RESEARCH ARTICLE

First Report of Two Unreported Fungi *Clonostachys eriocamporesii* and *Sporothrix euskadiensis* Isolated from Soil in Korea

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ABSTRACT

During an exploration of fungal diversity in Korean soil, this study isolated and identified two unrecorded fungal strains, designated KNU-HP-1804 and KNUF-20-007. Morphological and cultural features were examined for initial classification. Cultural and morphological characteristics of strains KNU-HP-1804 and KNUF-20-007 were matched with *Clonostachys eriocamporesii* CBS 647.91 and *Sporothrix euskadiensis* CBS 122138^{T}, respectively. To further pinpoint their identity and evolutionary relationships, molecular phylogenetic analyses were conducted using the internal transcribed spacer (ITS) region, translation elongation factor 1-alpha (*TEF*1), beta-tubulin (*TUB*2), and calmodulin (*CAL*). The neighbor-joining (NJ) phylogenetic tree using the concatenated sequences, and the cultural and morphological observations revealed KNU-HP-1804 as *C. eriocamporesii*, while KNUF-20-007 was identified as *S. euskadiensis.* To the best of our knowledge, this is the first report on *C. eriocamporesii* and *S. euskadiensis* in Korea.

Keywords: *Clonostachys eriocamporesii*, Cultural characteristics, Morphology, Phylogenetic analyses, *Sporothrix euskadiensis*

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INTRODUCTION

Fungi play an important ecological role as decomposers of organic matter. Fungi produce a wide range of enzymes that help regulate the balance of soil nutrients, greatly influencing plant productivity and diversity, and they also act as predators, pathogens, and parasites of many organisms [1,2]. The fungi in genus *Clonostachys*, belongs to the class Sordariomycetes, order Hypocreales, family Bionectriaceae, with 68 species reported worldwide [3]. The family Bionectriaceae is characterized by its herbicolous, corticolous, lichenicolous, fungicolous, and coprophilous nature [4]. The genus *Clonostachys* has recently been found in the bark and decaying leaves of dead trees, other fungi, nematodes, and insects, and comprises soil-borne species, mycoparasites, endophytes, epiphytes, and saprotrophs [4,5]. The *Clonostachys* species identified in Korea currently comprises three recognized species: *C. rosea, C. divergens,* and *C. farisona* [6–8]. The genus *Sporothrix* also belongs to the class Sordariomycetes, but is in the order Ophiostomatales, and family

Ophiostomataceae, with 56 species reported worldwide [9,10]. In 2016, a study confirmed that most species with *Sporothrix*-like asexual morphs do not constitute a monophyletic lineage in the genus *Ophiostoma*, and was reclassified into the genus *Sporothrix* [11]. The genus *Sporothrix* is widely distributed in different climatic zones of the world and has been reported from a variety of habitats associated with forest trees, soil, bark, beetles and mites [12]. In addition, the genus *Sporothrix* comprises soil-borne, saprophytic, endophytic, and phytophytic bacteria and includes S. *brasiliensis, S. chilensis, S. globose, S. luriei*, and *S. schenskii*, which have been reported as toxic pathogens of livestock [13–15]. Currently, there are 8 reported species in Korea under the genus *Sporothrix* [16–18].

The objective of this research was to isolate fungi from soil samples collected from Korea and to identify the isolated fungal species through morphological and molecular biological characteristics.

MATERIALS AND METHODS

Sample collection and fungal isolation

The fungal isolates used in this study were present in soil samples collected from Gunwi-gun, Gyeongbuk province (36°08'56.0"N 128°35'46.3"E) and Yangpyeong-gun, Gyeonggi province (37°28'03.7"N 127° 32'30.3"E) in Korea. The isolation of fungi from the soil samples followed by the serial dilution technique was described in a prior study [19]. Colonies displaying signs of germination were then transferred to new potato dextrose agar (PDA; Difco, Detroit, MI, USA) plates at a temperature of 25℃. The fungal strains KNU-HP-1804 and KNUF-20-007 were chosen for additional molecular analyses, as well as cultural and morphological evaluations. The stock culture for strain KNU-HP-1804 (NIBRFGC000502238) and strain KNUF-20-007 (NIBRFGC000507843) have been deposited at the National Institute of Biological Resources (NIBR) as a metabolically inactive culture.

