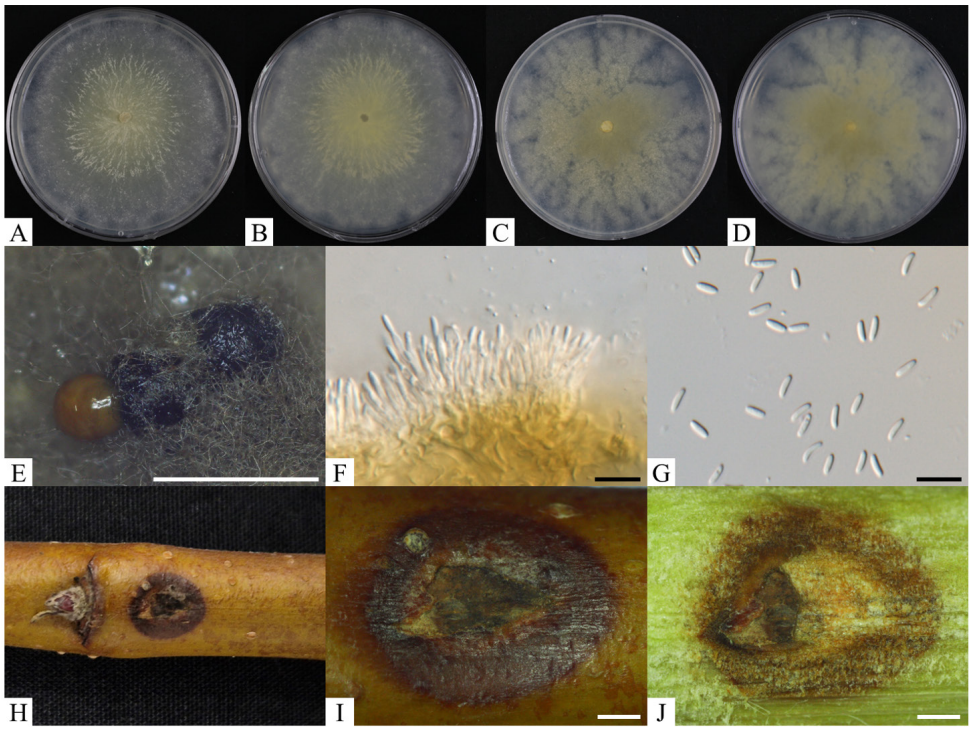

Fig. 1. Morphological characteristics of *Cytospora erumpens* KNUF-21-C5 isolated from apple trunk. A, B: obverse and reverse of colony on PDA at 25℃ for 7 days, respectively; C, D: obverse and reverse of colony on MEA at 25℃ for 7 days, respectively; E: pycnidia exudating conidial mass; F: conidiophores with conidiogenous cells; G: conidia; H–J: result of pathogenicity test for 2 weeks. White scale bars = 1 mm, black scale bars = 10 μm. PDA: potato dextrose agar; MEA: malt extract agar.

Molecular phylogenetic analysis of KNUF-21-C5

Partial sequences of the ITS regions, LSU, *ACT*, *TEF1*, and *RPB*2 were amplified, yielding sequences of 616, 1,262, 248, and 981 bp, respectively. The ITS sequence showed 99.8% similarity with *C. erumpens* CFCC 53163 and *C. leucostoma* CFCC 54140. The LSU sequence exhibited 100% similarity with *C. erumpens* CFCC 53163 and 99.4% similarity with *C. leucostoma* 54140 and *C. albodisca* CFCC 53161^{T}. The partial *ACT* sequence showed 98.4% similarity with *C. erumpens* CFCC 53163 and less than 93.2% similarity with other *Cytospora* species, including *C. leucostoma* CFCC 54140 and *C. albodsica* CFCC 53161^{T}. In the case of *TEF1*, 98.0% similarity with *C. erumpens* CFCC 53163 and 88.2% similarity with *C. leucostoma* CFCC 53140. The *RPB2* sequence revealed 98.5% similarity with *C. erumpens* CFCC 53163, and 94.6–95.9% similarity with *C. leucostoma* and *C. albodisca* strains. The ML phylogenetic tree was constructed based on the concatenated sequences of the ITS regions, LSU, *ACT*, *TEF1*, and *RPB2*. The KNUF-21-C5 strain clustered with *C. erumpens* CFCC 53163, forming a cluster distinct from other *Cytospora* species in the phylogenetic tree (Fig. 2).

