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New Report of Three Unrecorded Species in *Trichoderma harzianum* Species Complex in Korea

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ABSTRACT

The genus *Trichoderma* (Hypocreaceae, Ascomycota) consists of globally distributed fungi. Among them, *T. harzianum*, one of the most commonly collected *Trichoderma* species, had been known as a polyphyletic or aggregate species. However, a total of 19 species were determined from the polyphyletic groups of *T. harzianum*. Thus, we explored Korean *"T. harzianum"* specimens that were collected in 2013–2014. These specimens were re-examined based on a recent study with translate elongation factor 1-alpha (EF1 α) sequences to reveal cryptic *Trichoderma* species in Korea. As a result, four different species, *T. afroharzianum*, the other three species have not been reported in Korea. In this work, we describe these species and provide figures. **ARTICLE HISTORY**

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EF1α; phylogeny; taxonomy; *Trichoderma*; *Trichoderma harzianum* species complex

1. Introduction

The genus *Trichoderma* Persoon (type species: *T. viride* Pers) is a large group of ascomycetous fungi that contains more than 250 species [1]. Some of the *Trichoderma* species are economically important because they produce industrial enzymes and antibiotics [2,3]. They can be applied as biocontrol agents against plant pathogens [2–4]. Morphologically, their colonies are typically fast-growing and greencolored; conidiophores are loosely or compactly tufted and often form concentric rings; microscopically, the typical conidiophore is pyramidal with paired branches, terminating in one or a few phialides; and conidia are typically ellipsoidal or rarely globose [5].

T. harzianum Rifai is known as the most common species within the genus worldwide [6]. This species is commonly found on various substrates such as soil, wood, and other fungi [2-4,7-9]. Additionally, *T. harzianum* is an economically important species because of its remarkable bioactive abilities, particularly its cellulolytic enzymes and anti-fungal ability [2,4,10].

T. harzianum has been known as an aggregate species because they are very similar and are polyphyletic [6]. Thus, they are named the *T. harzianum*

species complex [6,11]. In 2015, their cryptic species were re-evaluated by phylogenetic analysis with translate elongation factor 1-alpha (EF1 α) sequences, and they were described as 14 new species [4]. Among them, the well-known biological control agent *T. harzianum* T22 was determined to be *T. afroharzianum*, and the well-known teleomorphic species of *T. harzianum*, "*Hypocrea lixii* Pat.", was re-named to be *T. lixii* (Pat.) P. Chaverri [4].

In Korea, *T. harzianum* was reported for the first time by Lee and Lee (1980), and approximately four species of the Harzianum group have been reported to date: *T. pleuroti, T. pleuroticola, T. harzianum,* and *T. tomentosum* [8,12,13]. Three out of these four species were identified with EF1 α sequences, but *T. harzianum* has not been tested in Korea to create a detailed phylogeny [8,12,13]. Thus, we expected that some Korean cultures identified as "*T. harzianum*" might be different species.

Both morphological and molecular biological methods have been used to research the taxonomy of *Trichoderma* [4,14]. However, species in the Harzianum group are morphologically very similar, and the internal transcribed spacer rDNA (ITS), the most universal fungal molecular barcode, has a species resolution for this group that is too low [4]. For

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molecular biological analysis, recent studies used several DNA regions: actin (ACT), calmodulin (CAL), ITS, RNA polymerase II (RPB2), and EF1 α [4,8,9,11,13,14]. Among them, EF1 α is the most recommended DNA region for single-region analysis, and it has shown high species resolution for phylogeny of *Trichoderma* [4,13].

Since 2013, we have studied the diversity of *Trichoderma* in Korea and collected many "*T. harzianum*" isolates. However, a poly-phylogenetic group was revealed by the phylogenetic analysis based on EF1 α . To determine the identities of recently collected "*T. harzianum*", the cultures in Korea University Culture Collection (KUC; Korea Univ., Seoul, Korea) were re-analyzed with EF1 α sequences. Among them, four species, *T. afroharzianum*, *T. atrobruneum*, *T. pyramidale*, and *T. harzianum*, were identified. Of these four species, only *T. harzianum* has previously been reported in Korea. These species were morphologically examined and described in detail, *T. afroharzianum*, *T. atrobruneum*, *M. atrobruneum*, *T. atrobruneum*, *M. atrobruneum*, *T. atrobruneum*, *T. atrobruneum*, *T. atrobruneum*, *T. atrobruneum*, and *T. pyramidale*, were newly reported.