Cultural and morphological characterization

Different media were used to observe the culturological and morphological characteristics of the two strains. The strain KNU-HP-1804 was cultured for 7 days on PDA, oatmeal agar (OA; Difco, Detroit, MI, USA), and synthetic nutrient deficient agar (SNA; Nirenberg, 1976); while strain KNUF-20-007 was cultured for 8 days on PDA, OA, and 2% malt extract agar (MEA; Difco, Detroit, MI, USA), with the growth, color, morphology, and texture of the colonies formed on were observed [3,20]. Morphological features were observed and recorded using a light microscope (BX-50, Olympus, Tokyo, Japan).

Genomic DNA extraction, PCR amplification, and sequencing

The total genomic DNA was extracted from the strains KNU-HP-1804 and KNUF-20-007 using the HiGene™ Genomic DNA Prep Kit (Biofact, Daejeon, Korea) for molecular identification. KNU-HP-1804 was analyzed for the internal transcribed spacer (ITS) region, translation elongation factor 1-alpha (*TEF*1), and beta-tubulin (*TUB*2) sequence fragments, while the strain KNUF-20-007 was analyzed for the ITS region, *TUB*2, and calmodulin (*CAL*) sequence fragments. The primers used to amplify the molecular phylogenetic markers were ITS1F/ITS4 for ITS, EF1-983F/EF1-2218R for *TEF*1, CL2F/CL2R for *CAL*,

and T1/T22 and Bt2a/Bt2b for *TUB*2 [21–26]. The confirmation of amplification was conducted through electrophoresis utilizing 1.0% HP Agarose gels (BIOPURE, Cambridge, USA). The amplified products underwent purification via ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) before being sent to Macrogen (Seoul, Korea) for sequencing.

Phylogenetic analyses

The sequences obtained were analyzed for similarity utilizing the Basic Local Alignment Search Tool (BLAST) within the National Center for Biotechnology Information (NCBI) database (Table 1). Phylogenetic trees were constructed from the combined sequences of the ITS regions, *TEF*1, *TUB*2, and *CAL* employing the neighbor-joining (NJ) method in Molecular Evolutionary Genetics Analysis (MEGA) version 11.0 [27,28]. The evolutionary distance matrices for the NJ analysis were calculated in accordance with Kimura's two-parameter model, with bootstrap values derived from 1,000 replications [29].

Table 1. List of species and their GenBank accession numbers used for the phylogenetic analyses in this study

