

RESEARCH ARTICLE

Isolation and Identification of an Unreported Fungal Species in Korea and Novel Ice Nucleation Active Fungus: *Fusarium diversisporum*

Diane Avalos-Ruiz¹, Gwang-Jae Lim¹, Seong-Keun Lim¹, Leonid N. Ten¹, In-Kyu Kang², Seung-Yeol Lee^{1,3,*}, and Hee-Young Jung^{1,3}

¹School of Applied Biosciences, Kyungpook National University, Daegu 41566, Korea

²Department of Horticultural Science, Kyungpook National University, Daegu 41566, Korea

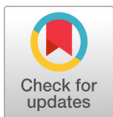
³Institute of Plant Medicine, Kyungpook National University, Daegu 41566, Korea

*Corresponding author: leesy1123@knu.ac.kr

ABSTRACT

In this study, the fungal strain KNUF-21-F39 was isolated from a declined apple tree (*Malus domestica*) in the Chungcheongbuk province in Korea. The strain KNUF-21-F39 presented a slow growth rate and a variety of macroconidia shapes and sizes ranging from ovoid to fusoid and 1- to 5-septate, primarily showing 3- and 4-septate, with “S”-shaped macroconidia rarely observed. The strain was identified based on morphological characteristics along with phylogenetic analysis performed using the internal transcribed spacer region (ITS) and partial sequences of translation elongation factor 1- α (*tef1*), RNA polymerase largest subunit (*rpb1*), and calmodulin (*cal*) genes. The fungal strain KNUF-21-F39 was identified as *Fusarium diversisporum*, which has not been previously reported in Korea. The ice nucleation activity (INA) of the strain was also evaluated, identifying the strain as positive for INA. This is the first report characterizing *F. diversisporum* as an IN-active fungal species.

Keywords: Apple tree, *Fusarium diversisporum*, Ice nucleation activity



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INTRODUCTION

Sordariomycetes is one of the largest classes of Ascomycota comprising 45 orders, 167 families, and 1,499 genera with a cosmopolitan distribution, and whose classification has changed considerably over the past decade [1-3]. The order Hypocreales, which belongs to the class Sordariomycetes, comprises 14 families, including Nectriaceae. This family includes endophytic, saprophytic, and pathogenic fungi observed in a wide range of regions [3]. The genus *Fusarium* of filamentous fungi is distributed worldwide and comprises at least 300 species [4,5]. First described by Link in 1809, *Fusarium* includes several important and well-known mycotoxigenic plant pathogens [4,6]. Additionally, this was the first fungal genus associated with ice nucleation activity (INA) [7]. INA is the ability to initiate water crystallization at temperatures above -38°C , the freezing point of water in its ultrapure state [8]. Ice-nucleating particles (INPs) can be derived from a variety of sources, such as desert dust, anthropogenic emissions, marine, and biological aerosols.

Biological INPs also include pollen, algae, lichens, and microorganisms like viruses, bacteria, and fungi [9]. In the case of *Fusarium*, INA has been reported above -12°C for several species, including *F. acuminatum*, *F. tricinctum*, and *F. avenaceum* [10]. *F. diversisporum* was first described in rotten potato tubers in the US [11] and has been found in wheat fields in Iran [12]. However, limited information is available regarding *F. diversisporum* and its characteristics. No study has been published examining *F. diversisporum* as a phytopathogenic fungus. Furthermore, this species has not been reported in Korea. This study aimed to morphologically and molecularly characterize an unreported *Fusarium* species in Korea, *F. diversisporum*, isolated from a declined apple tree and determine its INA.

MATERIALS AND METHODS

Sample collection and fungal isolation

The fungal isolate KNUF-21-F39 was obtained from the branches of a declined apple tree (*Malus domestica*) collected from Chungcheongbuk province ($36^{\circ}33'50.0''\text{N}$, $127^{\circ}46'35.0''\text{E}$), Korea. The strain was isolated and cultured on potato dextrose agar (PDA; Difco, Detroit, MI, USA) at 25°C . The strain was deposited into the general collection of the Korean Collection for Type Cultures (KCTC) (KCTC 56905).

