

## RESEARCH ARTICLE

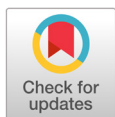
# First Report of *Xenorousoella triseptata* Isolated from Soil in Korea

Jung-Joo Ryu<sup>1</sup>, Seung-Yeol Lee<sup>1,2</sup>, In-Kyu Kang<sup>3</sup>, Leonid N. Ten<sup>1</sup>, and Hee-Young Jung<sup>1,2,\*</sup><sup>1</sup>School of Applied Life Science, Kyungpook National University, Daegu 41566, Korea<sup>2</sup>Institute of Plant Medicine, Kyungpook National University, Daegu 41566, Korea<sup>3</sup>Department of Horticultural Science, Kyungpook National University, Daegu 41566, Korea

\*Corresponding author: heeyoung@knu.ac.kr

## ABSTRACT

A fungal strain, designated KNUF-20-NI009, was isolated from soil collected from Gunsan-si, Jeollabuk-do, Korea. The isolate showed cultural features typical of the genus *Xenorousoella*. Colonies cultivated on malt extract agar were olivaceous-brown to pale olivaceous-white at the margins, with undersides of dark olivaceous to olivaceous-brown and a white margin. The conidia, with a size range of 2.7-5.1×1.6-3.3 μm ( $\bar{x}$ =3.6×2.6 μm, n=50), were globoid to ellipsoid in shape, hyaline when immature, becoming light brown to golden-brown when mature, and characterized by 1 or 2 guttules. Multi-locus sequence analysis based on a combined dataset of internal transcribed spacer regions (ITS), large subunit rDNA (LSU), small subunit rDNA (SSU), translation elongation factor 1- $\alpha$  (TEF1 $\alpha$ ), and RNA polymerase II largest subunit (RPB2) sequences revealed KNUF-20-NI009 to be a strain of *Xenorousoella triseptata*. This is the first report of this species in Korea.

**Keywords:** Morphological characteristic, Phylogenetic analysis, *Xenorousoella*

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## INTRODUCTION

As initially described, the fungal family Roussoellaceae Liu et al. comprised the three genera *Neorousoella* Liu et al., *Roussoella* Sacc., and *Roussoellopsis* Hino and Katum [1]. Subsequently, based on the multigene analysis of limited taxa, Jaklitsch and Voglmayr [2] synonymized Roussoellaceae under Thyridariaceae. However, the taxonomic status of Roussoellaceae was later re-established by Tibpromma et al. [3], who considered Roussoellaceae and Thyridariaceae to be separate families within the Pleosporales. More recent studies employing additional taxa and combining morphological and phylogenetic analyses have validated this familial distinction [4-8]. Currently, the family Roussoellaceae comprises the following 12 genera: *Appendispora* Hyde; *Cytoplea* Bizz. and Sacc.; *Elongatopedicellata* Zhang et al.; *Immotthia* Barr; *Neorousoella*, *Pararousoella*, and *Pseudoneoconiothyrium* Wanas et al.; *Pseudorousoella* Mapook and Hyde; *Roussoella* Sacc.; *Roussoellopsis* Hino and Katum.; *Setoarthopyrenia* Mapook and Hyde; and *Xenorousoella* Mapook and Hyde [9]. Among these, the genus *Xenorousoella* was initially proposed by Mapook et al. in 2020 [7]. Phylogenetic analysis based on a combined dataset of internal transcribed

spacer (ITS) regions, small subunit rDNA (SSU), large subunit rDNA (LSU), translation elongation factor 1- $\alpha$  (TEF1 $\alpha$ ), and RNA polymerase II second largest subunit (RPB2) genes revealed that *Xenorousoella* forms a separate branch and groups with *Pseudorousoella* species. The genus differs, however, from *Pseudorousoella* in having smaller ascospores and asci, a thin peridium, and larger ellipsoid to obovoid biserial ascospores that are 3-septate with irregular longitudinal striations and lacking a gelatinous sheath. In contrast, *Pseudorousoella* produces oval to ellipsoid uniseriate ascospores that are 1-septate, have reticulate spore wall ornamentation, and are encased in a hyaline gelatinous sheath. The name *Xenorousoella* is derived from the Greek terms *Xeno*, meaning separate, and *Rousoella*, meaning roussoella-like [7].