Species name	Strain number	GenBank accession number			
		ITS	*TEF*1	*TUB*2	*CAL*
Clonostachys fujianensis	CBS 12747^T	OQ910620	OQ982655	OQ944632	-
Clonostachys obovatispora	CBS 118752^T	OQ910649	OQ982680	OQ944661	-
Clonostachys epichloe	CBS 101037^T	OQ910581	OQ982618	OQ944593	-
Clonostachys miodochialis	CBS 997.69^T	OQ910646	OQ982677	OQ944658	-
Clonostachys divergens	JW190011	OQ910578	OQ982615	OQ944590	-
Clonostachys divergens	CBS 967.73B^T	OQ910575	OQ982612	OQ944587	-
Clonostachys samuelsii	CBS 699.97^T	OQ910812	OQ982832	OQ944822	-
Clonostachys samuelsii	CBS 701.97	OQ910814	OQ982834	OQ944824	-
Clonostachys rogersoniana	CBS 920.97^T	OQ910711	OQ982740	OQ944723	-
Clonostachys rogersoniana	CBS 668.70	OQ910710	OQ982739	OQ944722	-
Clonostachys penicillata	CBS 729.87^T	OQ910654	OQ982685	OQ944666	-
Clonostachys penicillate	CBS 148211	OQ910652	OQ982683	OQ944664	-
Clonostachys hongkongensis	CBS 118291^T	OQ910630	OQ982663	OQ944642	-
Clonostachys eriocamporesii	CBS 647.91	OQ910582	OQ982619	OQ944594	-
Clonostachys eriocamporesii	**KNU-HP-1804**	**PQ269427**	**PQ276740**	**PQ276750**	-
Fusarium acutatum	CBS 402.97^T	NR_111142	MT011051	MT010989	-
Sporothrix cf. *abietina*	CMW40454	MW581512	-	MW579723	MW579751
Sporothrix abietina	CBS 125.89^T	AF484453	-	KX590755	JQ511966
Sporothrix lunata	CMW10563^T	AY280485	-	AY280466	JQ511970
Sporothrix prolifera	KFL99WRJSI	MH283139	-	MH283355	MH283520
Sporothrix prolifera	CBS 251.88^T	KX590829	-	KX590770	KX590797
Sporothrix cantabriensis	CMW39766^T	KF951554	-	KF951544	KF951540
Sporothrix cantabriensis	CMW39767	KF951555	-	KF951545	KF951541
Sporothrix fusiformis	CMW9968^T	AY280481	-	AY280461	JQ511967
Sporothrix fusiformis	CMW7131	AY280497	-	AY280464	JQ511971
Sporothrix gossypina	ATCC 18999^T	KX590819	-	KX590761	KX590789
Sporothrix curviconia	CBS 541.84	KX590836	-	KX590777	JQ511968
Sporothrix rossii	CBS 116.78^T	NR_147597	-	KX590754	JQ511972
Sporothrix euskadiensis	CBS 122138^T	DQ674369	-	EF396344	JQ438830
Sporothrix euskadiensis	**KNUF-20-007**	**PQ269428**	**-**	**PQ276751**	**PQ276741**
Ophiostoma noisomeae	CBS 141065	KU639631	-	KU639628	KX590792

ITS: Internal transcribed spacer regions; *TEF1*: translation elongation factor-1α; *TUB2*: beta-tubulin; *CAL*: calmodulin.
^{T}Type strain; The strain used in this research is highlighted in bold.

RESULT AND DISCUSSION

Clonostachys eriocamporesii R.H. Perera & K.D. Hyde, Fungal Diversity 100: 199 (2020) [MB#556897]

Cultural and morphological characteristics of KNU-HP-1804

The colony grown on PDA at 25℃ for 7 days was observed to be growing 31.7–34.5 mm in diameter. The obverse side appears to be white with cottony aerial hyphae. The reverse side was observed to be yellowish white (Fig. 1A). Grown on OA at 25℃ for 7 days, the colony was observed to be 37.7–38.2 mm in diameter. On the obverse side was observed to be white and cottony, forming a crateriform in the center. The reverse side showed the same white color as the obverse side (Fig. 1B). Grown on SNA at 25℃ for 7 days, the colony was observed to be 24.7–25.5 mm, appeared to be yellowish on both obverse and reverse sides, and were observed to be rhizoid (Fig. 1C). The conidiophores, which produces conidia, were transparent, smooth-surfaced, monomorphic and sporodochial. The phialides were upright, cylindrical, tapers towards the apex and narrowly flask shaped. Phialides measured at 7.9–17.5 µm (avg. 10.9 µm, n = 20) (Fig. 1D). When incubated for one month on PDA and SNA, dark green, long, curled stuppeus conidia were observed. White pustules were also observed to first form on aerial hyphae, followed by light-green sporodochia which turns to dark-green sporodochia over time (Figs. 1E and F). Conidia produced at the tip of phialides were transparent unicellular with a thin, smooth surface, fusiform with a pointed tip, and were layered and chained like scales, measuring 4.5–7.0 × 1.7–3.4 µm (Figs. 1G and H). A comparison of the morphological features between strain KNU-HP-1804 and *Clonostachys eriocamporesii* CBS 647.91 was made in Table 2. Based on the table, the strain KNU-HP-1804 was indicated to be closely related to *C. eriocamporesii* CBS 647.91 [3].

