Diversity of Aspergillus, Penicillium, and Talaromyces Species Isolated from Freshwater Environments in Korea

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ABSTRACT

In order to elucidate the fungal diversity and community structure in freshwater environments, numerous fungal strains were isolated from freshwater, submerged soils, twigs, dead insects, etc. Among them, the present study has focused specifically on Aspergillus, Penicillium, and Talaromyces species, which produce diverse useful metabolites in general. Twelve strains of Aspergillus isolated were identified as A. japonicus (n = 5), A. tubingensis (3), A. niger (2), and A. flavus (2). 10 strains of which belong to Penicillium section Nigri, named black Aspergillus. Eight strains of Penicillium were identified as P. brasiliannum (n = 3), P. oxalicum (2), P. crustosum (1), P. expansum (1), and P. piscarium (1). Two different strains of Talaromyces were identified as T. pinophilus and T. versatilis. Thus far, Penicillium piscarium and Talaromyces versatilis have been unrecorded in Korea, for which we provide detailed morphological and molecular characteristics.

1. Introduction

Freshwater is a diverse and complex environment for microorganisms and provides several types of habitat for fungi, e.g., plant litters (such as fallen leaves and decaying woods), soil, aquatic insects, and aquatic plants [1]. In the ecosystem, fungi play a major role in regulating nutrients and carbon cycles by decomposing organic matter and producing secondary metabolites [2]. Compared to fungi inhabiting other easily accessible substrates, the diversity and community structure of fungi in aquatic ecosystems have received little attention.

The Trichocomaceae is a relatively large family of Ascomycetes with members frequently impinging upon human activities. The most well-known species of this family belong to the genera Aspergillus, Paecilomyces, Penicillium, Talaromyces, Trichocoma, etc. Aspergillus comprises a diverse and complex group of species in terms of morphological, physiological, and phylogenetic characters, which significantly impact on biotechnology, food production, indoor environments, and human health [3]. Penicillium is another group of the most widespread fungi, which survive in diverse habitats, ranging from soil over vegetation to air, indoor environments, and various food products [4]. Talaromyces was described by Benjamin in 1955 as a sexual state of Penicillium that produces soft-walled ascomata covered with interwoven hyphae [5], and recently Yilmaz et al. [6] re-classified all accepted species of this genus under seven sections. The three genera, Aspergillus, Penicillium, and Talaromyces, are ubiquitous in diverse environments, but their biodiversity in freshwater ecosystem remained unexplored [7–10]. In this study, we investigated the species diversity of Aspergillus, Penicillium, and Talaromyces in freshwater environment, and reported P. piscarium and T. versatilis as unrecorded species in Korea.

2. Material and methods

2.1. Fungal isolation

All strains were collected from algae, dead insect, herbaceous plant, soil, twig, and water in freshwater environments. Information on all strains used in this study is provided in Table 1. To isolate the fungal strains, we used a simple plating technique whereby each substrate is placed onto potato dextrose agar (PDA; Difco, Sparks, MD) and V8 agar (V8A) containing 8% V8 juice (v/v) and 1.5% agar (w/v) adjusted pH to 6.0 using 10 N NaOH, and
then incubated at 28°C in the dark. Mycelium growing 3 days after inoculation was checked under a microscope, and then each hyphal tip was transferred on a new PDA plate.

### 2.2. Morphological analysis

Inoculations were made from spore suspensions in a semi-solid agar solution containing 0.2% agar and 0.05% Tween-80 [11]. The strains were three point inoculated onto Czapek yeast extract agar (CYA; Difco), malt extract agar (MEA; Oxoid, Hampshire, UK), and dichloran 18% glycerol agar (DG18). All Petri dishes were incubated at 25°C under dark conditions for 7 days and CYA plates were additional incubated at 4 and 37°C. Colony diameters were measured after 7 days of incubation and colony characteristics recorded. For morphological identification, micro-and macro-morphology analyses were performed as described in Samson et al. [3], Visagie et al. [12], and Yilmaz et al. [6]. Conidiophores and conidia formed on three different media were transferred to a drop of distilled water on a slide glass, and covered with a cover slip. Slides were examined and photographed using a model DE/Axio Imager.A1 microscope (Carl Zeiss, Gottingen, Germany) equipped with a SteREO Discovery V12 stereomicroscope (Carl Zeiss).

