

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

Inbeom Heo^a, Kyeongyeon Hong^a, Hyejin Yang^a, Hyang Burm Lee^b, Young-Joon Choi^c and Seung-Beom Hong^a

^aAgricultural Microbiology Division, National Institute of Agricultural Science, RDA, Jeonju, Korea; ^bDivision of Food Technology, Biotechnology and Agrochemistry, Chonnam National University, Gwangju, Korea; ^cDepartment of Biology, Kunsan National University, Gunsan, Korea

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 *Aspergillus* section *Nigri*, named black *Aspergillus*. Eight strains of *Penicillium* were identified as *P. brasilianum* ($n=3$), *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.

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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

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

Fungal species	Isolate no.	Substrate	Geographic origin (year)	GenBank ITS/ β -tubulin
<i>Aspergillus japonicas</i>	KACC48322; W175	Algae	Sangju reservoir, Donam-dong, Sangju-si (2016)	MH567076/MH593507
<i>A. japonicas</i>	W181	Herbaceous plant	Sangju reservoir, Donam-dong, Sangju-si (2016)	MH567077/MH593508
	W185	Herbaceous plant	Sangju reservoir, Donam-dong, Sangju-si (2016)	MH567078/MH593509
	W395	Water	Geumsan creek, Geumsan-myeon, Wanju-gun (2016)	MH567079/MH593510
	W399	Water	Geumsan creek, Geumsan-myeon, Wanju-gun (2016)	MH567080/MH593511
<i>A. niger</i>	W154	Algae	Sangju reservoir, Donam-dong, Sangju-si (2016)	MH567081/MH593512
	W230	Soil	Seungchon reservoir, Seungchondong, Namgu, Gwangju-si (2016)	MH567082/MH593513
<i>A. tubingensis</i>	W205	Soil	Seungchon reservoir, Seungchondong, Namgu, Gwangju-si (2016)	MH567083/MH593514
	W299	Soil	Gama valley, Yong-myeon, Damyang-gun (2016)	MH567084/MH593516
	W320	Soil	Gama valley, Yong-myeon, Damyang-gun (2016)	MH567085/MH593517
<i>A. flavus</i>	W289	Water	Seungchon reservoir, Seungchondong, Namgu, Gwangju-si (2016)	MH567086/MH593518
	W293	Water	Gama valley, Yong-myeon, Damyang-gun (2016)	MH567087/MH593519
<i>Penicillium crustosum</i>	KACC48323; W007	Soil	Gangjeong Golyeong reservoir, Dalseong-gun, Daegu-si (2016)	MH567088/MH593520
<i>P. expansum</i>	KACC48324; W363	Water	near Sangyoung 2 bridge, Gui-myeon, Wanju-gun (2016)	MH567089/MH593521
<i>P. oxalicum</i>	W116	Water	Haman Changnyeong reservoir, Daesan-myeon, Uichang-gu Changwon-si (2016)	MH567090/MH593522
	W357	Twig	near Sangyoung 2 bridge, Gui-myeon, Wanju-gun (2016)	MH567091/MH593523
<i>P. brasilianum</i>	W212	Dead insect	Seungchon reservoir, Seungchondong, Namgu, Gwangju-si (2016)	MH567092/MH593524
	KACC48325; W213	Dead insect	Seungchon reservoir, Seungchondong, Namgu, Gwangju-si (2016)	MH567093/MH593525
	KACC48326; W216	Dead insect	Seungchon reservoir, Seungchondong, Namgu, Gwangju-si (2016)	MH567094/MH593526
<i>P. piscarium</i> ^a	ZEVCFG0000000024 (KACC48327; W208)	Soil	Seungchon reservoir, Seungchondong, Namgu, Gwangju-si (2016)	MH567095/MH593527
<i>Talaromyces pinophilus</i>	W195	Twig	Sangju reservoir, Donam-dong, Sangju-si (2016)	MH567096/MH593528
<i>T. versatilis</i> ^a	ZEVCFG0000000026 (KACC48328; W524)	Soil	Juklim reservoir, Sola-myeon, Yeosu-si (2017)	MH567097/MH593529

^aNew record in Korea.

