RESEARCH ARTICLE

Acrodontium burrowsianum and *Pestalotiopsis humicola*: Two Previously Unrecorded Fungal Species Isolated from Conifer Leaves in Korea

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ABSTRACT

Endophytic fungal strains were isolated from the leaves of two conifer species (*Juniperus rigida* and *Pinus densiflora*) in Korea and identified on the basis of their morphological and molecular characteristics. Internal transcribed spacer and large subunit regions of rDNA were used for the phylogenetic analysis, and the translation elongation factor 1-alpha (TEF) and RNA polymerase II second largest subunit (RPB2) genes were analyzed depending on the species. Two fungal species that were previously unrecorded in Korea were identified: *Acrodontium burrowsianum* and *Pestalotiopsis humicola*. Their morphological and phylogenetic characteristics are described herein.

Keywords: *Acrodontium burrowsianum*, Conifer, Endophytic Fungi, *Pestalotiopsis humicola*

OPEN ACCESS

pISSN : 0253-651X
eISSN : 2383-5249

Kor. J. Mycol. 2022 December, 50(4): 311-318
https://doi.org/10.4489/KJM.20220033

Received: November 09, 2022
Revised: December 15, 2022
Accepted: December 15, 2022

INTRODUCTION

Endophytic fungi are organisms that have a symbiotic relationship with plants and do not cause disease symptoms in their host when balance is maintained [1]. They are found in all plant tissues, such as roots, stems, and leaves, and are transmitted vertically or horizontally [2]. Endophytic fungi provide benefits to their host plants, such as tolerance to water stress [3], increased photosynthetic efficiency [4], and resistance to pathogens [5].

The diversity of endophytic fungi in symbiosis with conifers is affected by the host plant species and the geographic region [6]. In previous studies, endophytic fungi including the genus *Lophodermium* were found to be common endophytes in conifer species [6-8], and their close relationship with this host plant have been reported [9,10]. In this study, endophytes were isolated from the leaves of two conifer species (*Pinus densiflora* and *Juniperus rigida*) during a survey of the diversity of endophytic fungi in Korea. We identified two fungal species that were previously unrecorded in Korea and carried out their morphological and molecular characterization in the present study.

MATERIALS AND METHODS

Sampling, isolation and morphological characterization

Leaves of *Pinus densiflora* and *Juniperus rigida* were collected from Mt. Baegasan, Hwasungun, Jeollanamdo (35°41′52.0″N, 129°21′04.2″E) and Gyeongjusi, Gyeongsangbukdo (35°9′55.93″N, 127°10′18.38″E) in Korea, respectively. The samples were transported to the laboratory within 24 hours for the isolation of endophytic fungi. Randomly selected asymptomatic leaves were washed under running tap water and then surface sterilized through sequential treatment with 30% H_2O_2 and 70% EtOH for 1 minute each. After washing with sterile water, any remaining liquid on the leaves was removed using sterilized filter paper. After surface sterilization, the leaves were cut to a length 1.5 cm using surface-sterilized scissors and then placed on potato dextrose agar (PDA; Difco Lab., Detroit, USA) and malt extract agar (MEA; Kisan bio, Seoul, Korea) medium. Once the hypha had extended, the endophytic fungi were separated by subculturing on PDA medium. The pure isolated strain was inoculated onto PDA and MEA media and incubated for 7 days at 25°C in the dark. Conidia formation was induced using the slide culture method, and the hyphae and conidia were observed with an optical microscope.

Molecular characterization

For molecular identification of the species, genomic DNA was extracted using the HiGene™ Genomic DNA Prep Kit (BIOFACT, Daejeon, Korea). The internal transcribed spacer (ITS) and large subunit (LSU) regions of rDNA were amplified using the ITS1F/ITS4 [11] and LROR/LR16 primers [12], respectively. For further sequencing analysis, RNA polymerase II second largest subunit (RPB2) genes in *Acrodontium burrowsianum* were amplified using fRPB2-5F/fRPB2-7cR primers [13], whereas the translation elongation factor 1-alpha (TEF) gene in *Pestalotiopsis humicola* was amplified using the EF1-668F/EF1-1251R primers [14]. The PCR products were electrophoresed on a 1.5% agarose gel for 20 minutes. After confirmation of the bands, nucleotide sequence analysis of the genes was conducted by SolGent Co. (Daejeon, Korea). The species similarity of the nucleotide sequences was confirmed using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information, and phylogenetic trees were prepared using the maximum-likelihood method with MEGA11 [15]. The obtained sequences were submitted to the NCBI GenBank database (Table 1).

