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Discovery of Two *Chrysosporium* Species with Keratinolytic Activity from Field Soil in Korea

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ABSTRACT
In an ongoing survey of Korean indigenous fungi, two fungal strains (KNU16-74 and KNU16-99) belonging to the genus *Chrysosporium* were isolated from field soil in Gyeongnam, Korea. Morphological characterization and phylogenetic analysis using sequence of the internal transcribed spacer regions were carried out to confirm its precise identification. These strains were identified as *Chrysosporium indicum* (KNU16-74) and *Chrysosporium fluviale* (KNU16-99). To examine the keratin degradation efficiency of these two fungal species, human hair strands were incubated with fungus culture. Results revealed that these two fungal species have the ability to degrade keratin substrate. This is the first report of these two species in Korea.

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Chrysosporium fluviale; Chrysosporium indicum; keratin

1. Introduction

The fungal genus *Chrysosporium Corda* was first introduced by Corda in 1883 [1], but later it was reintroduced as *Chrysosporium* [2]. *Chrysosporium* spp. are ubiquitous, often keratinolytic anamorphic, and mostly inhabit soil or freshwater sediments, as well as feathers, skin, and hair of mammals, reptiles, and birds [3–6]. They are primarily characterized by whitish to pale colonies, conidia sessile or borne on a short stalk arising from the fertile hyphae, usually subglobose, pyriform or claviform, and released rhexolytically [7]. Based on the characteristics of the ascospore wall, enzymatic capabilities, and types of conidia, Currah [8] divided the order Onygenales into four different families: Onygenaceae, Gymnoascaceae, Arthrodermataceae, and Myxotrichaceae. Many *Chrysosporium* species have been already reported and their classifications have been studied [9]. As of 2016, 86 *Chrysosporium* species were recognized, excluding synonyms and invalid names [10]. These fungal species produce many useful metabolites, including keratinase, which is used widely in environmental protection, the chemical industry, and the medical and agricultural fields [11,12]. Although keratin waste is resilient in nature, it can be efficiently degraded by various microorganisms that secrete keratinolytic enzymes, a group of metallo- or serine proteases [13–15]. Keratin is an insoluble fibrous protein characterized by the presence of a high degree of cross-linkages by disulfide and hydrogen bonds [16]. They are grouped into hard keratin (e.g. hair, feather, nail, wool) having a high disulfide bond content and soft keratin (e.g. skin) with a low content of disulfide bonds. Keratin-rich wastes represent troublesome environmental contaminants and are produced in increasing quantities as waste from agro-industrial processes. Keratinolytic fungi have an ecological role in degradation of keratin substrates through their contribution to recycling the carbon, nitrogen, and sulfur from keratins [17–20]. Therefore, keratinolytic microorganisms are being considered as a biotechnological alternative for recycling and degradation of keratin waste.

The objective of this study was to assess the diversity of indigenous fungi in Korean soil based on the morphological characterization and molecular analysis of the sequences of the internal transcribed spacer (ITS) region. In addition, here, we examined the keratinolytic activity of these newly identified fungal species.

2. Materials and methods

2.1. Soil sampling and isolation of fungi

Soil samples were collected in March 2016 from crop field soil at various locations in Gyeongnam (35.102124° N, 128.023072° E) and (34.525836° N, 128.275765° E), Gyeongsangnam-do, Korea. Samples

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were taken from a depth of 10–15 cm, air dried, and stored in plastic bags at 4 °C until use. Fungi were isolated using a conventional dilution technique [21]. One gram of each soil sample was suspended in 9 mL of distilled water, and the prepared suspension was vortexed, serially diluted, and cultured on potato dextrose agar (PDA; Difco Laboratories Detroit, MI, USA) plates. The plates were incubated for 7 days at 25 °C, until fungal colony growth was observed. After that, single colonies on these plates were cultured by transferring them onto new PDA plates, supplemented with 100 mg/L chloramphenicol (a bacteriostatic agent) for 5–7 days at 25 °C until the fungal colony was observed. The pure cultures were maintained on PDA slants at 4 °C for future use.