### 2.3. Phylogenetic analysis

To extract genomic DNA from *Aspergillus*, *Penicillium*, and *Talaromyces* isolates, they were grown in liquid shake culture in malt extract broth medium (MEB; Oxoid) for 2–4 days at 25°C, from which mycelia were harvested by filtration and transferred to 1.5 ml tubes. The samples were frozen at −70°C, lyophilized, and finely ground. DNA was

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### Table 1. *Aspergillus*, *Penicillium* and *Talaromyces* strains isolated from freshwater environment in Korea.

| Fungal species           | Isolate no. | Substrate | Geographic origin (year) | GenBank ITS/b-tubulin               |
|--------------------------|-------------|-----------|--------------------------|------------------------------------|
| *Aspergillus japonicas*  | KACC84322; W175 | Algae     | Sangju reservoir, Donam-dong, Sangju-si (2016) | MHS67076/MHS93507                  |
| A. japonicas             | W181        | Herbaceous plant | Sangju reservoir, Donam-dong, Sangju-si (2016) | MHS67077/MHS93508                  |
|                          | W185        | Herbaceous plant | Sangju reservoir, Donam-dong, Sangju-si (2016) | MHS67078/MHS93509                  |
|                          | W395        | Water      | Geumsan creek, Geumsan-myeon, Wanjun-gun (2016) | MHS67079/MHS93510                  |
|                          | W399        | Water      | Geumsan creek, Geumsan-myeon, Wanjun-gun (2016) | MHS67080/MHS93511                  |
| A. niger                 | W154        | Algae      | Sangju reservoir, Donam-dong, Sangju-si (2016) | MHS67081/MHS93512                  |
|                          | W230        | Soil       | Seongchon reservoir, Seongchon-dong, Namgu, Gwangju-si (2016) | MHS67082/MHS93513                  |
| A. tubingensis           | W205        | Soil       | Seongchon reservoir, Seongchon-dong, Namgu, Gwangju-si (2016) | MHS67083/MHS93514                  |
|                          | W299        | Soil       | Gama valley, Yong-myeon, Damyang-gun (2016) | MHS67084/MHS93516                  |
|                          | W320        | Soil       | Gama valley, Yong-myeon, Damyang-gun (2016) | MHS67085/MHS93517                  |
| A. flavus                | W289        | Water      | Seongchon reservoir, Seongchon-dong, Namgu, Gwangju-si (2016) | MHS67086/MHS93518                  |
|                          | W293        | Water      | Gama valley, Yong-myeon, Damyang-gun (2016) | MHS67087/MHS93519                  |
| *Penicillium crustosum*  | KACC84323; W007 | Soil     | Gangjeong Golyeong reservoir, Dalseong-gun, Daegu-si (2016) | MHS67088/MHS93520                  |
| P. expansum              | KACC84324; W363 | Water     | near Sangyoung 2 bridge, Gumi-myeon, Wanjun-gun (2016) | MHS67089/MHS93521                  |
| P. oxalicum              | W116        | Water      | Haman Changnyeong reservoir, Daesan-myeon, Uichang-gu Changwon-si (2016) | MHS67090/MHS93522                  |
|                          | W357        | Twig       | near Sangyoung 2 bridge, Gumi-myeon, Wanjun-gun (2016) | MHS67091/MHS93523                  |
| P. brasilianum           | W212        | Dead insect | Seongchon reservoir, Seongchon-dong, Namgu, Gwangju-si (2016) | MHS67092/MHS93524                  |
|                          | KACC84325; W213 | Dead insect | Seongchon reservoir, Seongchon-dong, Namgu, Gwangju-si (2016) | MHS67093/MHS93525                  |
|                          | KACC84326; W216 | Dead insect | Seongchon reservoir, Seongchon-dong, Namgu, Gwangju-si (2016) | MHS67094/MHS93526                  |
| P. piscarium*            | ZEVCFG0000000024 | Soil | Seongchon reservoir, Seongchon-dong, Namgu, Gwangju-si (2016) | MHS67095/MHS93527                  |
| Talaromyces pinophilus   | W195        | Twig       | Sangju reservoir, Donam-dong, Sangju-si (2016) | MHS67096/MHS93528                  |
| T. versatilis*           | ZEVCFG0000000026 | Soil | Juklim reservoir, Sola-myeon, Yeosu-si (2017) | MHS67097/MHS93529                  |