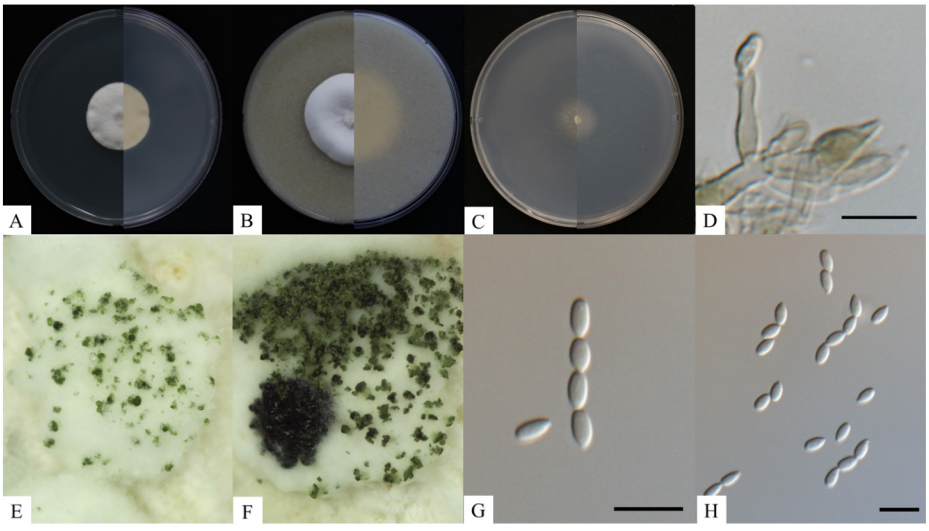

Fig. 1. Cultural and morphological characteristics of *Clonostachys eriocamporesii* KNU-HP-1804. Cultures were grown at 25℃ for 7 days. A–C: Front and reverse colony on potato dextrose agar (PDA), oatmeal agar (OA), synthetic nutrient deficient agar (SNA), respectively; D: Conidiophores; E: Sporodochia after 1 month; F: Sporodochia after 2 months; G, H: Conidia. Scale bars = 10 µm.

Table 2. Morphological characteristics of KNU-HP-1804 and comparisons between *Clonostachys eriocamporesii* CBS 647.91

Characteristics		*Clonostachys eriocamporesii* KNU-HP-1804[a]	*Clonostachys eriocamporesii* CBS 647.91[b]
Colony on PDA	Size	27 mm	32–41 mm
	Color	White, reverse concolorous	White, reverse concolorous
	Shape	Margin entire, aerial mycelium moderate, felty to cottony	Margin entire, aerial mycelium moderate, felty to cottony
Colony on OA	Size	37–38 mm	30–45 mm
	Color	White, reverse concolorous	White, reverse concolorous
	Shape	Margin entire, aerial mycelium moderate, felty to cottony	Margin entire, aerial mycelium moderate, felty to cottony
Colony on SNA	Size	23 mm	27–32 mm
	Color	Yellowish-white, reverse concolorous	White, reverse concolorous
	Shape	Rhizoid	Margin entire, aerial mycelium moderate in center, floccose
Sporodochia	Shape	White pustules, light green to dark green	White pustules, dark green
Conidiophores	Size	7.9–17.5 μm	9.5–17.5 μm
	Shape	Monomorphic, sporodochial	Monomorphic, sporodochial
Conidia	Size	4.5–7.0 × 1.7–3.4 μm	5.9–8.6 × 2.7–3.8 μm
	Shape	Hyaline, narrowly clavate, arranged in chain	Aseptate, greenish hyaline, ellipsoidal, narrowly clavate, arranged in chain

PDA: potato dextrose agar; OA: oatmeal agar; SNA: synthetic nutrient deficient agar.
[a] Fungal strain used in this study; [b] Source of description [3].