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 additionally 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

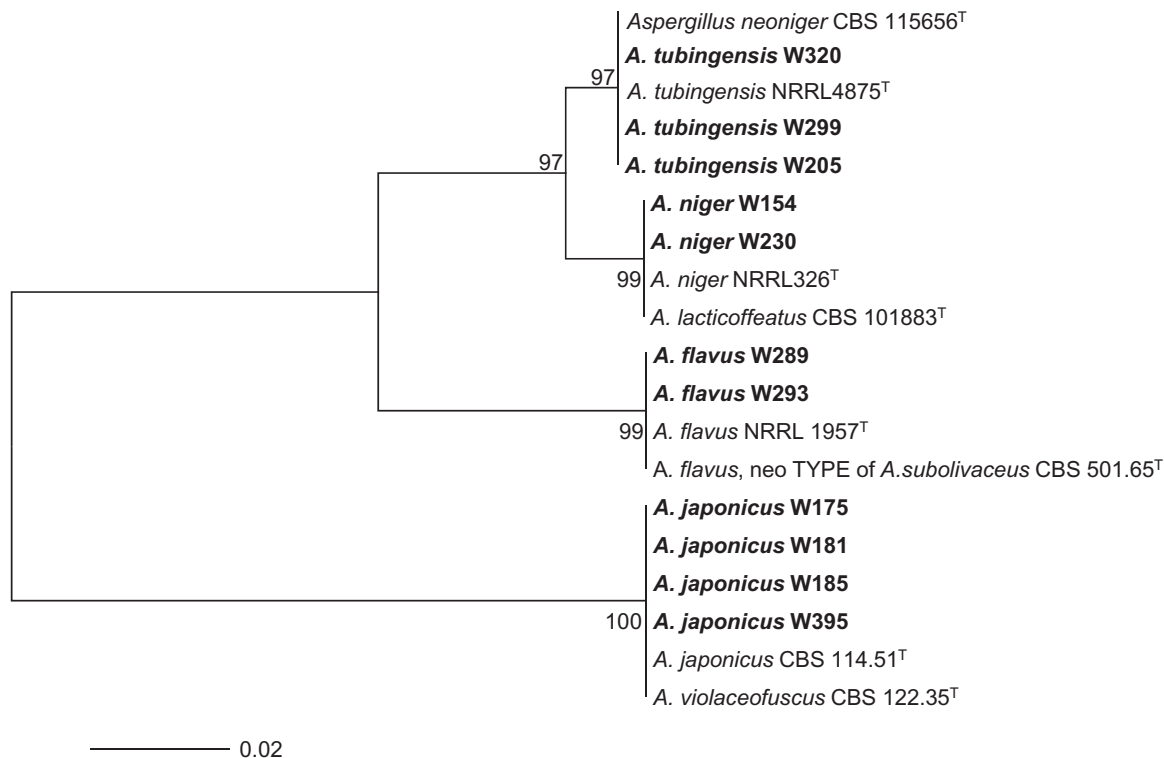


Figure 1. Phylogenetic tree depicting taxonomic position of *Aspergillus* species isolated from freshwater environment based on a concatenate alignment of ITS and *BenA* sequences. For the comparison of the isolated strains with the type strains, barcode sequences were obtained from Samson et al. [3].

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. japonicus* (W175, W181, W185, W399, W395). Five strains of *A. japonicus* strongly grouped with the type strain of this species (CBS114.51^T), 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 362.48^T, with the maximum bootstrapping (BS) value (Figure 2). The ex-type sequence of *P. brasilianum* (CBS253.55^T) 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 219.30^T, with maximum support. With no sequence difference, W363 grouped to *P. expansum* CBS 32548^T, but W7 grouped to *P. crustosum* CBS 115503^T. The Korean strains of *P. brasilianum* have been isolated from dead insects, and in accordance with the present result *P. brasilianum* 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