*New record in Korea.
extracted using Genomic Plus DNA Prep Kit (Inclone, Yongin, Korea). The internal transcribed spacer (ITS) region of ribosomal DNA was amplified using ITS1 and ITS4 primers [13], and β-tubulin (benA) gene was amplified using Bt2a and Bt2b [14]. The PCR products were purified and sequenced by a DNA sequencing service (Macrogen Inc., Seoul, Korea). The obtained nucleotide sequences were searched by using BLASTn available from the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). The resulting ITS and β-tubulin sequences were also compared with the authentic sequences published by Samson et al. [3], Visagie et al. [4], and Yilmaz et al. [6]. For phylogenetic analyses, the reference species of Aspergillus (n = 8), Penicillium (24), and Talaromyces (29) were selected in considering the isolation sources, as well as molecular and morphological characteristics [12,15,16], and retrieved from NCBI GenBank. To infer their phylogenetic relationship, a neighbor-joining tree was constructed using MEGA6 [17], with Tamura-Nei model and gamma distributed substitution rate. The reliability for each group was evaluated by bootstrap analysis of 1000 replications.

3. Results and discussion

All strains of Aspergillus, Penicillium, and Talaromyces isolated from freshwater environments in Korea have been initially identified in terms of both BLASTn-based comparison and phylogenetic analysis of ITS and β-tubulin sequences, and then confirmed by examining their morphology with reference to Varga et al. [16], Visagie et al. [12], Visagie et al. [15], and Yilmaz et al. [6].

Four species of Aspergillus have been identified from 12 isolates (Figure 1); A. tubingensis (W205, W299, W320), A. niger (W154, W230), A. flavus (W289, W293), and A. japonicas (W175, W181, W185, W399, W395). Five strains of A. japonicas strongly grouped with the type strain of this species (CBS114.51T), to which the sequence similarities were 100% for the former four isolates, but 99.8% for W395. The strain W208 grouped with P. piscarium CBS 362.48T, with the maximum bootstrapping (BS) value (Figure 2). The ex-type sequence of P. brasili um (CBS253.55T) grouped with three Korean strains, W212, W216 (98.7% similarity), and W213 (99.1% similarity), with 88% supporting. Two strains, W116 and W357, grouped with P. oxalicum CBS 219.30T, with maximum support. With no sequence difference, W363 grouped to P. expansum CBS 32548T, but W7 grouped to P. crustosum CBS 115503T. The Korean strains of P. brasili um have been isolated from dead insects, and in accordance with the present result P. brasili um is referred to as an opportunistic pathogen of insects [18]. According to Murali et al. [19], P. oxalicum is a plant growth-promoting fungus which is isolated mainly from soil adhering to the roots of pearl
Penicillium expansum is a psychrophilic blue mold that is common in soil throughout the world [20]. Penicillium crustosum is found in various substrates, such as air, soil, etc. [21]. Penicillium piscarium, which is commonly found in soil [22], has been so far unrecorded in Korea [23].