2.2. Morphological characterization

Morphological characteristics of isolates KNU16-74 and KNU16-99 were observed on potato dextrose agar (PDA), Czapek yeast extract agar (CYEA), malt extract agar (MEA), oat meal agar (OMA), and yeast extract sucrose agar (YESA). The strains were inoculated at three points on 9 cm petri dishes and incubated at 25 °C in the dark for 7 days. All the media were prepared as described by Samson et al. [22]. The diameter of the colonies on the different agar media was measured after incubation and the degree of sporulation was examined. Colony color (front and back sides) was described according to Kornerup and Wanscher [23]. Photomicrographs were taken with an HK 3:1 CMOS digital camera (KOPTIC, Seoul, Korea) attached to an Olympus BX50F-3 microscope (Olympus Optical Co. Ltd., Tokyo, Japan) and a scanning electron microscope (SEM; Carl Zeiss, Cambridge, MA, USA).

2.3. Genomic DNA extraction, sequencing, and phylogenetic analysis

Genomic DNA was extracted from isolates KNU16-74 and KNU16-99 using a DNeasy Plant Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The ITS region was amplified using primers ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) under the following conditions: initial denaturation 2 min at 95 °C, followed by 35 cycles of annealing for 30 s at 60 °C, extension for 60 s at 72 °C, and denaturation for 30 s at 95 °C, and one final step of extension for 7 min at 72 °C [24]. The amplified PCR products were sequenced using an ABI prism 3730 DNA analyzer (Applied Biosystems, Foster City, USA). The sequences were compared with reference ITS sequences from GenBank at the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST). The nucleotide sequences were deposited in GenBank and assigned accession numbers KY906216 and KY906217 for the isolates KNU16-74 and KNU16-99, respectively. Evolutionary distance matrices based on the neighbor-joining algorithm were calculated using Kimura's two-parameter model [25]. Phylogenetic relationships were analyzed using Molecular Evolutionary Genetic Analysis (MEGA 6) software [26]. Bootstrap analysis was performed with 1000 replications to determine the support for each clade.

2.4. Keratinolytic activity

Fifty grams of human hair was disinfected with ethanol (3%), washed thoroughly under running water, and then dried at 60 °C. The hair strands were cut into pieces of about 2 cm length and autoclaved at 121 °C for 20 min [27]. To assess the keratin substrate degradation, the selected microorganisms were cultured in 250 mL Erlenmeyer flasks containing 50 ml medium and 50 mg of keratin substrates. The flasks were inoculated with mycelium from each strain, incubated for 7 days at 25 °C. A basal solution of the following composition was applied (g/L): 0.1, KH_2PO_4; 0.01, $CaCl_2$; 0.1, $FeSO_4.7H_2O$; 0.005, $ZnSO_4$; pH 7.5 [28]. The control contained only basal mineral solution and keratin substrate without microorganisms. All the flasks were incubated in a rotary shaker at 100 rpm and 27 °C for 21 days. The cultivation experiments were performed in triplicate. The fungal cultures were filtered on Whiteman filter paper and dried at 60 °C for 48 h. The strand fragments with mycelium were analyzed by SEM.

The SEM examination was performed on hair samples after 21 days of incubation with fungal strains. The human hair strand samples recovered from culture medium of biodegradation experiments were placed on metallic support, aluminum standard stub, using a double-sided adhesive carbon tape. The SEM images were taken at an accelerating voltage of 12.5–15 kV and 250–50 Pa using a gaseous secondary electron detector. Micrographs of the samples were investigated at different magnifications to identify changes after the degradation process. For each sample, 8–10 micrographs were performed and the relevant images were presented.

2.5. Release of protein

After four weeks of incubation, the release of protein was calculated by following the method described by Lowry et al. [29]. Experimental values