Table 1. Strains and GenBank accession numbers used for phylogenetic analysis in this study.

Species	Strain	ITS	LSU	RPB2	TEF
Acrodontium burrowsianum	KNUE21E086	OP698004	OP698007	OQ130424	-
Acrodontium burrowsianum	CBS 147002	MZ064425	MZ064482	MZ078199	-
Acrodontium crateriforme	CBS 144.33	NR_152320	NG_057108	KX288399	-
Acrodontium crateriforme	CBS 985.70	KX287267	KX286954	KX288401	-
Acrodontium luzulae	CBS 839.71	NR_154720	NG_057110	KX288411	-
Acrodontium pigmentosum	CBS 111111	NR_154721	NG_057111	KX288412	-
Acrodontium fagicola	CBS 714.79	NG_057109	KX286960	KX288409	-
Ramularia gaultheriae	CBS 299.80	NR_172252	NG_058251	KX288569	-
Pestalotiopsis humicola	KNUE20E054	ON430569	ON430579	-	OP799546
Pestalotiopsis humicola	CBS 115450	KM199319	KM116208	-	KM199487
Pestalotiopsis humicola	CBS 336.97	KM199317	KM116230	-	KM199484
Pestalotiopsis diploclisiae	CBS 115587	NR_147552	NG_069220	-	KM199486
Pestalotiopsis malayana	CBS 102220	NR_147550	NG_069217	-	KM199482
Pestalotiopsis aggestorum	LC6301	KX895015	KX895129	-	KX895234
Pestalotiopsis colombiensis	CBS 118553	NR_147551	NG_069213	-	KM199488
Pestalotiopsis jinchanghensis	LC6636	KX895028	KX895135	-	KX895247
Pestalotiopsis scoparia	CBS 176.25	NR_145238	NG_069210	-	KM199478
Neopestalotiopsis mesopotamica	CBS 336.86	NR_145244	NG_069225	-	KM199555

ITS: internal transcribed spacer; LSU: large subunit; RPB2: RNA polymerase II second largest subunit; TEF: translation elongation factor 1-alpha.

RESULTS AND DISCUSSION

Acrodontium burrowsianum Crous, Persoonia 46: 365 (2021) [MB#839513] (Fig. 1)

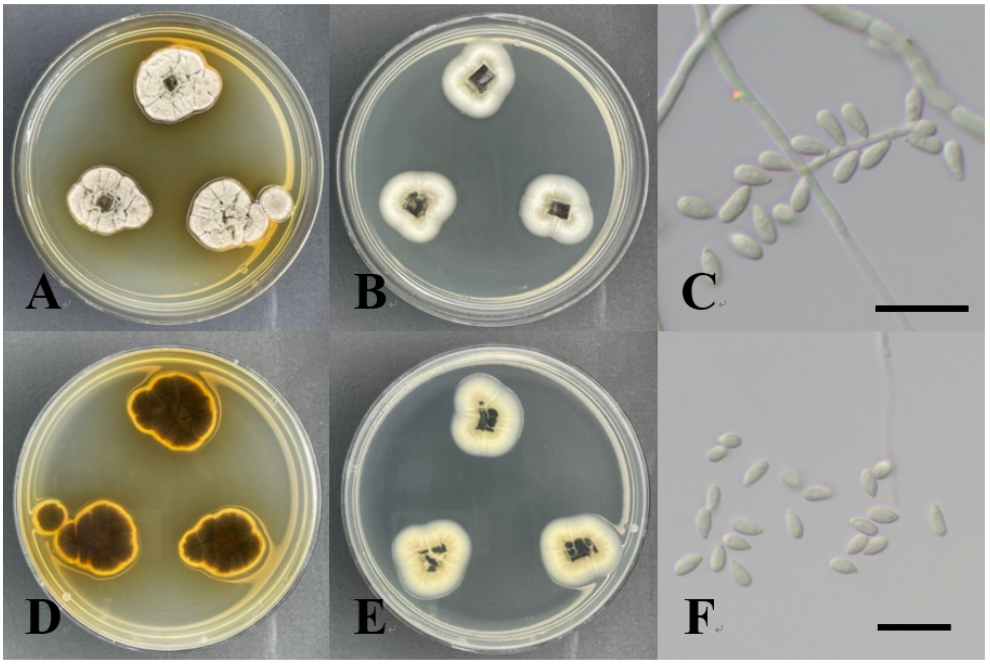

Fig. 1. Morphological characteristics of *Acrodontium burrowsianum* KNUE21E086. A, D: Grown for 7 days on potato dextrose agar (PDA) medium. B, E: Grown for 7 days on malt extract agar (MEA) medium. C: Conidia, conidiogenous cells and conidiophore. F: Conidia. Scale bars=10 μm.