The strain W195 was identical to T. pinophilus CBS 631.66T, which is well-known to be an endophytic fungi [24]. The strain W524 formed a well-supported group with the three sequences of T. versatilis, IMI378536, IMI134756, and IMI134755T, with no sequence difference (Figure 3). Talaromyces versatilis is a so far unrecorded species in Korea [23].

In total 12 species of Aspergillus, Penicillium, and Talaromyces, including two unrecorded specie in Korea, were isolated for a short period of 2 years (but all collected in 2016, except for one in 2017). It means that the species are more prevalent in freshwater environments, than we had anticipated, and somehow play a key role in maintaining the ecosystem. It seems most likely that freshwater fungi mainly contribute to degrade dead plant litters by producing celluloses and lignocelluloses [8], but also some of members may be involved in the degradation of animal parts such as insect exoskeletons, fish scales, and hair [25]. Other ecological groups are known as pathogens or endophytes of aquatic organisms [26]. Given the economic and industrial importance of these fungi, the freshwater environment is a good candidate to increase the discovery

Figure 2. Phylogenetic tree depicting taxonomic position of Penicillium species isolated from freshwater environment based on a concatenate alignment of ITS and BenA sequences. Type strains of all species in Penicillium section Lanata-Divaricata were obtained from Visagie et al. [12].
of species number in these groups, and to uncover their unknown roles.

4. Description of unrecorded species in Korea

*Penicillium piscarium* Westling (Figure 4).

**Cultural characters**

On CYA at 25°C, 38–43 mm growth; colonies more or less radially furrowed; conidial structures usually sparsely produced but some colonies have a section which has abundant conidiation; vegetative mycelium white, area in abundant conidiation is grey-green; exudate lacking or limited clear exudate reverse cream or beige. On MEA 25°C, 40–45 mm growth; conidial structures usually sparsely produced but some areas have abundant conidiation; vegetative mycelium white and conidiation area gray-green; exudate lacking. On DG18 at 25°C, 10–12 mm; no sporulation; mycelium white.

**Morphological characters**

Conidiophores borne terminally on long-trailing hyphae and irregular patterns ranging from mono- to bi-verticillate. Stipe finely rough. Metulae three to four, 8.8–11.1 × 2.5–3.7 μm. Phialides ampulliform, three to five per metulae, 9.8–13.1 × 1.9–2.7 μm. Conidia rough to echinulate, subglobose to broadly ellipsoidal, 2.7–3.6 × 2.0–2.8 μm. Sclerotia and teleomorph not observed.

**Isolate examined**

Republic of Korea, Jeollanam-do; Gwangju-si; Namgu; Seungchon-dong, in Seungchon reservoir.
Talaromyces versatilis P.F. Cannon, Bridge & Buddie. P.F (Figure 5).

Cultural characters

On CYA at 25°C, 33–34 mm growth; sporulation rare; vegetative mycelium white with pinkish exudate; reverse beige. At 37°C, 20–23 mm growth; colony white; reverse light brown. At 4°C, no growth. On MEA at 25°C, 40–44 mm growth; colony furcicolous; sporulation abundant with grey-green conidia; vegetative mycelium white; exudate clear, abundant. On DG18 at 25°C, 9–11 mm growth; no sporulation; mycelium white; exudate and diffusible pigment absent.

Morphological characters

Conidiophores bi-verticillate, usually arising as short branches from aerial hyphae (stipe shorter than 50 μm), or with penicilli borne terminally on longer trailing hyphae. Metulae three to six, divergent, 10–14 μm. Phialides acerose three to six per metulae, 10–14 μm. Conidia smooth, globose to subglobose, 2.4–2.8 × 2.0–2.4 μm. Sclerotia and teleomorph not observed.

Isolate examined

Republic of Korea, Jeollanam-do; Yeosu-si; Solanmyeon; Juklim-ri, in Juklim reservoir (34° 45′ 37″ N 127° 37′ 45″ E), ex soil under water, May 26 2017, Y.-J. Choi, ZEVCFG000000026 (KACC48328; W524).
Disclosure statement

No potential conflict of interest was reported by the authors.

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