Molecular phylogeny of KNU-HP-1804

To genetically identify fungal strain KNU-HP-1804, nucleotide sequences of ITS regions, *TEF*1, and *TUB*2 were acquired with the length 485, 724, and 1031 bp, respectively. The nucleotide sequences were registered to GenBank under the accession numbers PQ269427, PQ276740, PQ276750 for the ITS regions, *TEF*1, and *TUB*2, respectively. The ITS regions showed the highest similarity of 99.8% with *C. eriocamporesii* CBS 647.91, when compared with *C. epichloe* CBS 101037^{T} at a similarity of 93.2%. The *TEF*1 sequence showed a very high similarity of 99.0% with *C. eriocamporesii* CBS 647.91 compared to a similarity of 97.9% with *C. miodochialis* CBS 997.69^{T} while for *TUB*2, KNU-HP-1804 showed the highest similarity of 100% with *C. eriocamporesii* CBS 647.91. The NJ phylogenetic tree constructed based on the concatenated sequences of the ITS region, *TEF*1, and *TUB*2 (Fig. 2), showed that strain KNU-HP-1804 formed the same cluster as the reported *C. eriocamporesii* and was identified as *C. eriocamporesii* based on culture, morphological, and phylogenetic analyses. This report on *C. eriocamporesii* contributes to the existing knowledge of *Clonostachys* species, bringing the number of recorded species to 4 in Korea [6–8]. Species from the genus *Clonostachys* were reported to be entomopathogenic, with *C. eriocamporesii* reported to cause fast and high mortality in *Aedes aegypti* larvae and were found on invasive spotted lanternflies [30,31]. There were also several reports of *C. rosea* as opportunistic phytopathogens and naturally occurring entomopathogen or a promising control agent [8,30]. The mycorrhizal and saprophytic species of the genus *Clonostachys* are capable of destroying plant pathogens by secreting chitinolytic enzymes, proteolytic enzymes, and several antibiotics, and possess induced resistance that can protect plants from pathogen invasion [32,33]. It has been reported that *C. miodochialis* also produces metabolites that may be antagonistic to *F. acuminatum* and may be involved in degrading the cell wall without physical contact [34]. As such, biological control using the genus *Clonostachys* has been actively pursued, and further research on *C. eriocamporesii* is warranted.