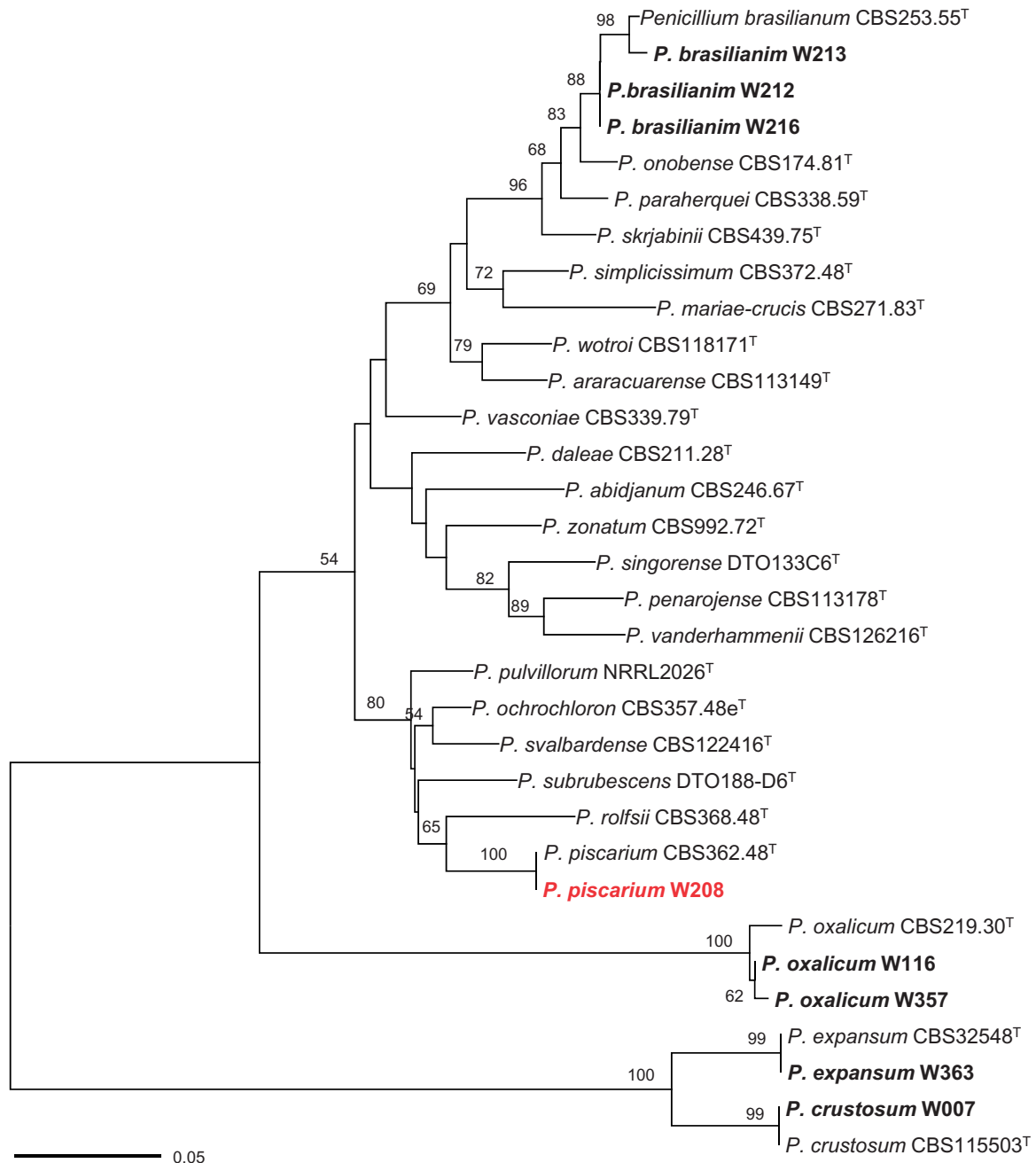


Figure 2. Phylogenetic tree depicting taxonomic position of *Penicillium* species isolated from freshwater environment based on a concatenate alignment of ITS and *BenA* sequences. Type strains of all species in *Penicillium* section *Lanata-Divaricata* were obtained from Visagie et al. [12].

millet. *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 631.66^T, 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 IMI134755^T, 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 species 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