2. Materials and methods

2.1. Analysis of phenotype

A total of seven Trichoderma strains were obtained from Korea University Collection (KUC, Seoul, Korea). They were deposited in National Institute of Biological Resources (NIBR, Incheon, Korea). T. harzianum SFC20151215-09M was received from Marine Fungal Resource Bank (http://mfrb.snu.ac.kr; Seoul, Korea). They were previously identified as members of Harzianum group using ITS sequence analysis. They were examined on three types of media: corn meal dextrose agar (CMD: cornmeal 20 g/L, glucose 20 g/L, agar 18 g/L), potato dextrose agar (PDA: DifcoTM potato dextrose agar 39 g/L) or synthetic nitrogen-poor or nutrient-poor agar (SNA, synthetischer nährstoffärmer agar: sucrose 0.2 g/L, glucose 0.2 g/L, KNO₃ 1 g/L, KH₂PO₄ 1 g/L, MgSO₄·7H₂O 0.5 g/L, NaCl 0.5 g/L, agar 12 g/L). All media were quantified as 20 mL in 90 mm-diameter plastic Petri dishes. To analyze morphologies of colony, 6 mm-diameter. plug of cultures were inoculated about 1.5 cm from the edge of the Petri dish and they were cultured at room temperature: $20 - 25 \degree C$ [9]. The observations were performed for 7 - 14 days. The colors were standardized using Munsell colors [15]. We photographed the colonies using NEX-5R digital camera (Sony, Tokyo, Japan). The microscopic characters were observed from 3% KOH mounts with Olympus BX51 light microscope (Olympus, Tokyo, Japan). Olympus DP20 microscopic camera (Olympus, Tokyo, Japan) was used to take pictures of conidiophore and conidia. More than 30 units of each element were measured, and 5% of the measurements from each end of the range were removed and they were noted in parenthesis. The isolates were deposited at NIBR.

2.2. Phylogenetic analysis

Genomic DNAs of seven strains were extracted with Accuprep Genomic DNA extraction kit (Bioneer, Daejeon, Korea). PCR reactions were carried out according to the previously described method [11]. The primer set: EF1-728F [16], and TEF1–rev were used [14]. The PCR products were sequenced by Sanger method with 3730xl DNA Analyzer (Life technology; Carlsbad, CA, USA) by Macrogen (Seoul, Korea).

The obtained EF1 α were aligned with the selected reference sequences including type specimen from GenBank using MAFFT 7.130 [17], and modified manually with MacClade 4.08 [18]. The data metrics contained 29 taxa and 686 characters and their suitable DNA evolution model were tested by MrModeltest 2.3 using the AIC criteria with default options [19]. The HKY+G model was chosen under the AIC criteria. Bayesian analysis was carried out with MrBayes 3.2.1 [20]. Phylogenetic tree was constructed according to previously described method [21].

3. Results

3.1. Phylogenetic identification of T. harzianum species complex

The sequences of the T. harzianum species complex were re-identified by phylogenetic analysis based on the EF1 α region (Figure 1). The tree contains 29 taxa of Trichoderma spp. Our sequences were classified into four clades with the reported species. KUC21203, KUC21207, and SFC20151215-M09 KUC21205, formed a clade with the type specimen of T. harzianum with 100% posterior probabilities (PP). KUC21213 and KUC21214 were identified as T. afroharzianum with high PP (100%). KUC21091 and KUC21123 were grouped with T. pyramidale and T. atrobrunneum, respectively, with high PP (100% and 99%, respectively). To confirm this result, we observed and described their detailed morphological features.

3.2. Taxonomy

Trichoderma afroharzianum P. Chaverri, F.B. Rocha, Degenkolb & I. Druzhinina, Mycologia 107(3): 580 (2014) (Figure 2).