Morphological characterization

The morphological characteristics of the designated strain KNUF-21-F39 were studied by culturing the strain on PDA and oatmeal agar (OA). The cultural characteristics of the strain and the colony color, texture, growth, shape, and size were examined after ten days of incubation at 25°C . The microscopic characteristics were observed using a light microscope (BX-50; Olympus, Tokyo, Japan) after 60 days.

Genomic DNA extraction, PCR amplification, and sequencing

Total genomic DNA of the fungal strain was extracted using the HiGene Genomic DNA Prep Kit (BIOFACT, Daejeon, Korea) following the manufacturer's instructions. The strain was identified using ITS1/ITS4 primers to amplify the internal transcribed spacer region of nrDNA (ITS) [13]. For the remaining partial gene sequences, EF1/EF2 were used as primers for the translation elongation factor 1- α (*tef1*) [14], RPB1-F5/RPB1-R8 and RPB1-F7/RPB1-G2r for the RNA polymerase largest subunit (*rpb1*) [15], and CAL-228f/CAL-2Rd for calmodulin (*cal*) [16,17]. The amplified PCR products were purified and sequenced (Bioneer Co., Daejeon, Korea), and the NCBI Basic Local Alignment Search Tool (BLAST) was used to search for sequence similarity percentages.

Phylogenetic analysis

The obtained sequences of ITS, *tef1*, *rpb1*, and *cal* were aligned with highly similar sequences identified using the BLAST search tool, and edited using BioEdit v.7.0.5.3 [18]. A phylogenetic tree was constructed

based on the neighbor-joining method [19], as described by the Kimura model [20] using MEGA version X [21] with bootstrap values based on 1,000 replications.

Ice nucleation activity evaluation

The strain KNUF-21-F39 was grown on PDA and potato dextrose broth at 25°C for seven days. Solid media-grown mycelium was removed from the PDA plates, suspended in 500 µL of sterile double-distilled water, and vigorously homogenized. Liquid cultures were filtered using an AVANTEC® 0.45 µm disposable membrane filter. A droplet freezing assay was conducted at -8°C based on previous studies [7,22] as described by Avalos-Ruiz et al. (2022) [23]. Frozen droplets were observed visually and the strain was considered IN-active if 4/5 or 5/5 droplets froze within one minute. Double-distilled water was used as a negative control, and *Pseudomonas syringae* pv. *syringae* strain KACC 21200 was used as the positive control. INA was confirmed using a tube-freezing assay; the strain was considered positive if freezing was observed after two minutes [23].

RESULTS AND DISCUSSION

Fusarium diversisporum Sherb., Memoirs of the Cornell University Agricultural Experimental Station 6:161 (1915) [MB#194315] (Fig. 1)

Specimen examined: Boeun-gun, Chungcheongbuk-do (36°33'50.0"N, 127°46'35.0"E) isolated from a declined apple tree (*Malus domestica*) branch.

