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## Pear Skin Stain Caused by *Mycosphaerella graminicola* on Niitaka Pear (*Pyrus pyrifolia* Nakai)

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**Pear skin stains on ‘Niitaka’ pears, which occur from the growing stage to the cold storage stage, reportedly negatively influence the marketing of pears. These stains on fruit skin are likely due to a pathogenic fungus that resides on the skin and is characterized by dark stains; however, the mycelium of this fungus does not penetrate into the sarcocarp and is only present on the cuticle layer of fruit skin. A pathogenic fungus was isolated from the skin lesions of infected fruits, and its pathogenicity was subsequently tested. According to the pathogenicity test, *Mycosphaerella* sp. was strongly pathogenic, while *Penicillium* spp. and *Alternaria* spp. showed modest pathogenicity. In this present study, we isolated the pathogenic fungus responsible for the symptoms of pears (i.e., dark brown-colored specks) and identified it as *Mycosphaerella graminicola* based on its morphological characteristics and the nucleotide sequence of the beta-tubulin gene. *M. graminicola* was pathogenic to the skin of ‘Niitaka’ pears, which are one of the most widely growing varieties of pears in South Korea.**

**Keywords :** *Mycosphaerella graminicola*, Niitaka, pear, pear skin stain

In South Korea, 282 thousand tons of pears were produced in 2013, making it the third most produced fruit after citrus fruits (649 thousand tons) and apples (494 thousand tons). Moreover, pears constitute the largest proportion of fruit exports with 18 thousand tons of export volume (KREI, 2014). Of the many pear varieties, ‘Niitaka’ (*Pyrus pyrifolia*

Nakai) pears feature excellent quality and cold storage and are thereby known as a promising pear variety for export. Thus, they account for approximately 83% of the total cultivated area in South Korea. They are an extensively consumed food for ancestral rites; in particular, pears are mostly consumed on the Korean Thanksgiving Day, around the harvest season of the pear, and followed by the New Year holiday, 3–4 months after the harvest. Furthermore, given the unavoidable long-term cold storage and the transportation of exported fruit, maintaining the quality of pears throughout the storage period is critical. Recently, pear skin stains that occurred during the cold storage and distribution processes have been known to result in poor marketability (Park et al., 2008). This discoloration might be due to a pathogenic fungus present on the skin of pears that results in dark brown stains on the pear’s skin and does not penetrate the flesh of the fruit (Kim, 1975; Nasu, 1998; Park et al., 2008). Therefore, it does not impact the quality of fruits *per se*, as ‘Niitaka’ pears are generally consumed after being peeled, but pears with these specks are generally not favored by consumers and often rejected by the inspection processes of quarantine and customs because of their unpleasant appearance. The types of contamination that often occur on pear skin include soot, flyspeck, and blotches, which are found during the growing and cold storage periods (Kim et al., 1999), and can be categorized into fruit skin blackening (Choi et al., 1995; Kim, 1975), fruit skin contamination (Kim et al., 1999), black stain (Yun et al., 2000), and sooty skin dapple (Park et al., 2008) depending on the symptoms of specks. Of these symptoms, sooty fruit skin has been reported, with black dark specks and brown specks on skins of ‘Niitaka’, European ‘Passe Crassane’ (Nasu, 1998) and Japanese ‘Gold Nijisseiki’ (Yasuda et al., 2005, 2007) pears. The pathogenic fungi known to be responsible for these symptoms (i.e. specks on the pear’s skin) are *Colletotrichum* spp. (Sadamatsu and Sanematsu, 1983), *Stenella* sp. (Nasu, 1998), *Gloeodes pomigena*

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(Hong et al., 2003; Yun et al., 2000), *Alternaria* sp., *Hyalodendron* sp., *Phomopsis* sp. (Yasuda et al., 2005), *Meira geulakonigii*, *Pseudozyma aphidis* (Yasuda et al., 2007), *Cladosporium* spp., *Leptosphaerulina* spp., and *Tripospermum* spp. (Park et al., 2008). Previously, *Gloeodes pomigena* (Hong et al., 2003) and *Cladosporium* spp. (Park et al., 2008) were reported as possible causative pathogenic fungi for sooty pear skin, particularly for the 'Niitaka' variety. However, this finding has been the subject of controversy, as the fungi were not clearly identified. Thus, we aimed to identify and report the pathogenic fungus responsible for the pear skin stain on 'Niitaka' pears in the present study.