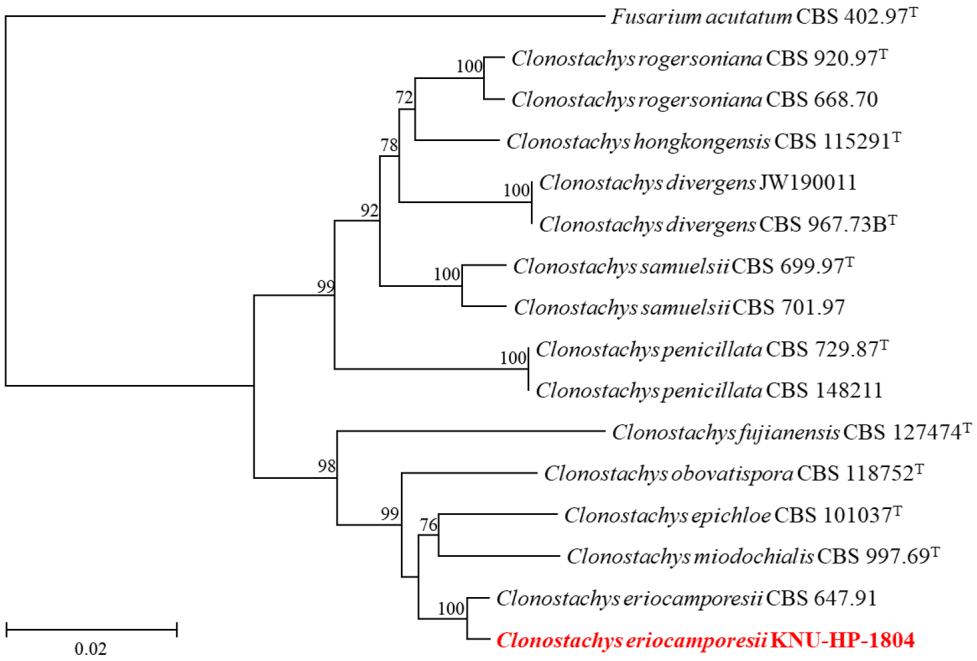

Fig. 2. Neighbor-joining phylogenetic analyses of KNU-HP-1804 based on concatenated sequence data of internal transcribed spacer (ITS) regions, translation elongation factor 1-alpha (*TEF*1) and beta-tubulin (*TUB*2) showing the phylogenetic position of the closest species in the genus *Clonostachys*. Bootstrap values greater than 70% (percentage of 1,000 replications) are shown at branching points. The strain isolated in this study was highlighted in bold and red, and bootstrap values were obtained from 1,000 replicates. *Fusarium acutatum* CBS 402.97^{T} was used as an outgroup. Bar = 0.02 substitutions per nucleotide position

Sporothrix euskadiensis (P. Romón, Z.W. de Beer, M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf., Studies in Mycology 83: 117 (2016) [MB#817572]

Cultural and morphological characteristics of KNUF-20-007

The colony grown on PDA for 8 days at 25℃ was observed to be growing 31.7–34.5 mm in diameter. On the obverse side, the colony appears yellowish-white with aerial hyphae that were filamentous (Fig. 3A). Grown on OA for 8 days at 25℃, the colony was observed to be 22.3–23.4 mm in diameter. The colony appears white and filiform on the obverse side, and pale white on the reverse side (Fig. 3B). Colony grown on MEA for 8 days at 25℃ measured to be 18.7–19.0 mm in diameter. The colony was filamentous, with both obverse and reverse sides being yellowish-white in color (Fig. 3C). The conidiophores were transparent, smooth-surfaced, with conidia hanging from the apex of an elongated cylindrical shape, and measured 6.1–23.4 µm (avg. 13.7 µm, n = 20) in length (Fig. 3D). The transparent unicellular conidia were clavate and cylindrical in shape, and measured to be 3.1–5.4 × 1.4–2.3 µm (n = 50) (Figs. 3E and F). A comparison of the morphological features between strain KNUF-20-007 and *S. euskadiensis* CBS 122138^{T} was made in Table 2. Based on the table, the strain KNUF-20-007 was indicated to be closely related to *S. euskadiensis* CBS 122138^{T} [11].