Morphological characteristics of strain KNUE21E086

Acrodontium burrowsianum was isolated from the leaves of *Juniperus rigida*. Colonies cultured for 7 days on PDA medium were 25-30 mm in diameter. The color of the front side of the colony was light brown at the center and changed gradually to a lighter smoke gray at the edge. The reverse side of the colony was dark brown in the center and yellow at the edge. The colony was higher in the central part, with hyphae extending radially, and the margin was irregular. Colonies cultured for 7 days on MEA medium were 20-25 mm in diameter. The front of the colony was smoke gray in color, whereas the reverse side was isabelline or cream colored. The colony was relatively flat in shape and attached to the medium, and the margin was irregular. The conidia, which were smooth and hyaline ellipsoids with an obtuse apex, were attached to the sides of the mycelium. The dimensions of the conidia were (2.64-) 3.79 (-4.65) × (1.44-) 1.92 (-2.25) μm.

Specimen examined: Gyeongjusi, Korea; 35°41′52.0″N, 129°21′04.2″E; May 26, 2021; isolated from the leaves of *Juniperus rigida*; National Institute of Biological Resources (NIBR) No. NIBRFGC000509082; GenBank No. OP698004 (ITS), OP698007 (LSU), and OQ130424 (RPB2)

Molecular characteristics of strain KNUE21E086

The BLAST results showed that the ITS sequence had 99.08% similarity with that of *Acrodontium burrowsianum* MZ064425, whereas the LSU sequence showed 99.83% similarity with that of *Acrodontium burrowsianum* MZ064482. The RPB2 sequence showed 98.97% similarity with the sequence from *Acrodontium burrowsianum* MZ078199. Maximum-likelihood phylogenetic analysis was performed by combining the ITS, LSU, and RPB2 sequences (Fig. 2). On the basis of its molecular and morphological characteristics, strain KNUE21E086 was identified as *Acrodontium burrowsianum.*

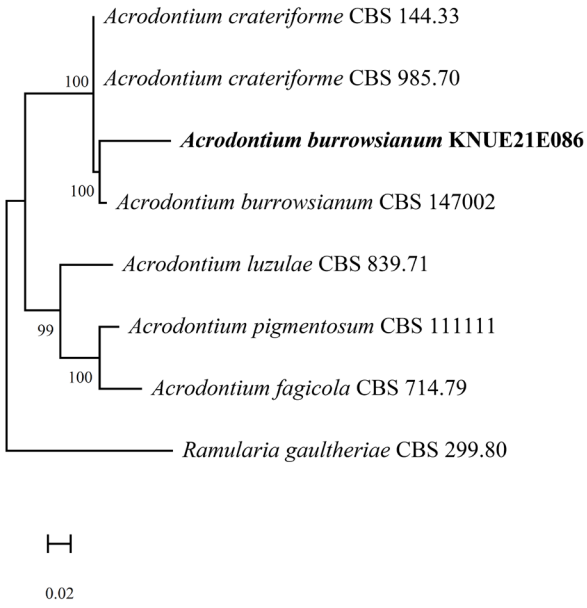

Fig. 2. Maximum likelihood phylogenetic analysis of combined internal transcribed spacer (ITS), large subunit regions (LSU) and RNA polymerase II second largest subunit (RPB2) sequences of *Acrodontium burrowsianum* KNUE21E086. *Ramularia gaultheriae* was used as an outgroup. Numbers on branches indicate bootstrap values (1,000 replicates). Fungal strain isolated in this study is in a bold.

Pestalotiopsis humicola Maharachch., K.D. Hyde & Crous, Studies in Mycology 79: 165 (2014) [MB#821661] (Fig. 3)