## Materials and Methods

**Pathogenic fungus collection and separation.** To identify the pathogenic fungus that causes sooty pear skin during the growing and post-harvest storage periods, infected 'Niitaka' pears were harvested over three years since 2008 from pear orchards located in Moga-myeon (Icheon-si), Miyang-myeon (Anseong-si), and Jukbaek-dong (Pyeongtaek-si) in Gyeonggi-do, South Korea; infected 'Niitaka' pears, harvested in 2012 and then stored in Anseong-si in Gyeonggi-do and Cheonan-si in Chungcheongnam-do were also collected. Sections ( $3 \times 3 \text{ mm}^2$ ) were cut from the lesions of infected fruit and then sterilized with 70% ethyl alcohol (EtOH) followed by three washes in sterilized distilled water (SDW); the samples were dried on a clean bench after removing excess water with sterilized filter papers. Once seeded on water agar, the samples were incubated in an incubator maintained at  $25^\circ\text{C}$  for three days. After three days, 2 mm of the end of the mycelium was cut and then transplanted on potato dextrose agar (PDA) for an additional three weeks of incubation in an incubator for fungal isolation. The single hyphae were separated under a microscope from the fungi originated from lesions. Each of the isolates was stored until the experiment of pathogenicity by inoculating on PDA. To study the incidence of each pathogen, we investigated the percentage of pathogen from the total isolates.

**Pathogenicity test.** Isolated strains were inoculated on PDA and then incubated over three weeks in order to induce sporogenesis. Ten milliliters of SDW were added to the cultured petri dish, and the spores were then collected by rubbing the colony surface with a sterilized brush. The collected spores were diluted to a concentration of  $10^6$  spores/mL and then used as the inoculum. Sterilized gauze was placed on the bottom of the plastic container ( $40 \times 20 \times 20 \text{ cm}^3$ ), and SDW was then added to saturate the inside.

The prepared pear skin was sterilized with 70% EtOH and placed into the container in order to be inoculated with the pathogenic fungus. Three different methods were used for inoculation: spraying inoculation using a spore suspension, inoculation using a wet paper disc with spores, and inoculation with a pipette. The pear skin was intentionally cut prior to the inoculation, except for the spraying method. Moreover, lesions ( $5 \times 5 \text{ mm}^2$ ) from the skin of infected pear fruit were collected and inoculated on young pear fruit skin in June, which is known as the growth and developmental stage of pear skin. Twenty days after the inoculation, the lesion tissue was detached and the symptoms on fruit skin were observed. Pathogenicity was investigated on the basis of reproducibility, magnitude, occurrence time etc. of disease symptoms after inoculation.

**Identification of an isolated fungus.** The isolated fungus was identified based on its fungal characteristics as well as via a comparison of the 16S ribosomal RNA (rRNA) and beta-tubulin gene nucleotide sequences. *Mycosphaerella* sp. was found to be the most abundant of the isolated fungi; thus, the optimal incubation temperature of *Mycosphaerella* sp. was assessed. The flora were cut using a cork borer (No. 2, DAIHAN, Korea) and then seeded on the middle of the PDA prior to sealing. *Mycosphaerella* sp. was incubated in the incubator for three weeks at different temperatures ranging from  $5^\circ\text{C}$  to  $35^\circ\text{C}$  with an interval of  $5^\circ\text{C}$ , and the diameters of the colony were then measured. In addition, the fungus was incubated on PDA for 30 days, and the mycelium and spores were then observed using both an optical microscope (de/mz16a, Leica, Germany) and an electron microscope (S-3500N, Hitachi, Japan) in order to investigate the morphological characteristics of the pathogenic fungus. To compare the nucleotide sequence of the beta-tubulin gene and the internal transcribed spacer (ITS) region, DNA was extracted from the freeze-dried fungus using the Nucleo spin<sup>®</sup> soil kit (MACHEREY-NAGEL, Germany). The sequence of the 16S rRNA gene was amplified using ITS1 (5'-CGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as described elsewhere (Staats et al., 2005). For the polymerase chain reaction (PCR, CM6019, Quante Biotech, England) conditions of ITS1/ITS4, the samples were denatured at  $94^\circ\text{C}$  for two minutes followed by an additional denaturation at  $94^\circ\text{C}$  for 40 seconds. The sample was annealed at  $60^\circ\text{C}$  for one minute and then extended at  $72^\circ\text{C}$  for one minute over 30 cycles. The final DNA synthesis condition was  $72^\circ\text{C}$  for five minutes. For the PCR conditions of Bt2a/Bt2b, the samples were denatured at  $94^\circ\text{C}$  for three minutes followed by an additional one