Since the original description of *Xenorousoella* in 2020 [7], there has been relatively little research conducted on this genus. In Korea, among species in the family Roussoellaceae, *Rousoella doimaesalongensis* has recently been isolated and reported [10]. Given the unknown virulence of *Xenorousoella* species, it is important to determine the suitable growth media, optimal pH, and temperature for these fungi. In this study, we describe a fungal strain isolated from soil collected from Gunsan-si, Jeollabuk-do, Korea, based on its cultural characteristics on three different media, asexual morphs, and the effect of temperature and pH on its mycelial growth. Finally, on basis of phylogenetic analysis, we identify the fungus as a strain of *Xenorousoella triseptata* (designated KNUF-20-NI009), which is reported in Korea for the first time.

## MATERIALS AND METHODS

### Collection of soil samples

The fungal strain KNUF-20-NI009 was isolated from soil collected from Gunsan-si, Jeollabuk-do (35° 49'02.2"N 126°23'36.4"E), Korea. Soil samples were extracted from the soil at a depth of approximately 15-30 cm. Before analysis, the samples were air-dried and then stored in a plastic bag at 4°C. Fungi were isolated using the serial dilution technique. Soil suspensions were prepared by the addition of 1 g of the soil sample to 9 mL of sterile distilled water, followed by vortexing and serial dilution to give dilutions of 10<sup>-3</sup>, 10<sup>-4</sup>, 10<sup>-5</sup>, and 10<sup>-6</sup>. Aliquots (1 mL) of each dilution were spread on potato dextrose agar (PDA; Difco, Detroit, MI, USA), followed by incubation for 2 to 3 days at 25°C [11]. Individual colonies developing on the agar were subcultured on fresh PDA plates and incubated at 25°C until the development of mycelium. Based on different cultural characteristics, isolated fungal strains were selected for further molecular analyses. In particular, an isolate designated KNUF-20-NI009 was maintained in 20% glycerol at -80°C for further studies. This strain has been deposited at the National Institute of Biological Resources (NIBR), Incheon, South Korea, with the accession number NIBRFGC000507836.

## Morphological characterization

For morphological observations, strain KNUF-20-NI009 was transferred onto PDA, malt extract agar (MEA), and oatmeal agar (OA) and incubated at 25°C for 14 days [12], after which, we measured growth, recorded colony characteristics, such as shape, color, and size, and observed conidiomata under a Dimis-M stereo microscope (Siwon Optical Technology Co Ltd., Anyang, Korea). For an examination of micro-morphological characteristics, the isolate was observed under a BX-50 light microscope (Olympus, Tokyo, Japan).

## Effects of temperature and pH on growth

To determine the mycelial growth of the isolate at different temperatures and pH, 6-mm-diameter mycelial plugs were extracted from the edge of 4-days-old PDA cultures using a sterile cork-borer and transferred to the center of 90-mm Petri dishes containing PDA. For an assessment of the effects of temperature, cultures were incubated at 5, 10, 15, 20, 25, and 30°C with three replicate plates per isolate being assessed at each temperature. The effect of pH on the growth rate of KNUF-20-NI009 was evaluated by adjusting the culture medium (PDA) to pH 4, 5, 6, 7, 8, and 9 with HCl or NaOH and incubating at 25°C. To determine growth rates, colony diameters were measured after incubating for 14 days.

## Genomic DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from fungal mycelia scraped from PDA plates using a HiGene™ Genomic DNA Prep Kit (Biofact, Daejeon, Korea) according to the manufacturer's instructions. The isolated DNA was amplified for the detection of five gene markers, namely, internal transcribed spacer (ITS) regions, small subunit rDNA (SSU), large subunit rDNA (LSU), translation elongation factor 1- $\alpha$  (TEF1 $\alpha$ ), and RNA polymerase II second largest subunit (RPB2) genes, using the primer pairs ITS1F/ITS4, NS1/NS4, LROR/LR5, EF1-983F/EF1-1567R, and fRPB2-5f/fRPB2-7cr, respectively [13-17]. The thermal conditions used for PCR amplification have been described previously [13,17]. The amplified PCR products thus obtained were purified using ExoSAP-IT reagent (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced commercially by Macrogen Co., Ltd. (Daejeon, Korea). Sequences obtained from the KNUF-20-NI009 isolate have been deposited in the National Center for Biotechnology Information (NCBI) GenBank and the DNA Databank of Japan (DDBJ) databases, with the accession numbers LC719282, LC723530, LC719283, LC723531, and LC723532 for ITS, SSU, LSU, TEF1 $\alpha$ , and RPB2 respectively.