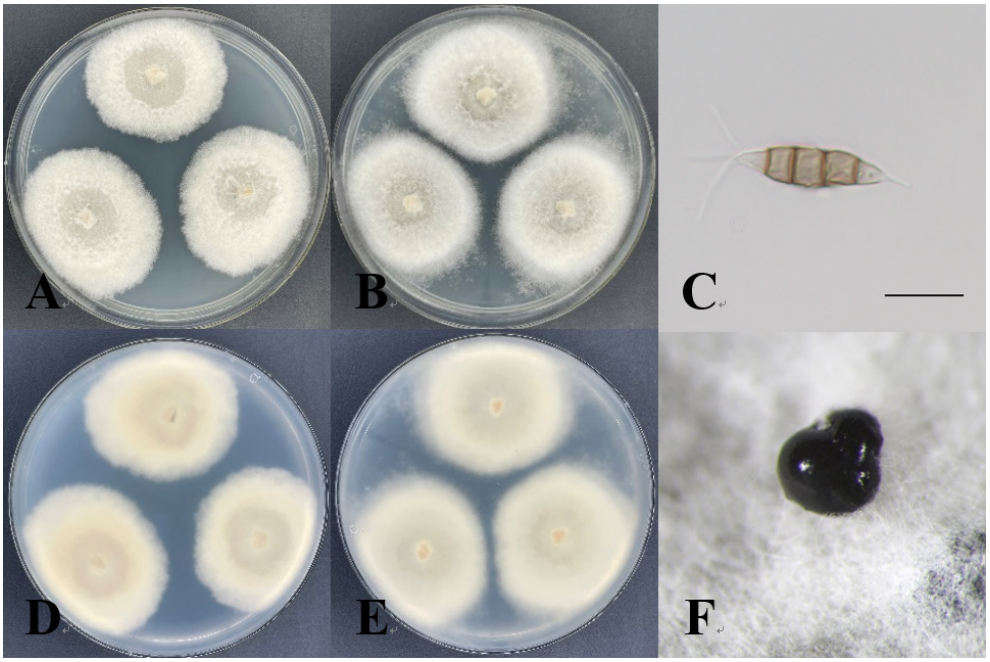

Fig. 3. Morphological characteristics of *Pestalotiopsis humicola* KNUE20E054. A, D: Grown for 7 days on potato dextrose agar (PDA) medium. B, E: Grown for 7 days on malt extract agar (MEA) medium. C: Conidia (scale bar=10 μm). F: Conidiomata sporulating on PDA.

Morphological characteristics of strain KNUE20E054

Colonies on PDA and MEA media were 42-44 and 40-42 mm in diameter, respectively, and had regular margins. White aerial mycelia were observed. The colony was bright ivory in color from the front side and ivory from the reverse side. When cultured on PDA medium, black pycnidial conidiomata were observed. The conidia were brown in color, with a size of (15.59-) 17.53 (-20.76)×(4.81-) 5.03 (-5.53) μm. The 5-cell-shaped conidia had septa and 2-3 tubular apical appendages.

Specimen examined: Mt. Baegasan, Hwasungun, Jeollanamdo, Korea; 35°9′55.93″N, 127°10′18.38″E; May 16, 2020; isolated from the leaves of *Pinus densiflora*; NIBR No. NIBRFGC000508505; GenBank No. ON430569 (ITS), ON430579 (LSU), and OP799546 (TEF).

Molecular characteristics of strain KNUE20E054

As determined by BLAST analysis, the ITS sequence of strain KNUE20E054 had 99.66% similarity with that of *Pestalotiopsis humicola* KM199319, and its LSU sequence showed a similarity of 99.68% with that of *Pestalotiopsis humicola* KM116230. The TEF sequence had 96.23% similarity with that of *Pestalotiopsis humicola* KM199484. Maximum-likelihood phylogenetic analysis was performed by combining the ITS, LSU, and TEF sequences (Fig. 4). On the basis of its molecular and morphological characteristics, strain KNUE20E054 was identified as *Pestalotiopsis humicola*.