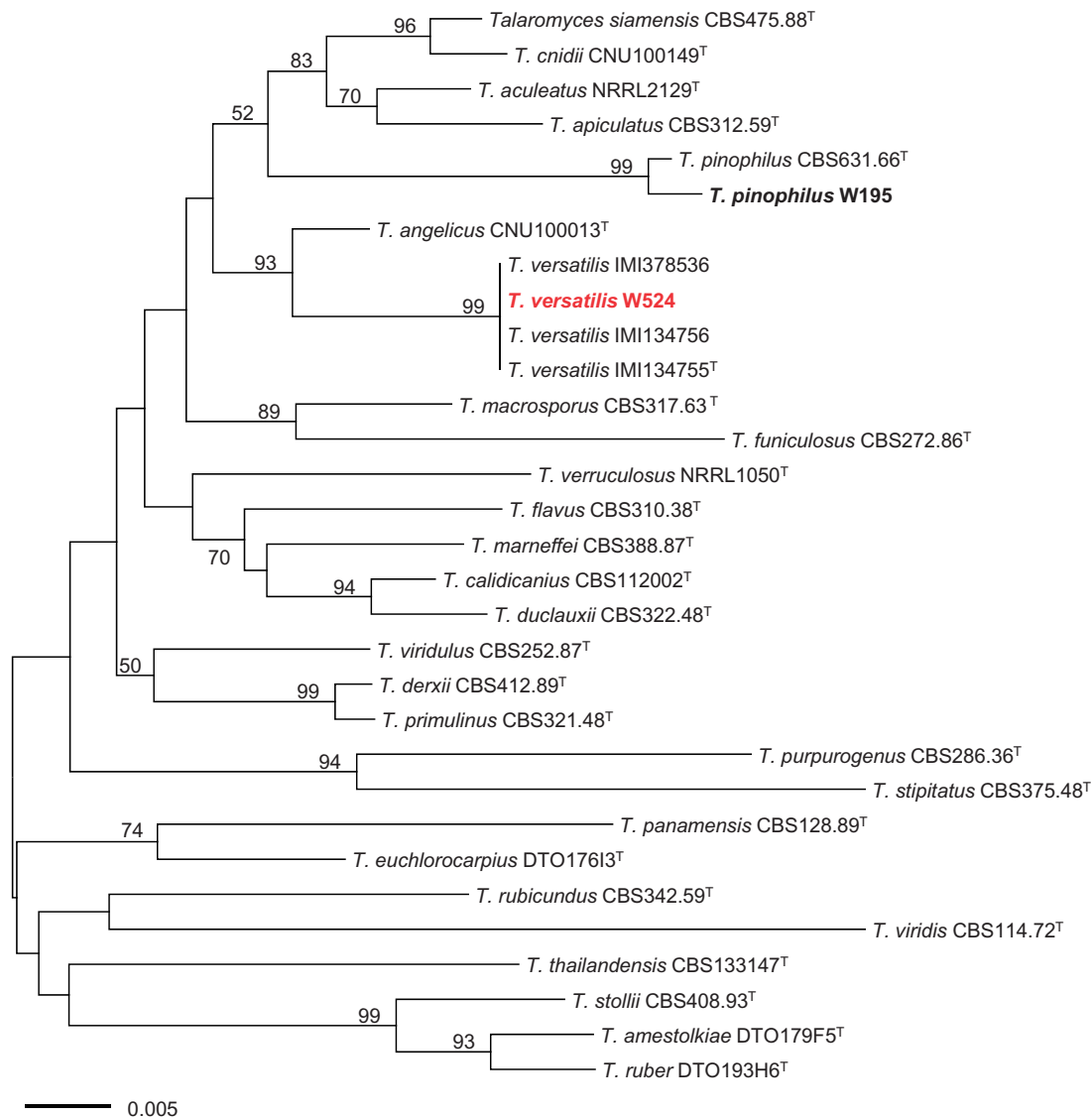


Figure 3. Phylogenetic tree depicting taxonomic position of *Talaromyces* species isolated from freshwater environment based on a concatenate alignment of ITS and *BenA* sequences. Type strains of all species in *Talaromyces* section *Talaromyces* were obtained from Yilmaz et al. [6].