## Phylogenetic analysis

Datasets of the ITS, SSU, LSU, TEF1 $\alpha$ , and RPB2 sequences were used to search for similar sequences in the NCBI GenBank database, and comparable sequences from related species were retrieved for phylogenetic analysis (Table 1). Phylogenetic tree was constructed based on a combined alignment of multi-genes using the neighbor-joining (NJ) method with the Kimura 2-parameter model [18] in the MEGA 7 software program, with bootstrap analysis of 1,000 replications [19].

**Table 1.** List of species used in the phylogenetic analysis along with their GenBank accession numbers.

Fungal species	Strain	Accession numbers				
		ITS	LSU	RPB2	TEF1 $\alpha$	SSU
<i>Pararousoella mukdahanensis</i>	HKAS 101766	MH453489	MH453485	MH453482	MH453478	-
<i>Pararousoella rosarum</i>	MFLUCC 17-0796*	MG828939	MG829048	-	MG829224	NG_061294
<i>Pararousoella mangrovei</i>	MFLUCC 17-1542	MH025951	MH023318	MH028250	MH028246	-
<i>Pseudoneoconiothyrium rosae</i>	MFLUCC 15-0052*	MG828922	MG829032	-	-	MG829138
<i>Pseudoneoconiothyrium euonymi</i>	CBS 143426*	MH107915	MH107961	MH108007	-	-
<i>Pseudorousoella chromolaenae</i>	MFLUCC 17-1492*	MT214345	MT214439	-	MT235769	MT214393
<i>Pseudorousoella elaeicola</i>	MFLUCC 15-0276a*	MH742329	MH742326	-	-	-
<i>Pseudorousoella elaeicola</i>	MFLUCC 15-0276b	MH742330	MH742327	-	-	-
<i>Pseudorousoella elaeicola</i>	MFLUCC 17-1483	MT214348	MT214442	MT235808	MT235772	-
<i>Rousoella arundinacea</i>	CPC 35554	MT223838	MT223928	-	MT223723	-
<i>Rousoella intermedia</i>	CBS 170.96	KF443407	KF443382	KF443394	KF443398	KF443390
<i>Xenorousoella triseptata</i>	MFLUCC 17-1438*	MT214343	MT214437	MT235804	MT235767	MT214391
<b><i>Xenorousoella triseptata</i></b>	<b>KNUF-20-NI009</b>	<b>LC719282</b>	<b>LC719283</b>	<b>LC723532</b>	<b>LC723531</b>	<b>LC723530</b>
<i>Occultibambusa bambusae</i>	MFLUCC 13-0855*	KU940123	KU863112	KU940170	KU940193	-

Bold characters indicate data from this study.

CBS: Centraalbureau voor Schimmelcultures, The Netherlands; CPC: Culture collection of Pedro Crous, Netherlands; HKAS: Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand.

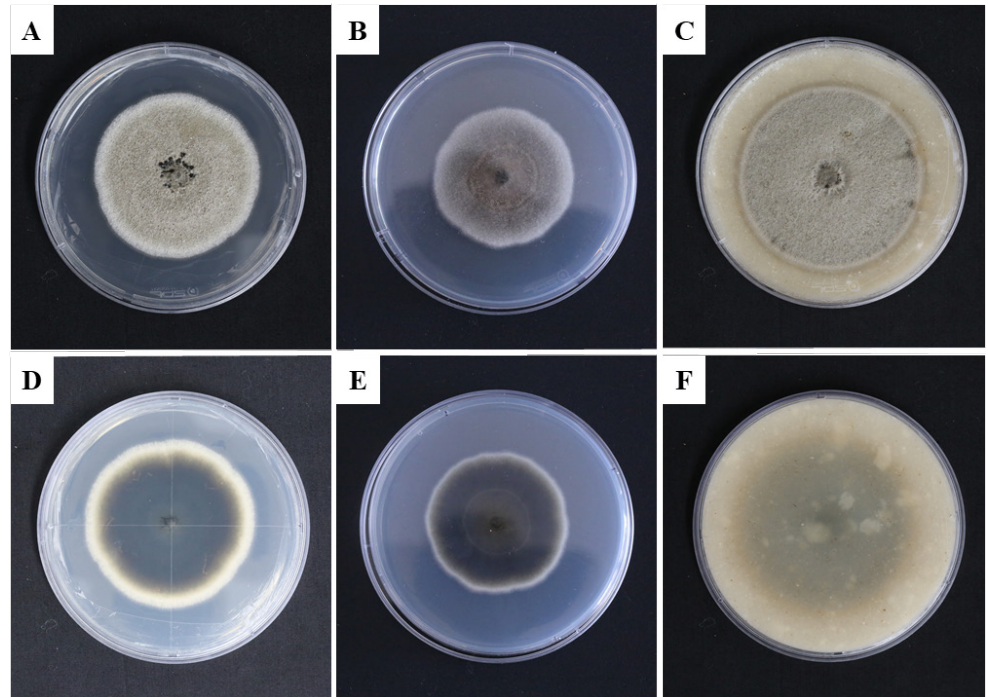
\* Ex-type culture.