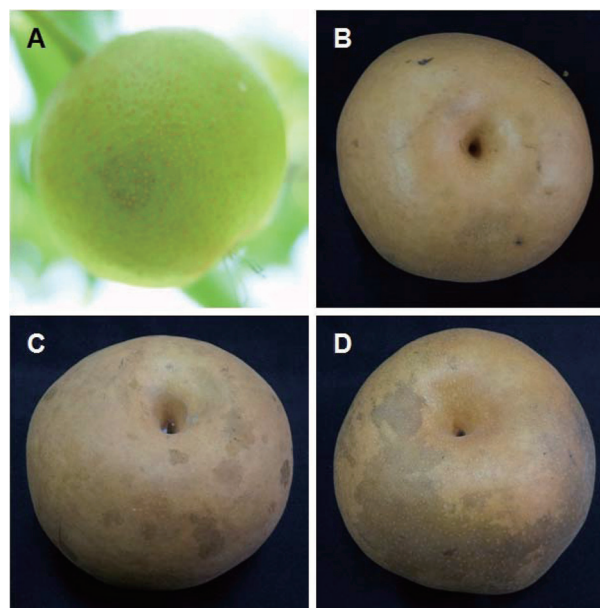
minute denaturation at 94°C. The sample was then annealed at 56°C for 1.5 minutes and extended for two minutes at 56°C for 30 cycles. The final DNA synthesis condition was 72°C for 10 minutes. The amplified products of PCR were separated on a 1.2% agarose gel using electrophoresis, and nucleotide sequence was then determined. The taxonomic relationships between isolated fungi were analyzed using Kimura's 2-parameter distance of Neighbor-joining method.

## Results

**Symptoms of disease.** Pear skin stains were observed from early July, which was the young fruiting period, and were bright in grey color (Fig. 1A). The circular grey-brown colored stains grew during the harvest period (Fig. 1B) and then changed to dark-brown colored stains of various sizes midway through the cold storage period (Fig. 1C). After the harvest, the small stains on the skins of diseased pears grew in size and darkened as they merged with each other during the extended cold storage time; in some cases, all of the fruit skin were completely dark in color, as if they were stained with ink (Fig. 1D). However, the sarcocarp of seriously infected fruits and their lesion area were not affected, and the mycelium was only present on the cuticle layer of fruit skin (Fig. 2).

### Isolation of a pathogenic fungus and pathogenicity test.

The pathogenic fungi were isolated from the skin lesions of infected pear fruits, and their pathogenicity was then tested (Table 1). *Mycosphaerella* sp. was the most abundant of the isolated pathogenic fungi, comprising an average of 64.2% of the population, followed by *Penicillium* spp. at 13.2% and *Alternaria* spp. at 12.0%. The pathogenicity test indicated that *Mycosphaerella* sp. was strongly pathogenic, while *Penicillium* spp. and *Alternaria* spp. showed modest pathogenicity. In contrast, *Nigrospora* spp. and *Diaphoth* spp. did not show any pathogenicity. The pathogenicity of *Mycosphaerella* sp. was similar as that observed at the farm, while the psychrotroph *Penicillium* spp. showed decay symptoms. Although *Alternaria* spp. exhibited weak