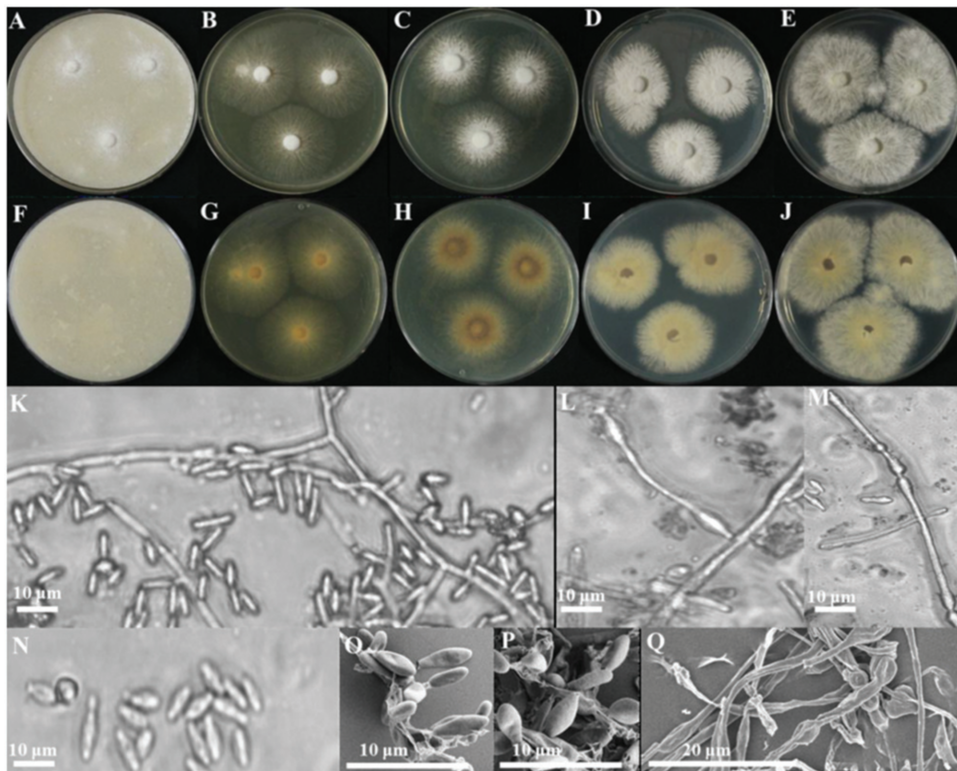

Figure 1. Morphological characteristics of *Chrysosporium indicum* (KNU16–74) grown for 7 days at 25 °C on potato dextrose agar (PDA), oatmeal agar (OMA), Czapek yeast extract agar (CYEA), yeast extract sucrose agar (YESA), and malt extract agar (MEA). (A–E) Obverse colony from left to right. (F–J) Reverse colony from left to right grown on OMA, MEA, CYEA, YESA, and PDA. (K) Hyphae. (L, Q) Intercalary conidia. (M) Racquet hyphae. (N–P) Conidia.

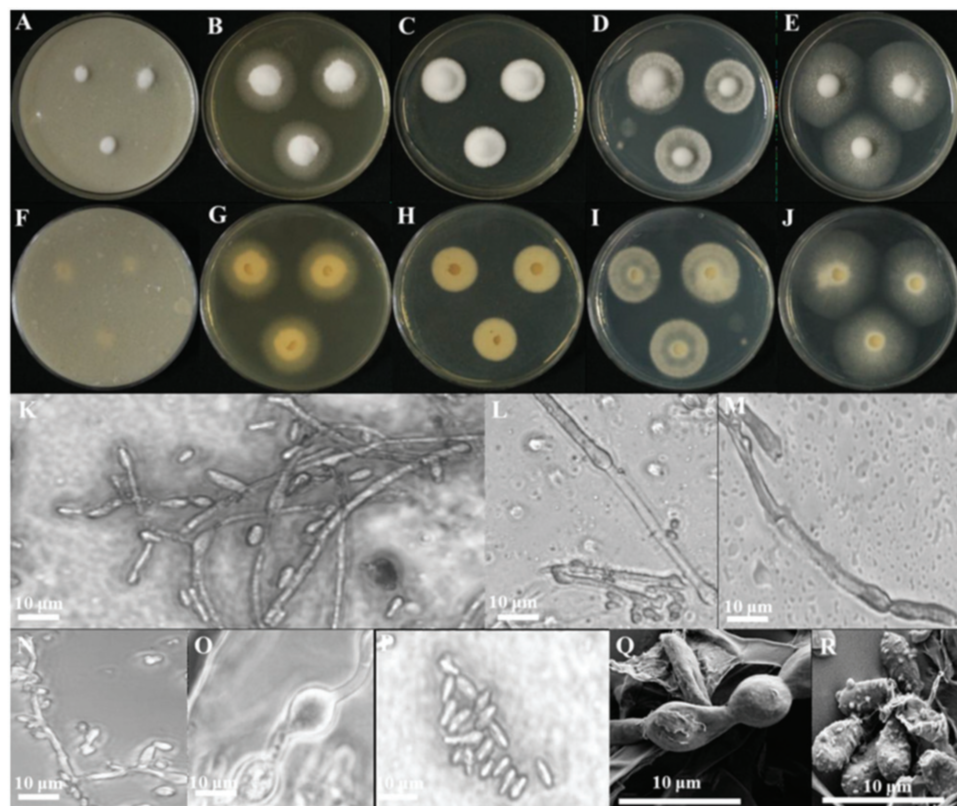

Figure 2. Morphological characteristics of *Chrysosporium fluviale* (KNU16–99) grown for 7 days at 25 °C on potato dextrose agar (PDA), oatmeal agar (OMA), Czapek yeast extract agar (CYEA), yeast extract sucrose agar (YESA), and malt extract agar (MEA). (A–E) Obverse colony from left to right. (F–J) Reverse colony from left to right grown on OMA, MEA, CYEA, YESA, and PDA. (K, N) Hyphae. (L, M) Racquet hyphae. (O, Q) Intercalary conidia. (P, R) Conidia.

were presented as mg of protein per mL of supernatant.