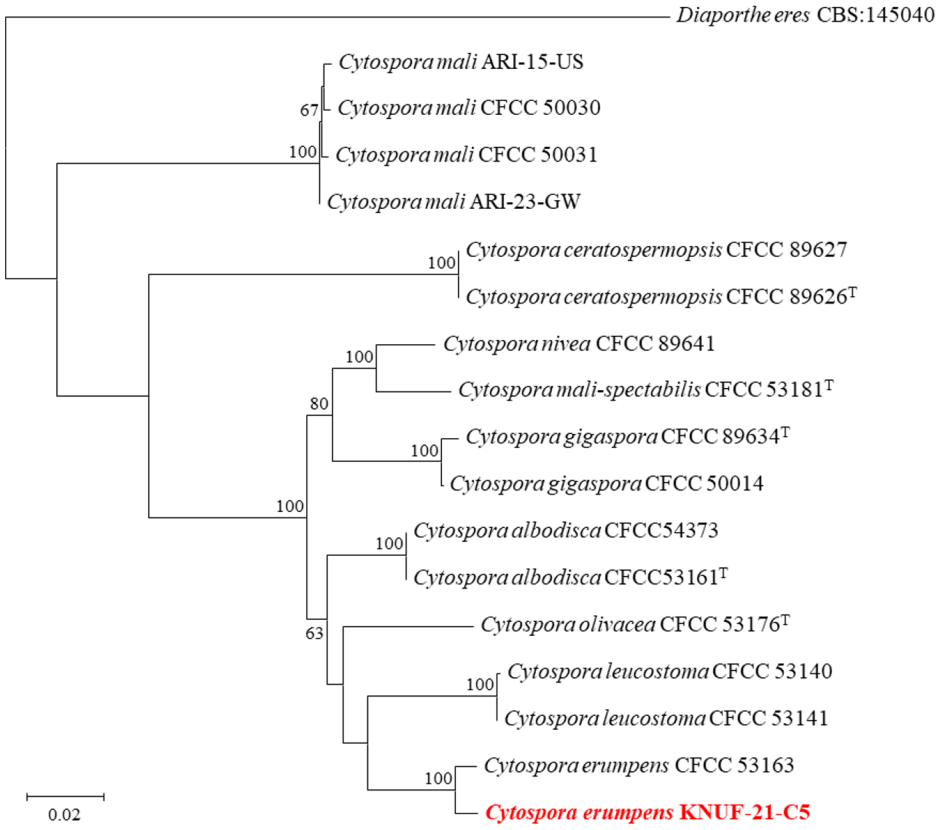

Fig. 2. Maximum-likelihood phylogenetic tree based on concatenated sequences of ITS regions, LSU, *ACT*, *TEF1*, and *RPB2*, showing the relationship between the strain KNUF-21-C5 with closest *Cytospora* species. The numbers above the branches indicate bootstrap values (> 60%) obtained from 1,000 replicates. *Diaporthe eres* CBS 145050 was used as an outgroup. The isolated strain in this study is indicated in red and bold. Bar = 0.02 substitutions per nucleotide position. T type strain. ITS: internal transcribed spacer regions; LSU: large subunit of 28S rRNA; *ACT*: actin; *TEF1*: translation elongation factor 1-alpha; *RPB2*: RNA polymerase II subunit.

Pathogenicity test of KNUF-21-C5

Discoloration and browning symptoms were observed on the bark at the inoculation sites two weeks post-inoculation (Fig. 1H–J). A fungal strain was re-isolated from lesions at the inoculation site, and its cultural and morphological characteristics were identical to those of the strain used for inoculation. In addition, the partial sequence of the re-isolated strain matched that of KNUF-21-C5 (data not shown). The pathogenicity of KNUF-21-C5 was confirmed on apple twig under laboratory conditions, though it showed weak pathogenicity.