Colony on CMD, aerial mycelium abundant; cottony conidia typically abundant in aerial mycelium,



Figure 1. The 50% majority-rule consensus tree which contains 29 taxa and 686 characters obtained from the Bayesian analysis based on EF1 α Numbers above branches indicate posterior probabilities. Fungal cultures collected in this study are in bold. Sequences of type specimens are indicated by asterisk symbols (*).

dark olive green (5GY3/4) to light olive green (5GY5/4) colored; olive (5Y4/3) pigment diffused. On PDA aerial mycelium abundant, cottony, radiating; conidia abundant and disposed in concentric rings; dark olive green (5GY3/4) to light olive green (5GY5/4) colored; olive (5Y4/3) pigment diffused. On SNA aerial mycelium sparse, conidia forming abundantly in broad concentric bands, pale yellowish green (5GY6/4) colored; diffusing pigment not observed. No distinctive odor on all media Conidiophores pyramidal; branches opposing, somewhat widely spaced, terminating in a whorl of 2-5 phialides, whorls cruciate or nearly verticillate. Phialides lageniform to ampulliform, $(4.6-)4.7 - 7.9(-10.2) \times (2.2-)2.3 - 3.5(-3.7) \ \mu m$ (mean $6.2 \times 2.9 \,\mu$ m), phialides length/width ratio (1.9-)2 - 2.2(-2.8) (mean 2.1), base 1.2 - 2.4(-2.7)µm wide. Conidia subglobose to ovoid, (2.2-) $2.5 - 3.2(-3.4) \times (1.8 -)2.2 - 2.8 \,\mu\text{m}$ (mean $2.8 \times$ 2.5 μ m), length/width ratio 1.1(-1.2) (mean 1.1); smooth, green. Chlamydospores not observed.

Specimens examined: Republic of Korea, Seoul, N37°35′13.8″, E126°49′03.4″, soil of wetland beside

Han River, February 19 2014, Seokyoon Jang (KUC21213, NIBRFGC000501544, Genbank Acc. No. KX912217; KUC21214, NIBRFGC000501545, Genbank Acc. No. KX912218).

Known distribution: Worldwide [3].

Note: *Trichoderma afroharzianum*, KUC21213, and KUC21214 was in good agreement with the description of another study [4]. They are monophyletic with the type strain of *T. afroharzianum* (GJS 04–186) with high support (100% of PP, Figure 1). Morphologically, this species is difficult to distinguish from other species in the *T. harzianum* species complex [4]. Molecular analysis with the EF1 α sequence is required to differentiate them.

Trichoderma atrobrunneum F.B. Rocha, P. Chaverri & W. Jaklitsch, Mycologia 107(3): 580 (2014) (Figure 3).

Colony on CMD, aerial mycelium abundant, cottony, with concentric rings; conidia forming abundant in the cottony pustules, greyish green (5G4/2) to pail green (5GY6/2). On CMD, aerial mycelium abundant, cottony, conidia forming abundantly, with concentric rings, greyish green (5G4/2) to pale



Figure 2. Trichoderma afroharzianum KUC21213. (A–C) Conidiophore. (D) Conidia. Scale bar $=10 \,\mu$ m. (E) Colonies on CMD (top); PDA (middle); and SNA (bottom).



Figure 3. Trichoderma atrobruneum KUC21223. (A–C) Conidiophore. (D) Conidia. Scale bar $=10 \,\mu$ m. (E) Colonies on CMD (top); PDA (middle); and SNA (bottom).