2.6. Substrate decomposition

The rate of hair decomposition was determined using the method of Chester and Mathison [30].

3. Results

3.1. Taxonomy

Chrysosporium indicum (Randhawa and Sandhu, 1966) [31]

Colony diameter (mm): 25 °C, 7 days; OMA 35–40, MEA 34–38, CYEA 30–34, YESA 33–37, PDA 35–3

Colony characters: 25 °C, 7 days; OMA: Colonies moderately deep, irregular form, margins defined, fimbriate, mycelium white in color, thin, granular surface texture, fringe, poor sporulation, soluble pigments absent, exudates absent (Figure 1(A, F)). MEA: Colonies translucent, circular form, margin entire, mycelium white in color, cottony texture, soluble pigments absent, exudates absent, reverse pale cream color (Figure 1(B, G)). CYEA: Colonies fairly deep, planar, margin uniform, mycelium white, powdery surface texture, fringe, poor sporulation, soluble pigment absent, exudates absent, reverse dark pale brownish-yellow at center and light at edge (Figure 1(C, H)). YESA: the colonies are reasonably fast-growing, thin, white in color, cottony texture, poor sporulation, exudates absent, and soluble pigment absent, reverse pale yellow (Figure 1(D, I)). PDA: Colonies passably deep, fast-growing, thin, mycelium white and light grey, cottony surface texture, margin regular, soluble pigments absent, exudate absent, reverse pale yellow and white edge (Figure 1(E, J)).

Micromorphology: Hyphae were hyaline, smooth and thin-walled, branched; 1.5–4.7 µm wide (Figure 1(K)); racquet hyphae present (Figure 1(M)). Conidia were 3.5–5.5 × 1.5–3.0 µm and were terminal and lateral, sessile, solitary, obovoid to ellipsoid sub-hyaline, cymbiform, smooth to slightly echinulate, thin-walled (Figure 1(N, O, P)). Intercalary conidia were present (Figure 1(L, Q)) and chlamydospores absent.

Species examined: South Korea, Gyeongnam, field soil, March 2016

NIBR (National Institute of Biological Resources, Korea) registration number: NIBRFG0000499372

Habitat: Field soil

Chrysosporium fluviale (Vidal et al. 2000) [32]

Colony diameter (mm): 25 °C, 7 days; OMA 24–28, MEA 26–30, CYEA 25–29, YESA 18–22, PDA = 19–23

Colony characters: 25 °C, 7 days; OMA: Colonies moderately slow growing, powdery to downy, flat, mycelium white in color, powdery surface texture, profusely sporulation, slightly raised, margin defined, frequently slightly undulate, absence of soluble pigment, exudates absent, reverse pale yellow in color (Figure 2(A, F)). MEA: Colonies comparatively fast growing, powdery surface structure, margin entire, circular form, dark white at center and translucent edge, sporulation dense, slightly undulate, absence of soluble pigment, exudates absent, reverse brownish orange shade (Figure 2(B, G)). CYEA: Colonies fairly deep, flat, margin defined, mycelium white, texture cottony, sporulation dense, soluble pigment absent, exudates absent, reverse pale cream color (Figure 2(C, H)). YESA: Colonies are flat, white to cream-colored with granular surface, margin regular, profuse sporulation absence of soluble pigment,

Table 1. Sequences used in this study, including isolate GenBank accession numbers.