## RESULTS AND DISCUSSION

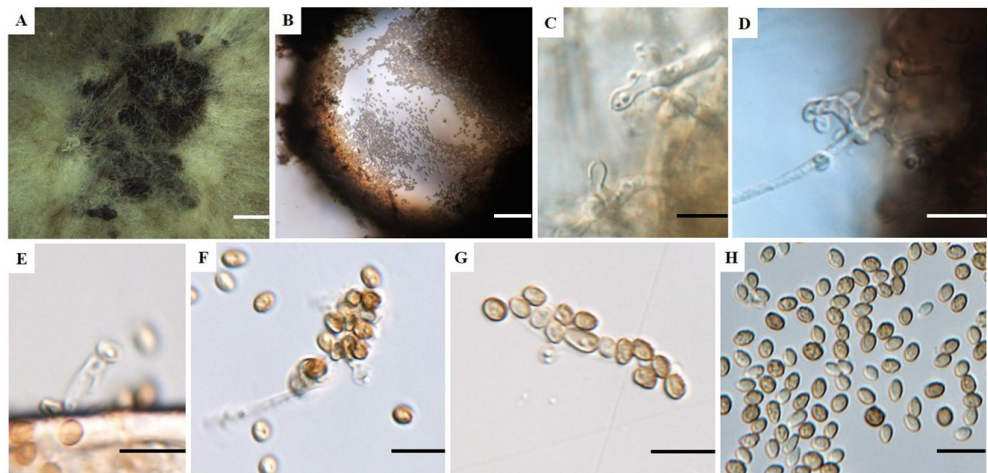
### Morphology of the KNUF-20-NI009 strain

To determine the morphological characteristics of strain KNUF-20-NI009 colonies, we cultured spore suspensions on PDA, MEA, and OA at 25°C for 14 days. Colony diameters on these media after the 2-week incubation were 55.9-68.0, 45.3-46.2, and 67.8-68.9 mm, respectively. On PDA, the color in the upper surface of the colonies was olivaceous gray, whereas the lower surface was olivaceous green to black in the center with a pale white margin (Figs. 1A and D). The colonies on MEA were olivaceous-brown to pale olivaceous-white at the margin on the upper surface, with undersides of dark olivaceous to olivaceous-brown and a white margin. (Figs. 1B and E). The colonies on OA were characterized by a rugged olivaceous-gray surface with a white to olivaceous margin (Figs. 1C and F). After 2 to 3 weeks on PDA medium, the isolate KNUF-20-NI009 showed pycnidial conidiomata, solitary or gregarious, scattered, globose to subglobose, dark brown to black. The pycnidial wall was 13-17  $\mu\text{m}$  wide (Figs. 2A and B) and the conidiophores were reduced to conidiogenous cells. These cells were 4-7.9  $\times$  2.6-4.8  $\mu\text{m}$  ( $\bar{x}$ =5.5  $\times$  3.6  $\mu\text{m}$ , n=20) in size, ampulliform to doliiform, proliferating percurrently at the apex, integrated, discrete, hyaline, and smooth (Figs. 2C-G). Conidia were aseptate, globoid to ellipsoid, with hebetate apex, thick-walled, hyaline when immature, becoming light brown to golden-brown when mature, with 1 or 2 guttules, and measured 2.7-5.1  $\times$  1.6-3.3  $\mu\text{m}$  ( $\bar{x}$ =3.6  $\times$  2.6  $\mu\text{m}$ , n=50) (Fig. 2H). However, we were unable to compare the asexual morphological characteristics of KNUF-20-NI009 with those of *Xenorousoella*

*triseptata* MFLUCC 17-1438, as these features have yet to be reported for this species. Based on asexual morphology, we compared the isolate with other phylogenetically close reference strains [7]. The conidia diameters of *Rousoella euonymi* (= *Pseudoneoconiothyrium euonymi*) and *Pseudoneoconiothyrium rosae* are (6-)7(-8) × (4-)5-6 μm and 7-10 × 4-8 μm, respectively [20-22]. Strain KNUF-20-NI009 can accordingly be distinguished from *R. euonymi* and *P. rosae* by its comparatively smaller conidia. Morphological comparisons with the previously reported closely related species are shown in Table 2.