Figure 4. *Trichoderma harzianum* KUC21203. (A-C) Conidiophore. (D) Conidia. Scale bar $=10 \,\mu$ m. (E) Colonies on CMD (top); PDA (middle); and SNA (bottom).

olive (5Y6/4) or pale green (5GY6/2). On SNA, conidia forming abundant in the cottony pustules, dark olive green (5GY3/4) to pale olive green (5GY6/4) colored, conidiation sometimes rare. No distinctive odor or pigment on all media. Conidiophores pyramidal, with opposing, somewhat widely spaced branches, terminating in a whorl of 2-5 phialides, whorls cruciate or nearly verticillate. Phialides ampulliform to lageniform, (3.3-)3.9-7.1 (-9.5) $\times 2.5 - 3.4(-3.7)$ µm (mean 5 \times 3 µm), phialides length/width ratio (1.3-)1.5-2.1(-2.7) (mean 1.7), base (1.1-)1.2 - 2.0(-2.1) µm wide. Conidia subglobose to ovoid, $(2.2-)2.3 - 3.8(-4.3) \times (2.4-)$ 2.5 - 3.4(-3.5) µm (mean 2.7×2.4 µm), length/ width ratio (1-)1.1(-1.2) (mean 1.1), smooth, green. Chlamydospores not observed.

Specimens examined: Republic of Korea, Gangwondo, Pyoengchang-gun, Odaesan National Park, N37°44′08.3″, E128°35′23.3″, topsoil of fir forest, April 2013, Seokyoon Jang (KUC21123, NIBRFG C000501546, Genbank Acc. No. KX912192).

Known distribution: Europe [3], North America [3], and Republic of Korea.

Note: *Trichoderma atrobrunneum* KUC21123 was in good agreement with the description from another study, but a diffusing pigment was not observed [4]. KUC21123 and the type strain of *T. atrobrunneum* (GJS 92-110) are monophyletic with

high support (99% of PP, Figure 1). In microscopic morphology, species in the *T. harzianum* species complex are very similar for detailed identification [4]. To identify it from other *Trichoderma* species, we recommend matching the molecular barcode based on the EF1 α sequence.

Trichoderma harzianum Rifai, Mycol. Pap. 116:38 (1969) (Figure 4).

Colony on CMD, aerial mycelium abundant, cottony pustules forming around inoculation; conidia forming abundantly, greyish green (5G4/2) colored. Colony on PDA, aerial mycelium abundant; conidia forming abundantly, with broad concentric rings, greyish green (5G4/2) to pale olive (5Y6/4) or pale green (5GY6/2). On SNA conidia forming abundantly, broad concentric rings; aggregated with pustules, pale green (5GY6/2) colored. No distinctive odor or pigment on all media. Conidiophores pyramidal with opposing branches, widely spaced branches, terminating in a whorl of 2-5 phialides, whorls cruciate or nearly verticillate. Phialides ampulliform to lageniform, $(4.4-)5-9.1(-12.2) \times$ (3-)3.2 - 4.3(-4.7) µm (mean 6.9×3.7 µm), phialides length/width ratio (1.5-)1.6 - 2.1(-2.6) (mean 1.9), base (1.4-)1.6-2.6(-2.9) µm wide (mean 2.0 μ m). Conidia subglobose to ovoid, (2.4–) $2.8 - 3.8(-4.3) \times (2.4 -)2.5 - 3.4$ (-3.5) µm (mean $3.3 \times 2.9 \,\mu\text{m}$), length/width ratio (1-)1.1(-1.2)



Figure 5. *Trichoderma pyramidale* KUC21091. (A) Conidiophore. (B) Conidia. Scale bar $=10 \,\mu$ m. (C) Colonies on CMD (top); PDA (middle); and SNA (bottom).

(mean 1.1), smooth, green. Chlamydospores not observed.

Specimens examined: Republic of Korea, Seoul, N37°35'13.8", E126°49'03.4", soil of wetland beside Han River, February 19 2014, Seokyoon Jang (KUC21203, NIBRFGC000499791, Genbank Acc. No. KX912207; KUC21205, NIBRFGC000501548, Genbank Acc. No. KX912209; KUC21207, NIBRFG C000501548, Genbank Acc. No. KX912211); Gyeo ngsangnam-do, Sacheon-si, N34°59'10.9", E128° 2'39", mud flat, 15 December 2015, Myung Soo Park (SFC20151215-M09).

Known distribution: Europe [4], North America [4], and Republic of Korea.

Note: *Trichoderma harzianum* has been reported and described in Korea [12], but it was recently re-evaluated and described in other studies [4]. KUC21203, KUC21205, and KUC21207 have relatively smaller conidia than previous descriptions in Korea [12], but *T. harzianum* was well-matched with descriptions of the type specimen [4].