Species	Collection Number	GenBank accession no.
Aphanoascus hispanicus	IMI 335379	AJ439438
Aphanoascus punsolae	IMI 334818	AJ439440
Aphanoascus durus	FMR 5651	AJ439434
Aphanoascus clathratus	IMI 329400	AJ439436
Aphanoascus pinarensis	FMR 4221	AJ439433
Aphanoscus foetidus	CBS 452.75	AJ439448
Aphanoascus Canadensis	UAMH 4574	AJ439435
Aphanoascus Keratinophilus	IMI 319010	AJ133436
Aphanoascus fulvescens	NBRC 8390	JN943431
Aphanoascus verrucosus	NBRC 32382	JN943439
Chrysosporium indicum	CBS 117.63	NR145203
Chrysosporium indicum	CBS 117.63	AJ005369
Chrysosporium indicum	FMR 6021	AJ439446
Chrysosporium minutisporosum	IMI 379912	AJ131689
Chrysosporium fluviale	FMR 6005	AJ005367
Chrysosporium submersum	FMR 6088	AJ131686
Chrysosporium siglerae	UAMH 6541	AJ131684
Chrysosporium lucknowense	IMI 112798	AJ131682
Chrysosporium mephiticum	CBS 320.86	AJ131683
Chrysosporium keratinophilum	IFO 7584	AJ131681
Chrysosporium shanxiense	EB1601M.3	KX462170
Chrysosporium europae	CBS 321.86	AJ007843
Chrysosporium lobatum	CBS 666.68	AJ131688
Chrysosporium pilosum	FMR 2157	AJ390385
Chrysosporium undulatum	IMI 375884	AJ007845
Chrysosporium carmichaelii	CBS 643.79	AJ007842
Chrysosporium vallenarense	ATCC 64421	AJ390389
Chrysosporium vespertilium	RV 27093	AJ007846
Chrysosporium pseudomerdarium	UAMH 4330	AJ390386
Chrysosporium filiforme	CBS 187.82	AJ131680
Chrysosporium synchronum	IMI 282433	AJ390386
Chrysosporium guarroi	CCFVB CH10	EU018451
Nannizziopsis vriessii	RKI 04-0104	HF547869
Nannizziopsis chlamydospora	UTHSC 04-2056	HF547870
Nannizziopsis draconii	CCFVB CH12	EU883993
Candida albicans	CBS 1905	AB018038
Chrysosporium indicum	KNU16-74	KY906216
Chrysoporium fluviale	KNU16-99	KY906217

ATCC: American Type Culture Collection, Maryland, USA; CBS: Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; CCFVB: Culture Collection of the Veterinary Mycology Group, Bellaterra, Barcelon, Spain; FMR: Facultat de Medicina de Reus, Spain; IFO: Institue of Fermenation, Osaka, Japan; IMI: International Mycological Institue, Egham, UK; RV: Institut de Medecine Tropicale "Prince Leopold", Antwerpen, Belgium; UAMH: University of Alberta Microfungus Collection and Herbarium, Edmonton, Canada.

exudates absent, reverse cream color (Figure 2(D, I)). PDA: powdery to downy, fairly dense, abundantly sporulated, mycelium light white, margin defined, frequently slightly undulate, absence of soluble pigment and exudates, reverse pale yellow in color (Figure 2(E, J)).

Micromorphology: Hyphae were hyaline, closely septate, smooth-walled, 1–3.5 µm wide, straight or slightly curved, branched (Figure 2(K, N)) and with racquet hyphae present (Figure 2(L, M)). Conidia were terminal and lateral, thin-walled and covered with minute warts, hyaline to sub hyaline). Conidia were over the main hyphae swollen protrusions, solitary, 3.5–4.8 × 1.5–3.0 µm (Figure 2(P, R)). Intercalary conidia present (Figure 2(O, Q)) but chlamydospores absent.

Species examined: South Korea, Gyeongnam, field soil, March 2016

NIBR (National Institute of Biological Resources, Korea) registration number: NIBRFG0000499379

Habitat: Field soil

3.2. Molecular phylogeny

To determine the phylogenetic relationship between KNU16-74 isolated from the field soil and its related species, ITS regions (ITS1-5.8s-ITS2) were analyzed. Moreover, molecular identification was carried out using the MEGA6 program by comparing the ITS sequences of the study isolates with those of other strains available in GenBank (Table 1). ITS-5.8S rDNA supports the greatest confidence for