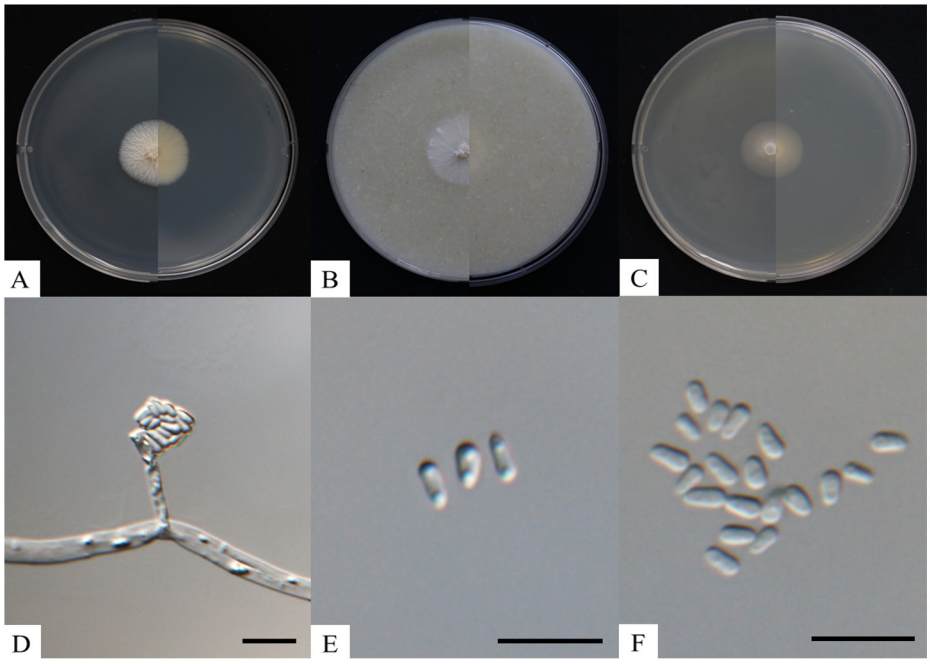

Fig. 3. Cultural and morphological characteristics of *Sporothrix euskadiensis* KNUF-20-007. Cultures were grown at 25℃ for 8 days. A-C: Front and reverse colony on potato dextrose agar (PDA), oatmeal agar (OA) and 2% malt extract agar (MEA), respectively; D: *Sporothrix*-like conidiophore; E, F: Conidia. Scale bars = 10 μm.

Table 3. Morphological characteristics of KNUF-20-007 and comparisons between *Sporothrix euskadiensis* CBS 122138[T]

Characteristics		*Sporothrix euskadiensis* KNUF-20-007[a]	*Sporothrix euskadiensis* CBS 122138[Tb]
Colony on PDA	Size	31.7–34.5 mm	N/A
	Shape and color	Yellowish-white, reverse concolorous, filamentous, hyphae aerial	N/A
Colony on OA	Size	22.3–23.4 mm	N/A
	Shape and color	White, reverse side concolorous, filiform	N/A
Colony on MEA	Size	18.7–19.0 mm	14.4 mm
	Shape and color	Yellowish-white, filamentous, entire margin	Yellowish-white, entire margin
Conidiophores	Size	6.1–23.4 μm	10.2–10.8 μm
	Shape	Transparent, smooth-surfaced, conidia hanging from apex of an elongated cylindrical shape	N/A
Conidia	Size	3.1–5.4 × 1.4–2.3 μm	2.2–3.0 × 1.2–1.8 μm
	Shape	Clavate, cylindrical	Clavate

PDA: potato dextrose agar; OA: oatmeal agar; MEA: malt extract agar.
[a] Fungal strain used in this study; [b] Source of d escription [11]; [T]Type strain; N/A: not available.

Molecular phylogeny of KNUF-20-007

To genetically identify the fungal strain KNUF-20-007, nucleotide sequences of ITS regions, *CAL*, and *TUB*2 were acquired with the length 611, 521, and 256 bp, respectively. The nucleotide sequences were registered to GenBank under the accession numbers PQ269428, PQ276751, PQ276741 for the ITS regions, *TUB*2, and *CAL*, respectively. The ITS regions revealed the highest similarity of 99.8% with *S. euskadiensis* CBS 122138[T], compared to a similarity of 99.6% with *S.* cf. *abietina* CMW40454, and 99.5% with *S. rossii* CBS 116.78[T]. Furthermore, the *CAL* sequences showed a very high similarity of 99.5%