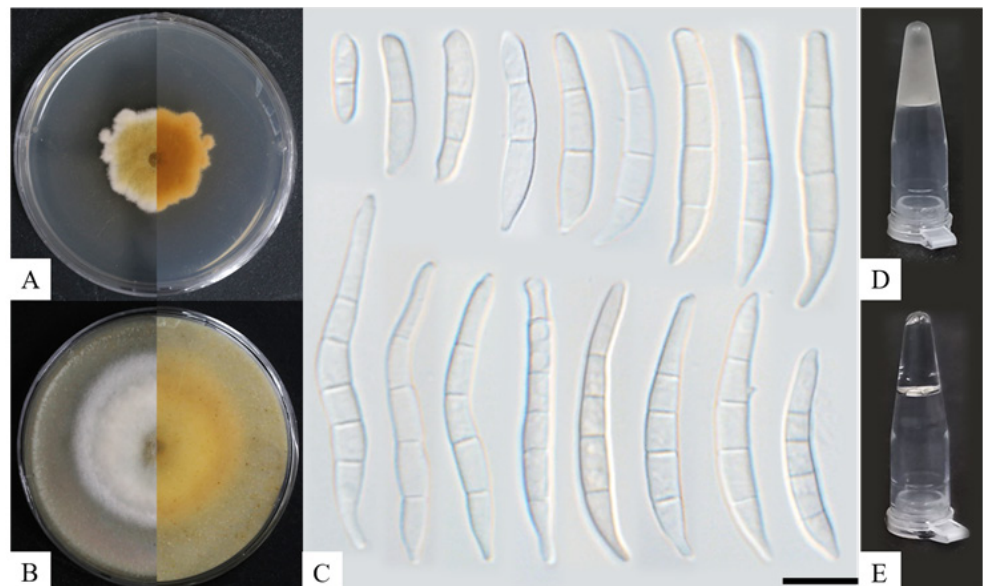


Fig. 1. Morphological characteristics and ice nucleation activity of *Fusarium diversisporum* KNUF-21-F39. A, Colony formation on potato dextrose agar (PDA) after ten days. B, Colony formation on oatmeal agar after ten days. C, Macroconidia observed after 60 days of growth in PDA. D, Ice nucleation activity of the strain observed using the tube-freezing assay. E, Ice nucleation negative control (without freezing). Scale bars: 10 µm.

Morphological characteristics of KNUF-21-F39

The strain KNUF-21-F39 showed a slow growth rate, reaching colonies with diameters ranging from 30–35 mm after ten days of incubation on PDA at 25°C, and 52–62 mm after ten days on OA. The colonies on PDA were irregularly shaped and dark-yellow to orange in color, with white margins and whiteish aerial mycelium that gave the colonies a fluffy appearance (Fig. 1A). Colonies on OA had a white fluffy appearance, with a ring-like outer border (Fig. 1B). The morphology of the strain was compared to previous descriptions [24]. The results indicated that the strain KNUF-21-F39 had a slower growth rate; however, the colony characteristics on PDA were consistent with those observed in previous studies. Macroconidia presented several shapes and sizes, from ovoid to fusoid, 1- to 5-septate, with “S”-shaped macroconidia rarely observed. Macroconidia primarily presented 3- to 4-septate morphology (Fig. 1C). Sizes were 11.9–48.4×2.8–8.2 (22.3±15.3×4.4±2.2), n=5 (1-septate, 6.3%), 18.4–30.9×2.8–4.6 (24.6±3.4×3.6±0.6), n=14 (2-septate, 17.5%), 21.0–38.8×2.9–4.7 (30.2±4.1×3.7±0.5), n=32 (3-septate 40.0%), 29.9–41.4×3.2–4.6 (35.4±3.4×4.0±0.4), n=26 (4-septate, 32.5%), and 38.2–48.8×3.2–4.3 (33.8±17.7×3.2±1.6), n=5 (5-septate, 3.7%) (Table 1). No chlamydo spores or microconidia were observed. The culture and morphological characteristics of the isolated strain KNUF-21-F39 suggested that the fungus was closely related to *F. diversisporum*.

Table 1. Morphological characteristics of KNUF-21-F39 and previously reported *Fusarium diversisporum*.