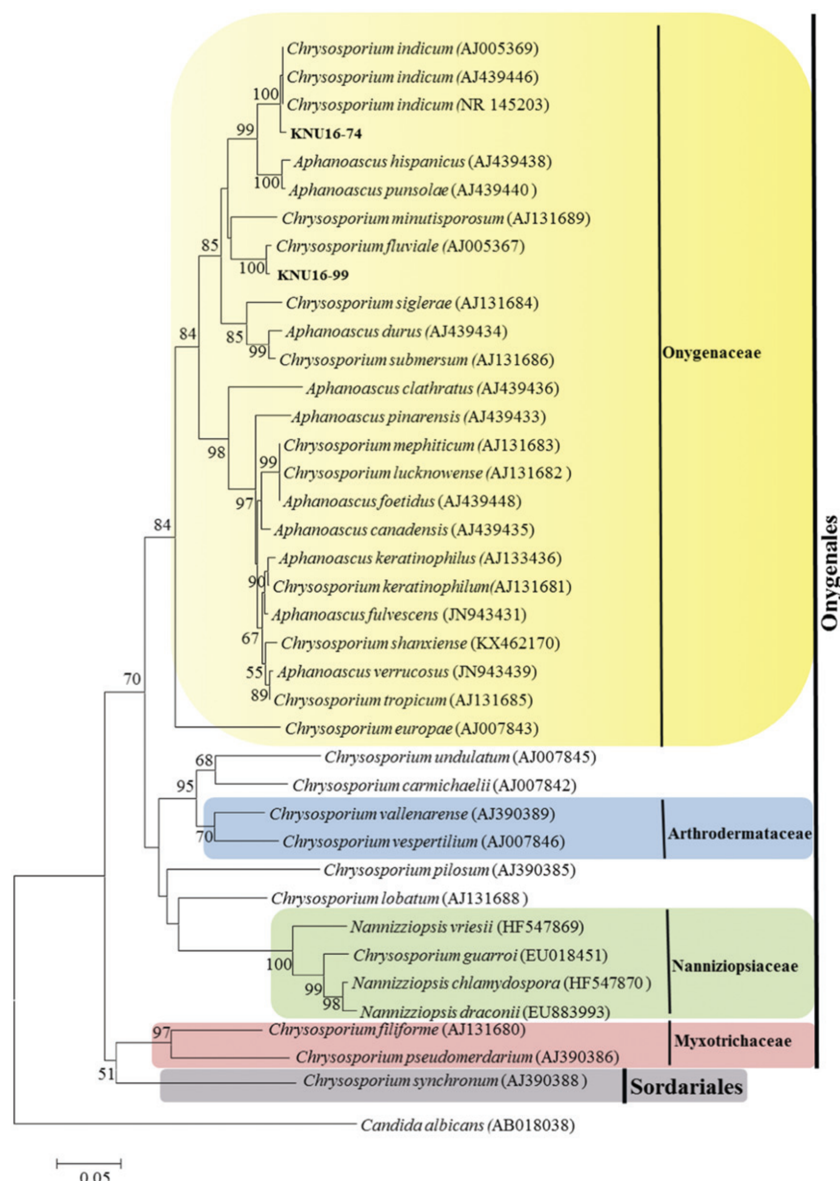

Figure 3. Neighbor-joining tree based on ITS sequences for *Chrysosporium indicum* KNU16–74, *Chrysosporium fluviale* KNU16–99 and some related species; *Candida albicans* used as out-group. The phylogenetic tree was constructed using the MEGA6 program. The sequence obtained in the study is shown in boldface. Numerical values (>50) on branches are the bootstrap values as percentages of bootstrap replication from an analysis of 1,000 replicates. The scale bar represents the number of substitutions per site.

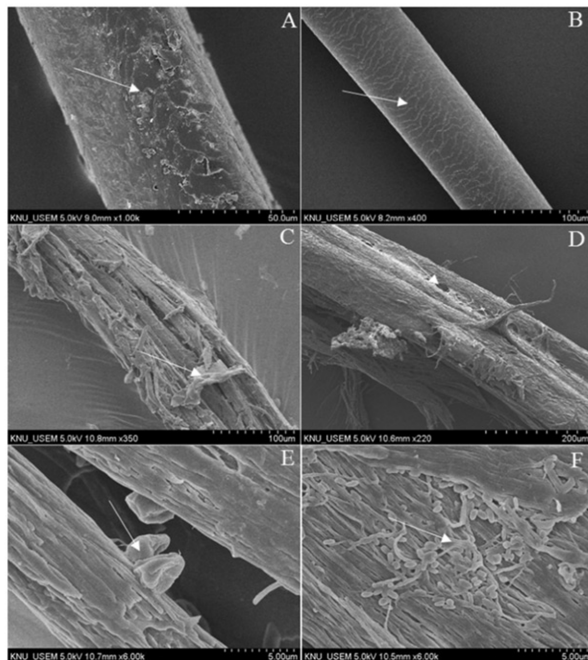

Figure 4. SEM images of human hair strands inoculated with keratinolytic fungi (KNU16–99) grown on agitated liquid cultures (micrographs of 21-d culture). (A) Crude hair strand. (B) Normal hair strand after cleaning operation. (C) Tunnel (arrow) fungal filaments detached from strand surface: (D) Tunnel perpendicular on strand axis. (E) Cable hyphae (arrow); tunnel perforating hair strand. (F) Fungal spores covering the hair strand.