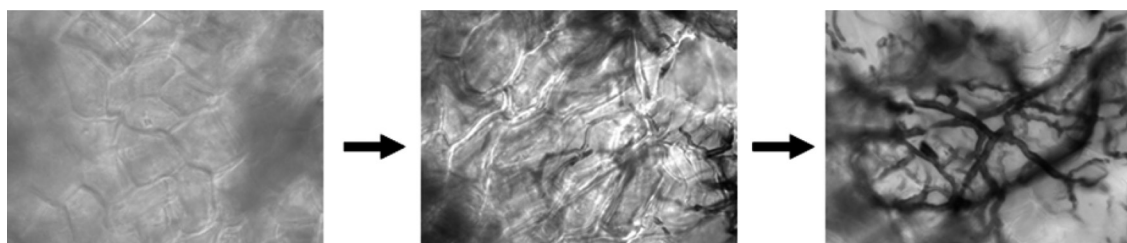
**Fig. 1.** Typical symptoms of pear skin stains on the 'Niitaka' pear. (A) Symptoms were bright in grey color on the young pear skin in a fruitlet stage. (B) The circular grey-brown colored stains grew on the pear skin during the harvest period. (C) The grey-brown colored stains were changed to dark-brown colored stains during cold storage period. (D) The dark-brown stains were merged with each other during the extended cold storage period.

**Table 1.** Incidence and pathogenicity of fungi isolated from pear skin stains on 'Niitaka' pears collected from the cold storage in January

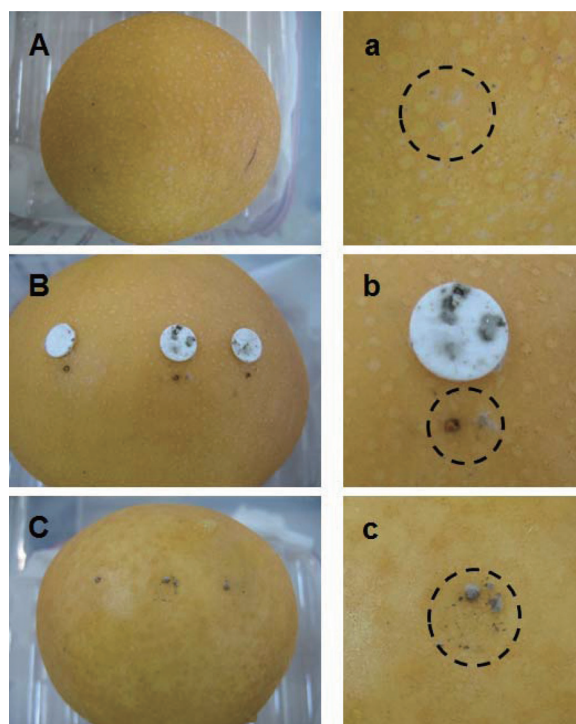
Causal Agents	Incidence (%) <sup>z</sup>	Pathogenicity <sup>y</sup>
<i>Mycosphaerella</i> sp.	64.2	+++
<i>Penicillium</i> spp.	13.2	++
<i>Alternaria</i> spp.	12.0	+
<i>Nigrospora</i> spp.	5.0	-
<i>Diaphoth</i> spp.	3.3	-

<sup>z</sup>means of two replications

<sup>y</sup>strong +++, medium ++, weak +, and no -

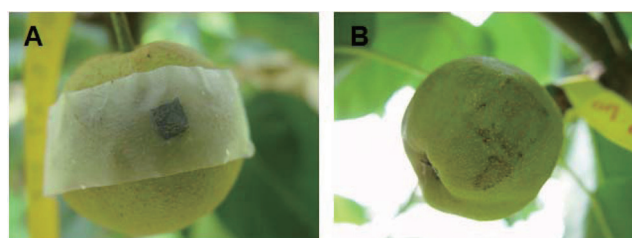


**Fig. 2.** Invasion process of *Mycosphaerella* sp. into the cuticle layer responsible for pear skin stains in 'Niitaka' pears.



**Fig. 3.** Pathogenic test of *Mycosphaerella* sp. on pear skin. (A) Spraying inoculation using a spore suspension on the skin of pear. (B) Inoculation using wet paper disc on the skin of pear. (C) Inoculation with a pipette drops in damaged skin of pear. (a, b and c) Extension of fungus (in dotted circle) from A, B, and C in each.