Characteristics		<i>Fusarium diversisporum</i> KNUF-21-F39 ^a	<i>Fusarium diversisporum</i> ^b
Colony	Size (diam.)	3.0–3.5 cm in 10 days at 25°C on PDA, 5.2–6.2 cm in OA.	4.8–6.5 cm in 10 days at 25°C on PDA.
	Color	Dark yellow-orange in the center with white borders	Yellowish pink, clay color, dark yellow-brown
	Shape	Irregular	N/A
	Texture	Fluffy and whiteish aerial mycelium	Abundant aerial mycelium, fluffy to cottony, whiteish
Macroconidia	Shape and septate	Various shapes and sizes; ovoid to fusoid, rarely “S” shaped, from 1-septate to 5-septate, mostly 3- and 4-septate (1-septate: 6.3%, 2-septate: 17.5%, 3-septate: 40.0%, 4-septate: 32.5%, 5-septate: 3.7%).	Primary conidiophores: ovoid to fusoid, slightly falcate, 0- to 3-septate but occasionally up to 9-septate, relatively wide. Of secondary conidiophores: falcate, slightly bent, slender, with an elongated and curved apical cell and a pedicellate basal cell, mostly 3- to 5-septate.
	Size (µm)	1-septate 11.9–48.4×2.8–8.2 (22.3±15.3×4.4±2.2), n=5 2-septate 18.4–30.9×2.8–4.6 (24.6±3.4×3.6±0.6), n=14 3-septate 21.0–38.8×2.9–4.7 (30.2±4.1×3.7±0.5), n=32 4-septate 29.9–41.4×3.2–4.6 (35.4±3.4×4.0±0.4), n=26 5-septate 38.2–48.8×3.2–4.3 (33.8±17.7×3.2±1.6), n=5	3-septate f. 28×4.3 (19–37×4.0–5.3) 5-septate f. 48×4.2 (37–61×4.1–5.5) 7-9-septate f. (60–100×4.2–5.5) 3-septate 36×3.2 (20–48×2.4–3.5) 5-septate 48×3.6 (35–70×2.7–4.5)

^a Fungal strain of this study, ^b Source of description [24].

diam.: diameter; PDA: potato dextrose agar; OA: oatmeal agar; N/A: not available in reference.

Molecular phylogeny of KNUF-21-F39

Sequences containing 566, 594, 1510, and 588 bp were obtained that corresponded to the ITS region and partial sequences of *tefl*, *rpb1*, and *cal* genes, respectively. All obtained gene sequences were registered in the National Center for Biotechnology Information (NCBI) with the following GenBank accession numbers (ITS: OP113837, *tefl*: OP186041, *rpb1*: OP186042, *cal*: OP186043). BLAST results showed, for the ITS region, a 99.82% similarity with several strains of *F. acuminatum*, such as N-51-1 (MT566456),

Scobey-MT (MK729582), and JAC13614 (MK432763); and *F. tricinctum* strains DSM100325_DISC_RLCS08 (MT453281) and QB23 (MT123097). A 99.46% of similarity was observed with *F. diversisporum* CBS 795.70 (=BBA 11129, MH859946). For the *tefl* partial sequence, 100.0% similarity was observed with several *F. avenaceum* strains, such as Fv5-9 (MK111429), G17XY4-45 (MG670378) and JA-0925 (KP170732); 99.83% with *F. tricinctum* RTN17 (LC500690), and *F. diversisporum* BBA 11129 (MZ921930). For the *rpb1* gene, 100.0% of similarity was observed with *F. diversisporum* BBA 11129 and 99.9% or less with several strains of *F. avenaceum* like LC6387 (MW024699) and LC6044 (MW024695).

Examination of the similarity of ITS, *tefl*, and *rpb1* indicated that the isolate KNUF-21-F39 belonged to the genus *Fusarium* and, more specifically, to the *F. tricinctum* species complex. However, it was not possible to precisely identify the strain at the species level. For the *cal* gene, 100.0% similarity with *F. diversisporum* BBA 11129 (MZ921621) and 98.85% similarity with *F. avenaceum* ITEM7630 (LR215911) and ITEM13593 (LR215892) was observed, allowing the differentiation between *F. avenaceum* and *F. diversisporum* that was not attained before. The phylogenetic tree based on *cal* partial sequences showed that the strain KNUF-21-F39 clustered together with *F. diversisporum* BBA 11129 (Fig. 2). Therefore, the strain KNUF-21-F39 was identified as *F. diversisporum*.