The genus *Cytospora* includes several plant pathogens that cause Cytospora canker diseases, primarily affecting woody hosts with a wide distribution and broad host range [9]. Traditionally, the identification of *Cytospora* species that cause Cytospora canker has been based on morphological characteristics and host affiliation [20]. However, recent studies have shown that several *Cytospora* species such as *C. chrysosperma* and *C. punicae* infect a range of hosts rather than being host-specific [21]. *C. erumpens* was first described as causing cankers and dieback on twigs and branches of crack willows (*Salix fragilis*) in

Russia in 2017 [20]. Subsequently, *C. erumpens* was isolated from canker lesions on bird cherries (*Prunus padus)*, peaches (*P. persica*), and apples (*Malus* spp.) in China [7,22,23]. The pathogenicity of *Cytospora* species on apple varieties and wild apples was evaluated, including *C. erumpens* [23]. The results showed that the pathogenicity of *Cytospora* species varied depending on the variety, and *C. erumpens* was strongly pathogenic to apples in the leaf test, with relatively weak pathogenicity to branches [23]. According to Lin et al., *C. erumpens* is regarded as a synonym of *C. leucostoma* [24]. However, recent research has shown that *C. erumpens* forms a cluster distinct from *C. leucostoma* strains in phylogenetic trees [23]. Moreover, the strain of *C. erumpens* was differentiated from *C. leucostoma* CFCC 53140 based on cultural and morphological characteristics, such as colony color, ostiole diameter, and conidial size [23]. In this study, the strain KNUF-21-C5 exhibited distinctive cultural characteristics compared to *C. leucostoma* CFCC 53140 (Table 2). Additionally, the strain clustered with *C. erumpens* CFCC 53163, but was distinct from *C. leucostoma* CFCC 53140 (Fig. 2). Therefore, *C. erumpens* KNUF-21-C5 differed from *C. leucostoma*.

Cytospora canker is one of the most significant diseases in apple trees, and over 20 *Cytospora* species have been reported in apples, including *C. chrysosperma*, *C. leucostoma*, *C. nivea*, and *C. pyri* [6,25]. According to a survey conducted from 2015 to 2024, the incidence of apple Cytospora canker has sharply increased during the last three years, and it has been confirmed that *C. mali* is predominant in apple orchards in Korea [25]. Recently, the distribution of fungi has been affected by global warming, and the distributions of *C. chrysosperma, C. mali,* and *C. nivea* are expected to shift toward the northeastern regions of China [26]. For this reason, other *Cytospora* species are presumed to inhabit apple orchards in Korea, although *C. mali* is predominant. In this study, KNUF-21-C5 was isolated from the trunks of apple trees in Korea. Based on its cultural and morphological characteristics, and phylogenetic analysis, the strain KNUF-21-C5 was identified as a previously undescribed species, *C. erumpens*. To our knowledge, this is the first report of *C. erumpens* isolated from apple trunks in Korea.

Table 2. Cultural and morphological characteristics of the isolated strain KNUF-21-C5 with reference to *Cytospora* species

Characteristics		*C. erumpens* KNUF-21-C5[a]	*C. erumpens* MFLUCC 16-0580[Tb]	*C. leucostoma* CFCC 53140[c]	*C. albodisca* CFCC 53161[Td]
Colony	PDA	Flat, lacking aerial mycelium, irregular, yellow, white margins, 90 mm at 3 days.	N/A	Flat, greenish-olivaceous to grey olivaceous, 90 mm at 4 days.	Sparse in the center, compact margin, felt, white to dark herbage green, 90 mm at 5 days.
	MEA	Flat, lacking aerial mycelium, dense, olivaceous-yellow, white margins, 90 mm at 7 days.	Dense, circular, margin rough, white, lacking aerial mycelium, 85 mm at 7 days.	N/A	N/A
Conidiophores		Straight, hyaline, unbranched at the base, or occasionally branched.	Unbranched or occasionally branched at the base.	Hyaline, branched at the base or unbranched, cylindrical.	Cylindrical, hyaline, unbranched, straight to slightly sinuous.
Conidiogenous cells		Enteroblastic, phialidic, hyaline, cylindrical.	Enteroblastic, phialidic, hyaline.	Enteroblastic, phialidic, sub-cylindrical to cylindrical.	Enteroblastic, phialidic.
Conidia		Unicellular, hyaline, elongated-allantoid, slightly curved, smooth, 5.1–6.6 × 1.3–1.6 µm.	Unicellular, elongate-allantoid, hyaline, smooth, 5.6–6.7 × 1.3–1.7 µm.	Hyaline, elongate-allantoid, smooth, aseptate, 4.5–6.0 × 1.0–2.0 µm.	Hyaline, allantoid, little curve, rough, aseptate, biguttulate, 5–7 × 1–2 µm.

[a] fungal strain investigated in this study; [b] sources of description [20]; [c] sources of description [27]; [d] sources of description [21]; [T] type strain.
PDA: potato dextrose agar; MEA: malt extract agar; N/A: not available in previous study.

CONFLICT OF INTERESTS

The authors declare that they have no potential conflicts of interest.

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