**Fig. 1.** Cultural characteristics of KNUF-20-NI009 after 14 days of cultivation at 25°C. Colonies on (A, D) potato dextrose agar; (B, E) malt extract agar; (C, F) oatmeal agar. Upper (A, B, C) and lower (D, E, F) surfaces.



**Fig. 2.** Morphological characteristics of KNUF-20-NI009. (A) Conidiomata on potato dextrose agar (PDA); (B) Section through conidiomata; (C-G) Conidiogenous cells and developing conidia; (H) conidia. Scale bars: A=500 μm, B=50 μm, C-H=10 μm.

**Table 2.** Comparison of morphological characteristics of strain KNUF-20-NI009 and closely related species.

Characteristics		KNUF-20-NI009 <sup>a</sup>	<i>Xenorousoella triseptata</i> <sup>b</sup>	<i>Rousoella euonymi</i> <sup>c</sup> (= <i>Pseudoneoconiothyrium euonymi</i> )	<i>Pseudoneoconiothyrium rosae</i> <sup>d</sup>
Colony	Color	On MEA, olivaceous brown to pale olivaceous-white at the margin, reverse dark olivaceous to olivaceous-brown with white margin	On MEA, white, pale grayish brown, olivaceous to olivaceous brown creamy-white in reverse, olivaceous-brown to brown, olivaceous to olivaceous-brown at the margin	On MEA, olivaceous gray with patches of pale olivaceous gray, reverse pale olivaceous gray	On MEA, olivaceous gray with patches of pale olivaceous gray, reverse pale olivaceous gray
	Size	59.3 mm diam in 14 days	N/A	60 mm diam in 14 days	5-10 mm diameter after one week at 16°C
	Shape	Circular with aerial mycelium, rugged surface slightly undulate	Circular, mycelium slightly raised, undulate	Flat, spreading, with moderate aerial mycelium and even, lobate margins	Undulate, white, with dense, flat mycelium on the surface, pale yellow in reverse
Conidia	Size (µm)	3-4.8 × 1.7-3.2	N/A	(6-)7(-8) × (4-)5-6	7-10 × 4-8
	Shape and color	Aseptate, globose to ellipsoid, apex hebetate, thick-walled, hyaline when immature, becoming light brown to golden-brown when mature, with 1-2 guttules	N/A	Solitary, ellipsoid, guttulate, aseptate, apex obtuse, base 2 µm diam, bluntly rounded, thick-walled, becoming warty, golden-brown to red-brown	Initially hyaline, smooth and thin-walled, rough-walled when mature, globose to ellipsoid, aseptate, guttulate, and light brown to dark brown
Conidiogenous cells(µm)		Ampulliform to doliiform, proliferating percurrently at apex, integrated, discrete, hyaline, and smooth, 4-7.9×2.6-4.8	N/A	Lining the inner cavity, hyaline, smooth, ampulliform to doliiform, proliferating percurrently at apex, 5-12×5-7	Ampulliform, enteroblastic, annellidic with distinct percurrent proliferation, sometimes cylindrical, elongate neck, integrated, discrete, smooth, and hyaline.

N/A: not available.

<sup>a</sup> Fungal strain studied in this paper, <sup>b</sup> Mapook A et al.[7], <sup>c</sup> Crous PW et al. [21], <sup>d</sup> Hyde KD et al. [20].