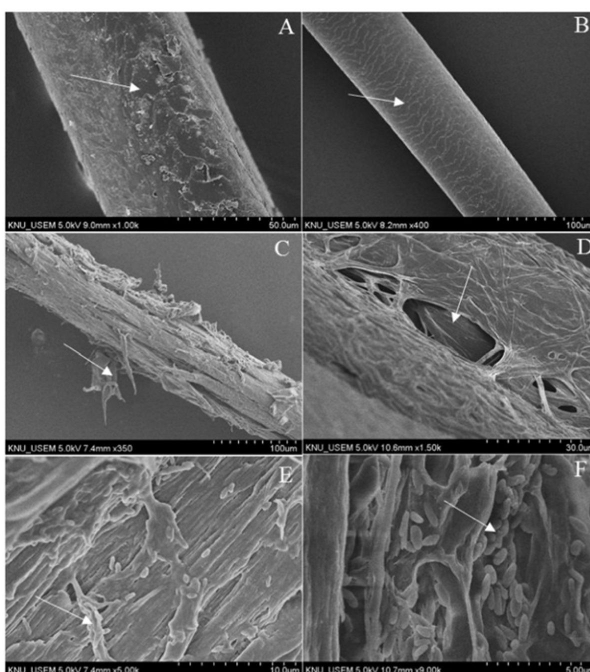

Figure 5. SEM images of human hair strands inoculated with keratinolytic fungi (KNU16–74) grown on agitated liquid cultures (micrographs of 21-d culture). (A) Crude hair strand. (B) Normal hair strand after cleaning operation. (C) Tunnel (arrow) fungal filaments detached from strand surface. (D) Tunnel perpendicular on strand axis. (E) Cable hyphae (arrow); tunnel perforating hair strand. (F) Fungal spores covering the hair strand.

Table 2. Protein released and percentage of human hair decomposition during the growth of *C. indicum* and *C. fluviale* after 4 weeks of incubation.

S. N	Tested isolates	Protein released (μg/mL)	Percentage decomposition
1	*Chrysosporium indicum*	202.6	23.2
2	*Chrysosporium fluviale*	197.4	21.7

molecular identification of most fungal species [33]. NCBI BLASTn search with homology analysis revealed that KNU16-74 shared 100% sequence identity with *Chrysosporium indicum* CBS 117.63 (GenBank accession numbers AJ005369, ITS) and *Chrysosporium indicum* FMR 6021 (GenBank accession No. AJ439446, ITS), clustering together in the same clade (Figure 3). The isolate of KNU 16-99 has 100% sequence identity with *Chrysosporium fluviale* FMR 6005 (accession No. AJ005367, ITS).

3.3. Keratin degradation

An experiment was carried out to assess the keratin waste degradation efficacy of our study isolates. Results revealed that both of our study isolates (KNU16-74 and KNU16-99) were able to degrade keratin waste (Figures 4, 5). Crude hair and normal hair strand after cleaning operation was observed through SEM analysis. Degradation was not observed in the control condition (Figure 4(A, B)). Tunnel fungal filaments detached from hair strand surface were observed for the isolate KNU16-74 (Figure 4(C, D)). Furthermore, hair strand was found to be covered with fungal spores (Figure 4(E, F)) for the isolate KNU16-74. In addition, attachment of fungal spores and filaments was observed in KNU16-99-treated hair strand (Figure 5). Degradation of keratin in the case of crude and normal hair was not observed in control (Figure 5(A, B)). However, KNU16-99-inoculated hair strands were found degraded (Figure 5(C, D, E, F)). In addition, the efficacy of keratinolytic activity was evaluated by release of protein and percentage of decomposition of human hair (Table 2). Among the tested isolates, *C. indicum* released protein amounting to 202.6 μg/mL while *C. fluviale* released 197.4 μg/mL protein. *C. indicum* decomposed human hair by 23.2%, followed by *C. fluviale* at 21.7%.

4. Discussion

In an indigenous fungal diversity survey, the soil samples were collected from the crop field soil of Gyeongnam, Korea. Morphologically distinct fungal isolates were isolated using dilution technique. In this study, we found two distinct isolates that have not been reported previously in Korea. We designated these isolates as KNU16-74 and KNU16-99. The conidia, structure of hyphae, colony

Table 3. Morphological characteristics of KNU16-74 and the reference species *Chrysosporium indicum*.