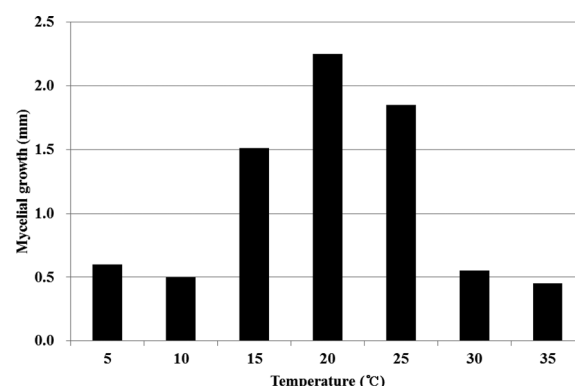
pathogenicity, it caused decay symptoms as opposed to fruit skin stains. The most commonly isolated *Mycosphaerella* sp. was inoculated on fruit skins with different inoculation methods in order to test its pathogenicity (Fig. 2). The *Mycosphaerella* sp. cultured in medium was diluted with SDW to a concentration of  $10^6$  spores/mL and then sprayed on the skin (Fig. 3A); the fruit skins showed small fungi (Fig. 3a). When a paper disc was soaked in the suspension and then placed on the fruit skin (Fig. 3B), a pathogenic fungus was found on the inoculated spot (Fig. 3b). Similarly, a pathogenic fungus was evident when the damaged pear skin was directly inoculated with the suspension (Fig. 3C and 3c). Collectively, pear skin stains were considered to be primarily due to *Mycosphaerella* sp. Young pear fruits were inoculated with infected tissues that were sooty in appearance, which were evident during the cold storage period and observed Fungal colony (Fig. 4). Infected tissues ( $5 \times 5 \text{ mm}^2$ ) were attached to the surfaces of fruits and fixed with tape for 20 days (Fig. 4A). Twenty days after inoculation, the infected tissues were removed, and the young pear fruits showed a typical symptomatic stain appearance (Fig. 4B). Based on these observations, fruits are likely in-



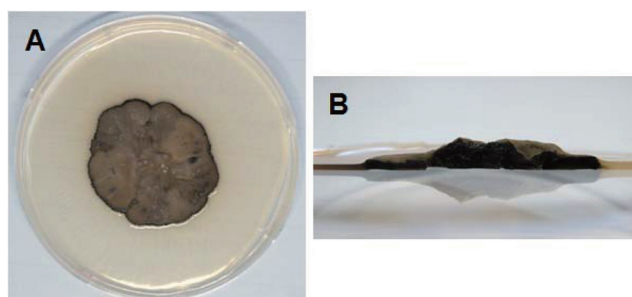
**Fig. 4.** Pathogenic test of *Mycosphaerella* sp. in a pear orchard. (A) Inoculation of a pathogen with lesion ( $5 \times 5 \text{ mm}^2$ ) from infected fruit skin in June. (B) Typical symptoms of pear skin stains after 20 days of inoculation.

fectured via infected tissues from the previous harvest.

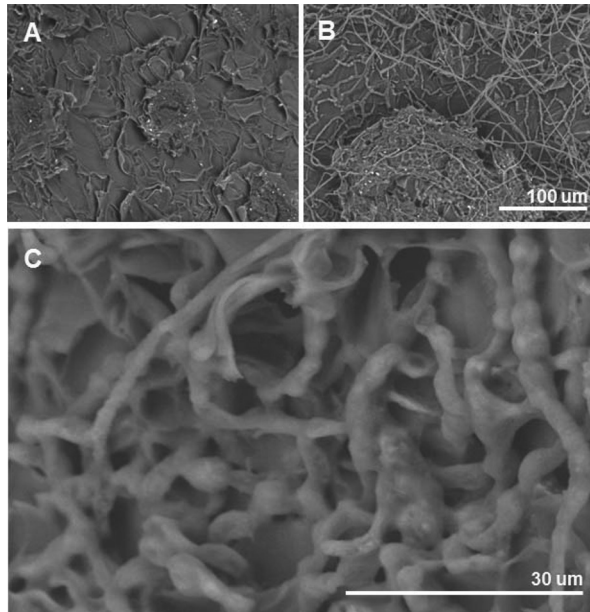
**Microbiological characteristics.** *Mycosphaerella* sp. was incubated at different temperatures over three weeks on PDA. The results showed that the mycelium of *Mycosphaerella* sp. was elongated to a maximum of 2.25 mm at  $20^\circ\text{C}$ , while they grew to 1.85 mm and 1.51 mm at  $25^\circ\text{C}$



**Fig. 5.** Effect of temperature on the mycelial growth of *Mycosphaerella* sp. isolated from the pear skin stains of the 'Niiitaka' pear. The mycelial growth was measured after three weeks of incubation on potato dextrose agar.