Temperature and pH are important factors affecting the growth and survival of living organisms. To determine the effects of the two factors on KNUF-20-NI009 growth, we cultured the isolate in a PDA medium, which is generally and widely used for the cultivation of fungi. We accordingly established that the isolate grows optimally at a temperature of 25°C, reaching a colony diameter of 59.3 ± 2.1 mm. No growth was observed at 5°C, whereas the colonies obtained at 10°C were characterized by a white upper surface and olivaceous black to the light beige lower surface. At 15°C and 20°C, the colonies developed a rugged upper surface, olivaceous in the center with white margins, whereas the lower surface was olivaceous to iron-gray with a pale white margin. When grown at 25°C, the upper surface of colonies was olivaceous gray in the center and the lower surface was olivaceous green to black with a pale white margin. Contrastingly, at 30°C, we observed colonies with a cottony pale white surface colored olivaceous beneath, with the lower surface being olivaceous green to iron-gray with a white margin. Generally, we found that incubation at temperatures below 20°C and above 30°C resulted in an increase of white mycelia and colonies with a pale white margin.

With respect to pH, we established that growth of the isolate was maximal at pH 7 ( $63.3 \pm 0.6$  mm) and minimal at pH 4 ( $35 \pm 2.6$  mm), with the optimum pH for growth being neutral or slightly alkaline. Under optimum pH conditions, colonies were mostly olivaceous green to pale white with a white margin, whereas, at pH 9, the colonies were olivaceous brown to pale white with a light brown to pale white margin. Details of colony diameters at different temperatures and pH values are presented in Table 3.

**Table 3.** Effects of temperature and pH on mycelial growth of *Xenorousoella triseptata* KNUF-20-NI009 cultured on potato dextrose agar (PDA).

Temperature	5°C	10°C	15°C	20°C	25°C	30°C	Optimum temperature (°C)
	0f	23.3±0.6e	31.3±1.2d	44.7±0.6c	59.3±2.1a	54±2.6b	25
pH	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	Optimum pH
	35±2.6d	58±1.0c	58.7±1.2bc	63.3±0.6a	61±1.7ab	59±1.0bc	7

All strains were cultured on a PDA medium at various temperatures and pH for 14 days.

Values are the means of three replicates and the means of three plates. The numbers represent the mean±SD of three replicates, except for the inoculum size, which is 6 mm in diameter.

Different letters in the same column indicate a significant difference at  $p < 0.05$ , as determined using Duncan's multiple range test ( $n=3$ ). (mycelial length=mm).