Character		Study isolate KNU16-74	*Chrysosporium indicum*[a]
Colony	Color	White; reverse pale yellow	White; reverse pale cream color
	Shape	Fringe, dense and felty, uniform, poor sporulation	Margin defined, fringe, regular, poor sporulation
	Size (mm)	PDA = 34 ∼ 36,MEA = 34 ∼ 35,OMA = 24 ∼ 26,YESA,34 ∼ 36,CYEA = 30 ∼ 32 after 7 days at 25oC	PDA = 40 ∼ 45 after 14 days at 25 °C.
Hyphae	Shape	Hyphae hyaline, straight and smooth, branch	Hyaline, smooth and thin walled, more frequently branched when fertile
	Size (μm)	1.5 ∼ 4.7 wide	1.5 ∼ 5 wide
	Racquet hyphae	Present	Present
Conidia	Shape	Obovoid to ellipsoid, cymbiform conidia, solitary, terminal and lateral conidia	Terminal and lateral conidia, swollen lateral branches, solitary, sub-hyaline, 1 Celled, cymbiform
	Size	(3.5 ∼ 5.5) × (1.5 ∼ 3.5)	(3.5 ∼ 7.5) × (1.5 ∼ 3) μm
	Wall	Smooth	Smooth
	Intercalary conidia	Present, infrequent	Infrequent
Clamydospores	Shape	Absent	Absent

[a]Source of description [2,33].

Table 4. Morphological characteristics of KNU16-99 and the reference species *Chrysosporium fluviale*.

Character		Study isolate KNU16-99	*Chrysosporium fluviale*[a]
Colony	Color	Front view white in color while reverse brownish orange	white, reverse brownish orange.
	Shape	margin entire, slightly raised, radially folded, dense sporulation	margin defined, slightly raised profusely sporulating
	Size (mm)	PDA = 45, MEA = 64, OMA = 42, YESA = 45 CYEA = 62 at 30 °C in 14 days	30 ∼ 40 diameter on YESA at 30 °C in 14 days
Hyphae	Shape	Hyaline, septate	Hyphae hyaline, closely septate
	Size (μm)	1 ∼ 3.5 wide	1 ∼ 3(∼5) wide
	Racquet hyphae	Presence	Presence
Conidia	Shape	Terminal and lateral, Obovate, clavate, ellipsoid or pyriform	Conidia hyaline to sub-hyaline, terminal and lateral, solitary, obovate, 1 celled
	Size (μm)	(3.5 ∼ 4.8) × (1.5 ∼ 3.0)	(3.5∼)4 ∼ 6.5(15) × (1∼)2 ∼ 3(∼3.5)
	Wall	covered with minute warts	regularly minutely warty
	Intercalary conidia	Absent	Very rare
Clamydospores	Structure	Absent	Rare

[a]Source of description [32,33,40].

characteristics, and infrequent intercalary conidia of KNU16-74 isolate matched with the description of *C. indicum* [1,34,35]. Apinis [35] reported that *C. indicum* is an anamorph of *Aphanoascus terrus*. Similarly, colony characteristics, structure of hyphae, racquet hyphae, conidia of KNU16-99 (Figure 2) reasonably fits with the description of *C. fluviale* [32,35,36]. The clear comparison between our study isolate and a previously described isolate has been drawn in Tables 3 and 4.

Our two newly recorded fungal isolates from Korea have the potential to degrade keratin waste. SEM micrograph shows detailed information on interaction between microorganism and structural elements of the hair strand. One may notice that both strains were able to develop sufficient structures related to biodegradation, such as hyphae sleeve covering the strand, hyphae network and mycelium attached to the surface, and hypha penetrating the cortex below the scale cuticle (Figures 4, 5). Cortex, medulla and cuticle are three main structural components of hair [37]. Micrographs revealed that both tested strains were able to develop adequate structures related to surface erosion and radial penetration. *C. indicum* has the capacity to degrade human hair [38]. This keratin degradation results shows that *C. fluviale* KNU16-99 and *C. indicum* KNU16-74 are also potential keratinase-producing strains. In addition, *C. indicum* was found to be the most promising isolate for protein release and human hair decomposition [39]. In our study, we also observed similar results (Table 2).

Conclusively, *C. indicum* and *C. fluviale* have been reported for the first time in Korea and shown to be potential keratin waste degraders. Further studies regarding keratinolytic activity by these fungal isolates would be worthwhile.

Disclosure statement

No potential conflict of interest was reported by the authors.

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