with *S. euskadiensis* CBS 122138^{T}, compared with a similarity of 97.8% with *S.* cf. *abietina* CMW40454. Based on the *TUB*2 sequences, strain KNUF-20-007 showed a 100% similarity with *S. euskadiensis* CBS 112138^{T}. A NJ phylogenetic tree was constructed using the concatenated ITS region, *CAL* and *TUB*2 sequences (Fig. 4). The NJ phylogenetic tree indicated that strain KNUF-20-007 was clustered with *S. euskadiensis* CBS 112138^{T}. Based on the morphological, cultural, and phylogenetic analysis, the strain KNUF-20-007 was identified as *S. euskadiensis*. Including this report on *S. euskadiensis,* currently there are 9 reported species of *Sporothrix* in Korea [16]. The genus *Sporothrix* is mostly a soil-borne species native to tropical regions, but it has been reported to be isolated from insects such as beetles and mites that live in the bark of trees. While there has been no reports of *S. euskadiensis* being naturally pathogenic or an entomopathogenic fungi; *S. schenckii,* which is reported to be an endophytic pathogen, has been isolated from decaying plants, rose bushes, and sphagnum moss, causing a chronic fungal infection known as sporotrichosis [15]. However, as many species of the genus *Sporothrix* are reported to be toxic pathogens of livestock, it is necessary to obtain additional isolates and conduct pathogenicity tests to characterize *S. euskadiensis*. This study contributed to increasing the diversity of endemic fungal species by discovering *C. eriocamporesii and S. euskadiensis*, which to the best of our knowledge, have not been reported in Korea. It also contributed to discovering their utilization value through further research and increasing the possibility of their application in various industrial fields.

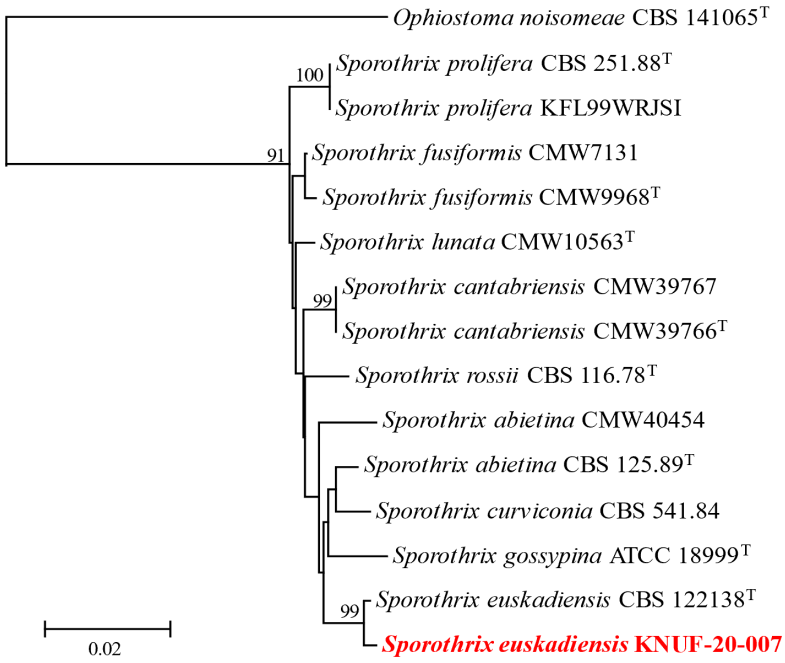

Fig. 4. Neighbor-joining phylogenetic analyses of KNUF-20-007 based on concatenated sequence data of internal transcribed spacer (ITS) regions, calmodulin (*CAL*) and beta-tubulin (*TUB2*) showing the phylogenetic position of the closest species in the genus *Sporothrix*. Bootstrap values greater than 90% (percentage of 1,000 replications) are shown at branching points. The strain isolated in this study was highlighted in bold and red, and bootstrap values were obtained from 1,000 replicates. *Ophiostoma noisomeae* CBS 141065^{T} was used as an outgroup. Bar = 0.02 substitutions per nucleotide position.

CONFLICT OF INTERESTS

No conflict of interest is declared by the author.

ACKNOWLEDGEMENTS

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