**Fig. 6.** Fungal colony of *Mycosphaerella* sp., the causal agent of pear skin stains on 'Niiitaka' pears. (A) Above view of the formed circular flora. (B) Cross-sectional view of the formed convex center (diameter  $4 \pm 0.8 \text{ cm}$ , height  $0.4 \pm 0.2 \text{ cm}$ ).



**Fig. 7.** Mycelia of *Mycosphaerella* sp., the causal agent of pear skin stains on ‘Niitaka’ pears. (A) Pear skin on a normal pear. (B) Pear skin on an infected pear. (C) Extension of colony from the skin of an infected pear (A and B:  $\times 600$ , Bar = 100  $\mu\text{m}$ , C:  $\times 2.5\text{k}$ , Bar = 30  $\mu\text{m}$ ).

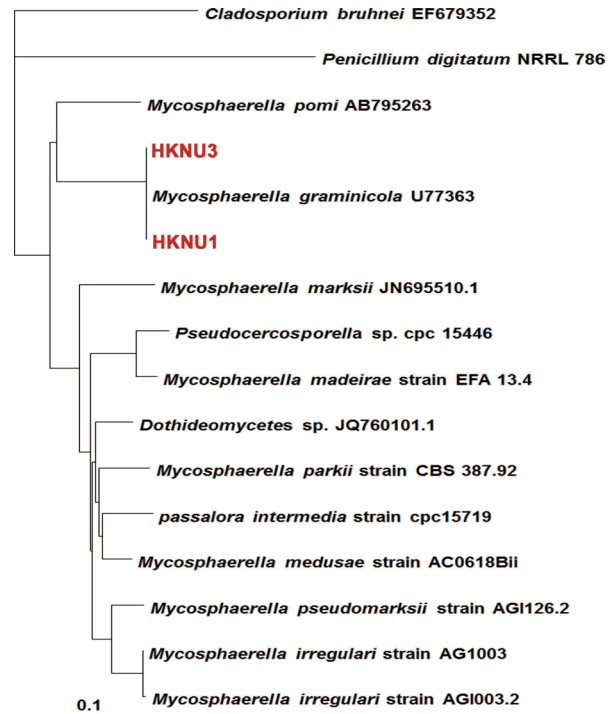
and 15°C, respectively (Fig. 5). The major causal fungus responsible for pear skin stains, *Mycosphaerella* sp., formed a circular colony with a convex center. It was dark brown in color and grew very slowly (Fig. 6). The mycelia of *Mycosphaerella* sp. were long and rod-shaped with a narrow septum that formed a continuum. It was milky white in color (Fig. 7 and Table 2).

**Identification of isolated fungi.** HKNU1 and HKNU3 were isolated from the lesions of infected pears. The results showed that HKNU1 and HKNU3 represented the most relevant and closest homogeny (Fig. 8 and Fig. 9). Taken together, these genes were identified as *Mycosphaerella graminicola* given their microbiological characteristics and similarity to nucleotide sequences of the ITS and beta-tubulin gene.

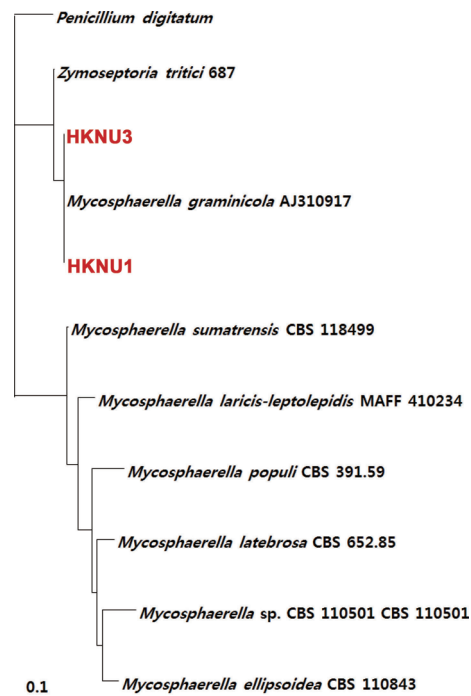
**Table 2.** Comparison of the morphology of the present isolate from a pear skin stain and *M. graminicola*