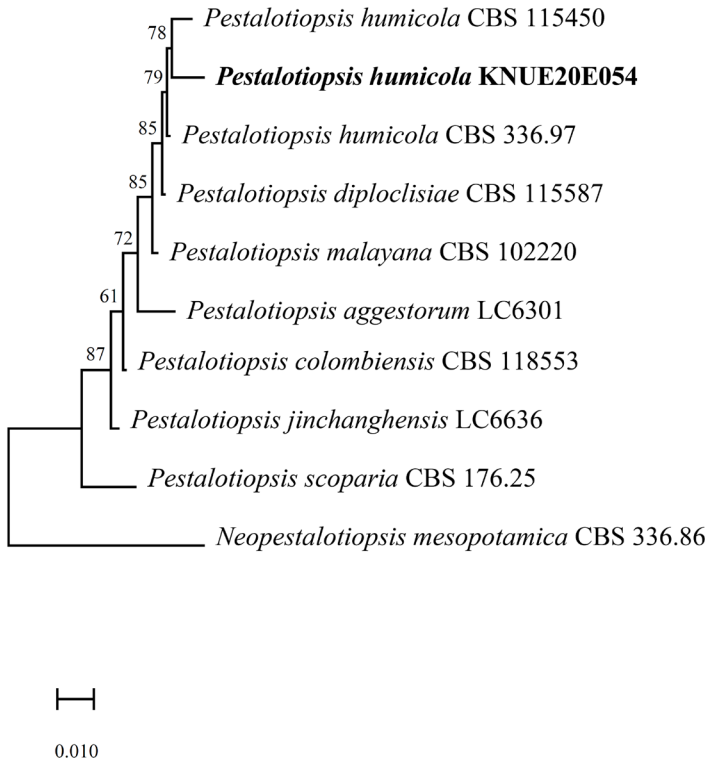

Fig.4. Maximum likelihood phylogenetic analysis of combined internal transcribed spacer (ITS), large subunit regions (LSU) and translation elongation factor 1-alpha (TEF) sequences of *Pestalotiopsis humicola* KNUE20E054. *Neopestalotiopsis mesopotamica* was used as an outgroup. Numbers on branches indicate bootstrap values (1,000 replicates). Fungal strain isolated in this study is in a bold.

Acrodontium burrowsianum was first isolated from the leaves of an unidentified Poaceae plant in the Republic of South Africa by Crous et al. [16] in 2021 (Table 2). Although closely related to *Acrodontium crateriforme*, it is known that *Acrodontium burrowsianum* has smaller conidia [16]. The conidia of the strain KNUE21E086 reported in this study are smaller than those of *Acrodontium crateriforme* and similar to the ones of *Acrodontium burrowsianum*. *Pestalotiopsis humicola* was first isolated from soil by Maharachch et al. in 2014 [17], and the symbiotic relationship of the fungus with plants was confirmed through that study. Although the conidial morphologies of the genera *Truncatella* and *Pestalotia* are similar to that of *Pestalotiopsis*, these three genera can be distinguished by their differences in the number of cells separated by a septum; that is, *Truncatella* has 4-cell, *Pestalotiopsis* has 5-cell, and *Pestalotia* has 6-cell conidia (Table 3). To the best of our knowledge, this is the first record of the species *Acrodontium burrowsianum* KNUE21E086 and *Pestalotiopsis humicola* KNUE20E054 in Korea.

Table 2. Morphological characteristis of *Acrodontium burrowsianum* KNUE21E086 isolated from *Juniperus rigida*.

Characteristics	*Acrodontium burrowsianum*	
	21E086	Crous [16]
Colony		
Culture condition	PDA, MEA, 25°C, 7 days	PDA, MEA, OA, 25°C, 2 week
Color	PDA surface light brown to smoke gray; reverse dark brown with yellow margin MEA surface smoke gray; Reverse isabelline margin	PDA and MEA surface smoke gray; reverse isabelline OA surface smoke gray with diffuse hazel pigment
Size	PDA 25-30 mm in diam MEA 20-25 mm in diam	reaching up to 30 mm diam
Shape	erumpent, irregular margin	erumpent, spreading, surface folded, with moderate aerial mycelium and smooth, lobate margin
Conidia		
Color	hyaline	hyaline
Size	(2.64-) 3.79 (-4.65)×(1.44-) 1.92 (-2.25) µm	3-4×1.5-2 µm
Shape	smooth, apex obtuse, ellipsoid	thin-walled, smooth, solitary, aseptate, ellipsoid, apex obtuse

PDA: potato dextrose agar; MEA: malt extract agar; OA: oatmeal agar.

Table 3. Morphological characteristis of *Pestalotiopsis humicola* KNUE20E054 isolated from *Juniperus rigida*.

Characteristics	*Pestalotiopsis humicola*	
	20E054	Maharachchikumbura [17]
Colony		
Culture condition	PDA, 25°C, 7day	PDA, 25°C, 7day
Color	front white, reverse ivory	Pale honey-coloured
Size	diameter of 42-44 mm	45-50 mm diam
Shape	regular margin, smooth edge, aerial mycelium	smooth edge, with sparse aerial mycelium on the surface with black, gregarious
Conidia		
Color	brown, septa darker than the rest of the cell, basal cell hyaline	brown, septa darker than the rest of the cell, basal cell hyaline
Size	(15.59-) 17.53 (-20.76)×(4.81-) 5.03 (-5.53) µm	(17-) 18.5-22 (-23)×5-7 (-7.5) µm, x±SD=20±1.4×6±0.4 µm
Shape	4-septate, ellipsoid, straight	fusoid, ellipsoid, straight to slightly curved, 4-septate, constricted at septum

PDA: potato dextrose agar; SD: standard deviation.

CONFLICT OF INTERESTS

No conflict of interest was reported by the author(s).

ACKNOWLEDGEMENTS

This work was supported by the Project on Survey and Discovery of Indigenous Fungal Species of Korea, funded by the NIBR.

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