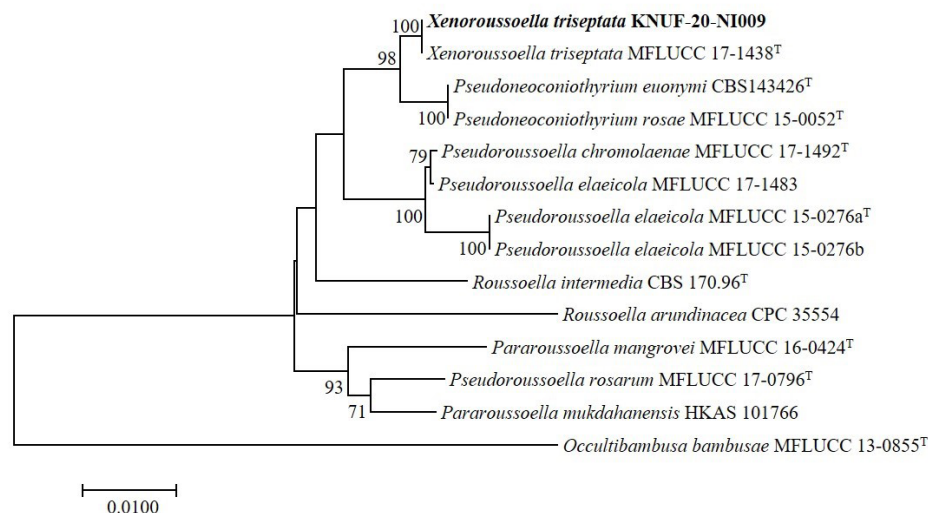
## Phylogenetic analysis

The taxonomic status of isolate KNUF-20-NI009 was determined based on an analysis of a combined dataset of the ITS region and LSU, SSU, RPB2, and TEF1 $\alpha$  gene sequences [7,22,23]. Amplification of the ITS, LSU, SSU, RPB2, and TEF1 $\alpha$  loci of the strain yielded fragments of 586, 837, 1,008, 1,041, and 921 bp, respectively. A BLAST search of the NCBI database revealed similarities of 99.41% between the ITS regions of KNUF-20-NI009 and *Xenorousoella triseptata* MFLUCC 17-1438 (MT214343). Similarly, analysis based on the partial LSU gene sequence revealed a 100% similarity with *Xe. triseptata* MFLUCC 17-1438 (MT214437) and 99.40% similarity with *Rousoella euonymi* CBS 143426 (MH107961) and *Pseudoneoconiothyrium rosae* MFLU 18-0117 (NG\_059868). Comparisons based on the SSU sequence indicated that KNUF-20-NI009 has identities of 100, 99.70, and 99.60% with *Xe. triseptata* DC9 (OL700219), *Rousoella intermedia* CBS 170.96 (KF43390), and *Rousoella padinae* MUT<ITA>:5503 (MN556315), respectively, whereas the RPB2 gene sequence of KNUF-20-NI009 showed 99.83% similarity with *Xe. triseptata* MFLUCC 17-1438 (MT235804), but only 85.77% similarity with *P. rosae* IT3796 (MN814846). Moreover, based on TEF1 $\alpha$  region sequence similarity, we identified *Xe. triseptata* MFLUCC 17-1438 (MT235767), with 99.73% similarity, as the closest relative to KNUF-20-NI009.

However, despite *Xe. triseptata* being identified as taxonomically closest to strain KNUF-20-NI009 based on all of the molecular markers assessed in this study, it is clear that the sequence of any one of these loci does not enable us to unambiguously identify the novel fungal strain. Accordingly, to attain a more precise identification, we performed multi-locus sequence analysis using the concatenated sequences of the ITS regions, and LSU, SSU, RPB2, and TEF1 $\alpha$  genes of strain KNUF-20-NI009 (Table 1). This combination of five molecular markers has previously been shown to be highly effective in resolving species in the family Rousoellaceae [7]. The neighbor-joining phylogenetic tree (Fig. 3) constructed based on analysis of these concatenated sequences demonstrated that strain KNUF-20-NI009 maps to a location distinct from

those of *Rousssoella*, *Pseudorousssoella*, and *Pseudoneoconiothyrium* species and that its phylogenetically closest neighbor is *Xe. triseptata*, as indicated by the fact that both the KNUF-20-NI009 and MFLUCC 17-1438 strains clustered together with a high bootstrap value of 100%.

Previous studies have reported that species of *Rousssoella* and *Rousssoellopsis* can typically be found on or



**Fig. 3.** Neighbor-joining phylogenetic tree based on combined dataset (ITS+LSU+SSU+RPB2 and TEF1 $\alpha$ ) showing the phylogenetic position of strain KNUF-20-NI009 among members of the family Rousssoellaceae and its closest relationship with *Xenorousssoella triseptata*. The tree was rooted using *Occultibambusa bambusae* MFLUCC 13-0855<sup>T</sup> as an outgroup. Bootstrap values greater than 70% (based on 1,000 replications) are shown at the branching points. The isolated strain is indicated in bold. Bar=0.01 substitutions per nucleotide position. ITS, internal transcribed spacer regions; LSU, large subunit rDNA; SSU, small subunit rDNA; RPB2, RNA polymerase II largest subunit; TEF1 $\alpha$ , translation elongation factor 1-alpha.

in the vicinity of a diverse range of plants, including monocotyledons, tall grasses, bamboos, and palms [1]. In particular, the single representative of the genus *Xenorousssoella*, *Xe. triseptata* was initially isolated from dead stems of the invasive weed *Chromolaena odorata* [7]. Contrastingly, in the present study, we isolated the KNUF-20-NI009 strain of this species from soil samples collected in Korea, thereby highlighting the wide geographical distribution and diverse habitats of this species. Although the cultural characteristics of *Xe. triseptata* on MEA medium and the morphological characteristics of its sexual stage have been described previously [7], until the present study, its asexual features had remained undetermined. By providing a detailed description of the asexual morphology of the KNUF-20-NI009 strain, the findings of this study complement our existing knowledge of this species. Moreover, we have established the cultural characteristics of the strain grown on three different media, determined the optimal temperature and pH for its cultivation, and evaluated the effects of various temperatures on its cultural characteristics. However, further investigations are needed to gain more in-depth insights into its ecological, etiological, and biological roles, particularly under the environmental conditions found in Korea.

In conclusion, our morphological and phylogenetic analyses in this study have revealed KNUF-20-



NI009 to be a strain of the fungal species *Xenorousoella triseptata*. To the best of our knowledge, this study is the first to report the isolation of this species in Korea.

## CONFLICT OF INTERESTS

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

## ACKNOWLEDGMENTS

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