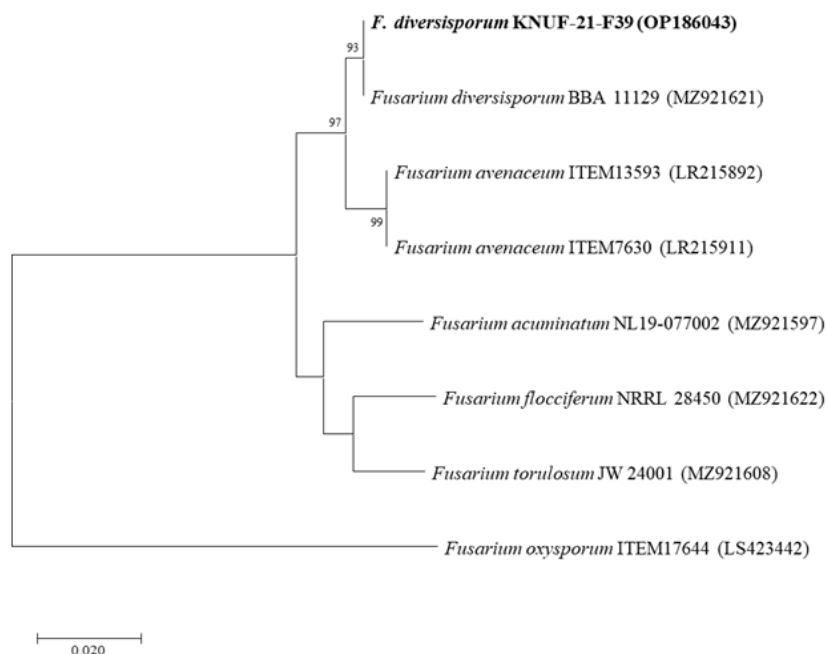


Fig. 2. Neighbor-joining phylogenetic tree based on *cal* partial sequences showing the position of *Fusarium diversisporum* KNUF-21-F39. *F. oxysporum* ITEM17644 was used as an out-group. The strain isolated in this study is marked in bold. Bootstrap values are based on 1,000 replications, and values below 70 are not shown. Bar, 0.02 substitutions per nucleotide position. Sequence accession numbers are presented in parentheses.

Ice nucleation activity of KNUF-21-F39

The strain showed high INA (5/5 frozen droplets) in both mycelial and mycelia-free suspensions (data not shown). INA was confirmed for KNUF-21-F39 using a tube-freezing assay, yielding a positive result for INA (Fig. 1D) compared to that observed with the negative control (Fig. 1E).

In 1992, two *Fusarium* species were reported to be IN-active: *F. acuminatum* and *F. avenaceum* [7]. Since then, *F. oxysporum*, *F. tricinctum* [25], *F. armeniacum*, *F. begoniae*, *F. concentricum*, and *F. langsethiae* [10] have been reported as IN-active species. However, information regarding the distribution and mechanisms of INA across *Fusarium* is lacking. In Korea, INA has been reported for microorganisms such as *Pseudomonas syringae* pv. *syringae* [26,27] and *F. tricinctum* [23]. In this study, the strain KNUF-21-F39 was collected from a declined apple tree (*M. domestica*) in Chungcheongbuk Province. The strain presented morphological and molecular characteristics similar to those previously reported for *F. diversisporum*. In addition, the strain presented the ability to act as an ice nucleator at -8°C .

Authors have suggested that the production of INA metabolites by some species of the genus *Fusarium* could be used for taxonomic classification [7]. Moreover, the production of INA metabolites can play an important environmental role and may be associated with the virulence and survival of the species [10]. Previous reports have stated that the INA of *P. syringae* can cause frost injury in sensitive plants [22]. Similarly, enhanced infection of plant roots by fusaria has been observed under freezing conditions [25], highlighting the importance of examining the INA of fungal species and strains along with their potential pathogenicity factors.

To our knowledge, this is the first report of *F. diversisporum* in Korea, and the first report of *F. diversisporum* as an IN-active fungus. With this study, we hope to expand the knowledge about fungal strains in Korea, as well as IN-fungal strains and their potential ecological roles.

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