Trichoderma pyramidale W. Jaklitsch & P. Chaverri, Mycologia 107(3): 580 (2014) (Figure 5).

Colony on CMD, aerial mycelium abundant in margin, cottony pustules forming around inoculation; conidia forming abundantly, greyish green (5G4/2) colored; olive (5Y4/3) pigment diffused. Colony on PDA, aerial mycelium abundant; conidia

forming abundantly, with broad concentric rings, greyish green (5G4/2) colored; olive (5Y4/3) pigment diffused. On SNA conidia forming abundantly, broad concentric rings; aggregated with large pustules (>1 mm), pale green (5GY6/2) colored; diffusing pigment not observed. No distinctive odor on all media. Conidiophores pyramidal with unpaired branches, branches often replaced by phialides, terminating in a whorl of 2-5 phialides, whorls cruciate or verticillate. Phialides lageniform or ampulliform, $(3.9-)4.3-9.3(-10.5) \times (2.5-)2.6-3.4(-3.6)$ μm (mean $6.3 \times 3 \,\mu$ m), phialides length/width ratio 1.6 - 2.7(-2.9) (mean 2.1), base 1.2 - 2.5(-2.9) µm wide, solitary or in whorls of 2 - 4(-5). Conidia glosubglobose or ovoid, (2.2-) 2.4-2.9bose, $(-3.2) \times (2.1-)$ 2.4 – 2.8 µm (mean 2.7 × 2.5 µm), length/width ratio (1.0-)1.1(-1.2) (mean 1.1); smooth green. Chlamydospores not observed.

Specimens examined: Republic of Korea, Seoul, N37°35′13.8″, E126°49′03.4″, soil of wetland beside Han River, February 19 2014, Seokyoon Jang (KUC21091, NIBRFGC000501547, Genebank Acc. No. KX912188).

Known distribution: Southern Europe [4] and Republic of Korea.

Note: *Trichoderma pyramidale* KUC21291 were in good agreement with the description from another study [4]. KUC21123 and the type strain of *T. pyramidale* (CBS 135574) are monophyletic with high support (100% of PP, Figure 1). The name "*pyramidale*" refers to the pyramidal structures of its conidiophores, but most of other species in the *T. harzianum* species complex have pyramidal conidiophores [4]. Other microscopic features of *T. pyramidale* are similar to those of the allied species. DNA sequences should be analyzed to identify this species.

4. Discussion

4.1. Phylogeny of the T. harzianum species complex

The topology of the tree (Figure 1) was not in total agreement with that of another study based on ACT, EF1a, CAL and ITS [4] because we used EF1 α sequences only. However, the EF1 α -only phylogeny can determine the cryptic species in the T. harzianum species complex [4]. The other study showed that a clade contained T. pleuroti and eight other species as the sister group of the T. harzianum species complex clade [4], but the T. pleuroti clade belonged to the T. harzianum clade in our phylogenetic tree. Despite the topological differences, the identification of each species was reliable because of the high support of each branch. Further study with Korean specimens of the T. harzianum species complex using a multi-gene phylogeny is needed to confirm the systemics of the Harzianum group.

4.2. T. harzianum in Korea

T. harzianum has been reported and described in Korea [12], but it was recently re-evaluated and described in another study [4]. KUC21203, KUC21205, and KUC21207 have relatively smaller conidia than previous descriptions in Korea [12], but they were in good agreement with the description of the type specimen [4]. Re-identification of the Korean type culture of *T. harzianum* is required.

A total of four species were revealed from a small quantity of cultures, which were previously identified as "*T. harzianum*" from the soil. The habitable range of the genus *Trichoderma* is very broad [2]. However, our study was limited to a few *Trichoderma* cultures isolated in narrow habitats. It is necessary to re-examine "*T. harzianum*" cultures from diverse habitats: soil, marine environments, plants and other fungi. We presume that more cryptic species will be revealed by further studies with greater numbers of strains from various sources.

Disclosure statement

No potential conflict of interest was reported by the authors.

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