Characteristics	Present isolate	<i>M. graminicola</i> <sup>z</sup>
Colony color	dark brown	dark brown
Conidia shape	hyaline and threadlike	hyaline and threadlike
Conidia color	milky white	milky white to buff

<sup>z</sup>described by Wiese (1987)



**Fig. 8.** Phylogenetic tree using internal transcribed spacer (ITS) sequences showing the relationships among *Mycosphaerella graminicola* isolated from the ‘Niitaka’ pear and the closely related *Mycosphaerella* species.



**Fig. 9.** Phylogenetic tree using beta-tubulin gene sequences showing relationships among *Mycosphaerella graminicola* isolated from the ‘Niitaka’ pear and the closely related *Mycosphaerella* species.

## Discussion

Pear skin stains that appeared during the growing period as well as the post-harvest cold storage period on 'Niiitaka' pears have been a serious concern, as they result in poor marketability of the pears in South Korea. Although multiple studies reported that various fungi, including *Colletotrichum* spp. (Sadamatsu and Sanematsu, 1983), *Stenella* sp. (Nasu, 1998), *Alternaria* sp., *Hyalodendron* sp., *Phomopsis* sp. (Yasuda et al., 2005), *Meira geulakonigii*, *Pseudozyma aphidis* (Yasuda et al., 2007), *Cladosporium* spp., *Leptosphaerulina*, and *Tripospermum* (Park et al., 2008), are responsible for these symptoms, few studies have examined and exactly identified the pathogenic fungi responsible for the stains on 'Niiitaka' pears. Thus, we isolated the pathogenic fungus from the lesions of infected pear skin, which was apparent during the growing period and cold storage period. We then identified the morphological features of the fungus, and analyzed the nucleotide sequence of the beta-tubulin gene; the results showed that the main pathogenic fungus responsible for these symptoms was the ascomycete *M. graminicola*. In South Korea, several diseases are reportedly caused by *Mycosphaerella* spp., such as circular leaf spot in the persimmon tree by *M. nawae* (Kwon and Park, 2004) and gummy stem blight in the muskmelon by *M. melonis* (Cho et al., 1997). However, studies that examined the plant diseases caused by *M. graminicola* have not been reported. However, *M. graminicola* (in the asexual stage) reportedly is the ascomycete fungus responsible for *Septoria tritici* blotch (STB), primarily in winter wheat but also occasionally in rye, triticale, and some grass species. STB is one of the most important foliar diseases that cause significant damage to wheat worldwide (Brown et al., 2001; Linde et al., 2002; Siah et al., 2007; Suffert et al., 2013). This fungus is difficult to control because populations contain extremely high levels of genetic variability. Furthermore, the biology of this fungus is very unusual for a pathogen (Goodwin et al., 2011). *M. graminicola* is a fungus known to degrade plant cell walls (Torriani et al., 2008), which results in necrotic lesions in the leaves and stems of wheat; the progress of the disease is known to be exacerbated in cold and humid conditions (Ponomarenko et al., 2011). Regarding these disease conditions and features, Goodwin et al. (2011) presumed that the pathogenicity of *M. graminicola* might be related to protein degradation rather than carbohydrate degradation in order to avoid the host's defensive mechanisms when infected in the biotrophic stage. In previous studies (Park et al., 2008), it was reported on the occurrence of pear skin stain during storage on the 'Niiitaka' pears, but the study did not address

the identification of the pathogen. This is the first report on pear skin stain symptoms caused by *M. graminicola* on pears in Korea. Therefore, future investigations are warranted, especially with regard to the specificity of the fungal pathogenicity against pear skin and the underlying mechanisms of the inability of the mycelium to penetrate the sarcocarp.

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