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

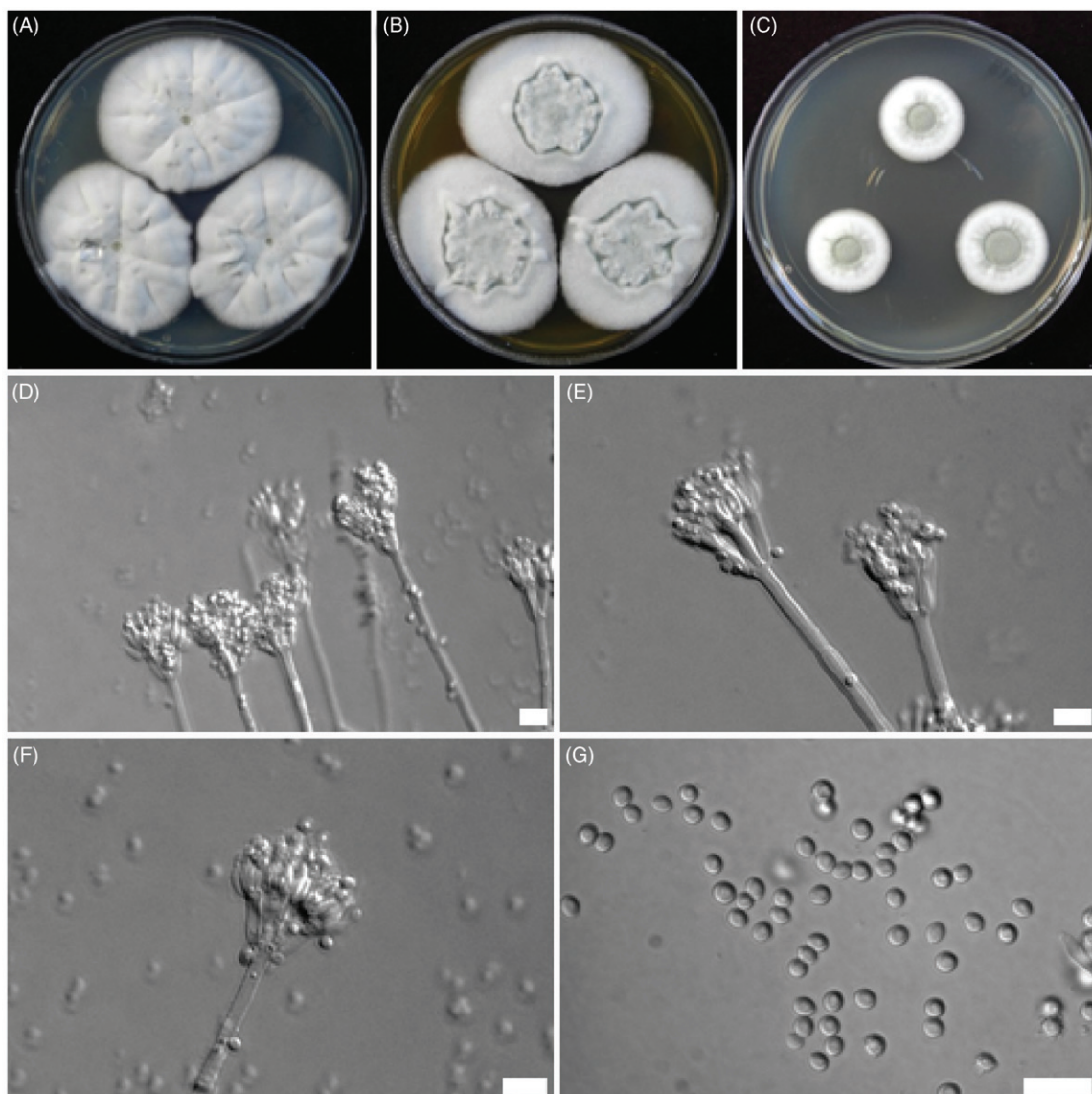


Figure 4. Morphological characteristics of *Penicillium piscarium* KACC 48329. (A–C) Colonies on CYA (A), MEA (B) and DG18 (C) after 7 days growth, (D, E, F) penicilli, (G) conidia (scale bar = 10 μ m).

(35° 03' 59'' N, 126° 45' 51'' E), ex soil on water, Jun 29 2016, Y.-J. Choi, ZEVCFG000000024 (KACC48327; W208).

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 funiculose; 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 μ m), or with penicilli borne terminally on longer trailing hyphae. Metulae three to six, divergent, 10–14 μ m. Phialides acerose three to six per metulae, 10–14 μ m. Conidia smooth, globose to subglobose, 2.4–2.8 \times 2.0–2.4 μ m. Sclerotia and teleomorph not observed.

Isolate examined

Republic of Korea, Jeollanam-do; Yeosu-si; Solamyeon; 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).

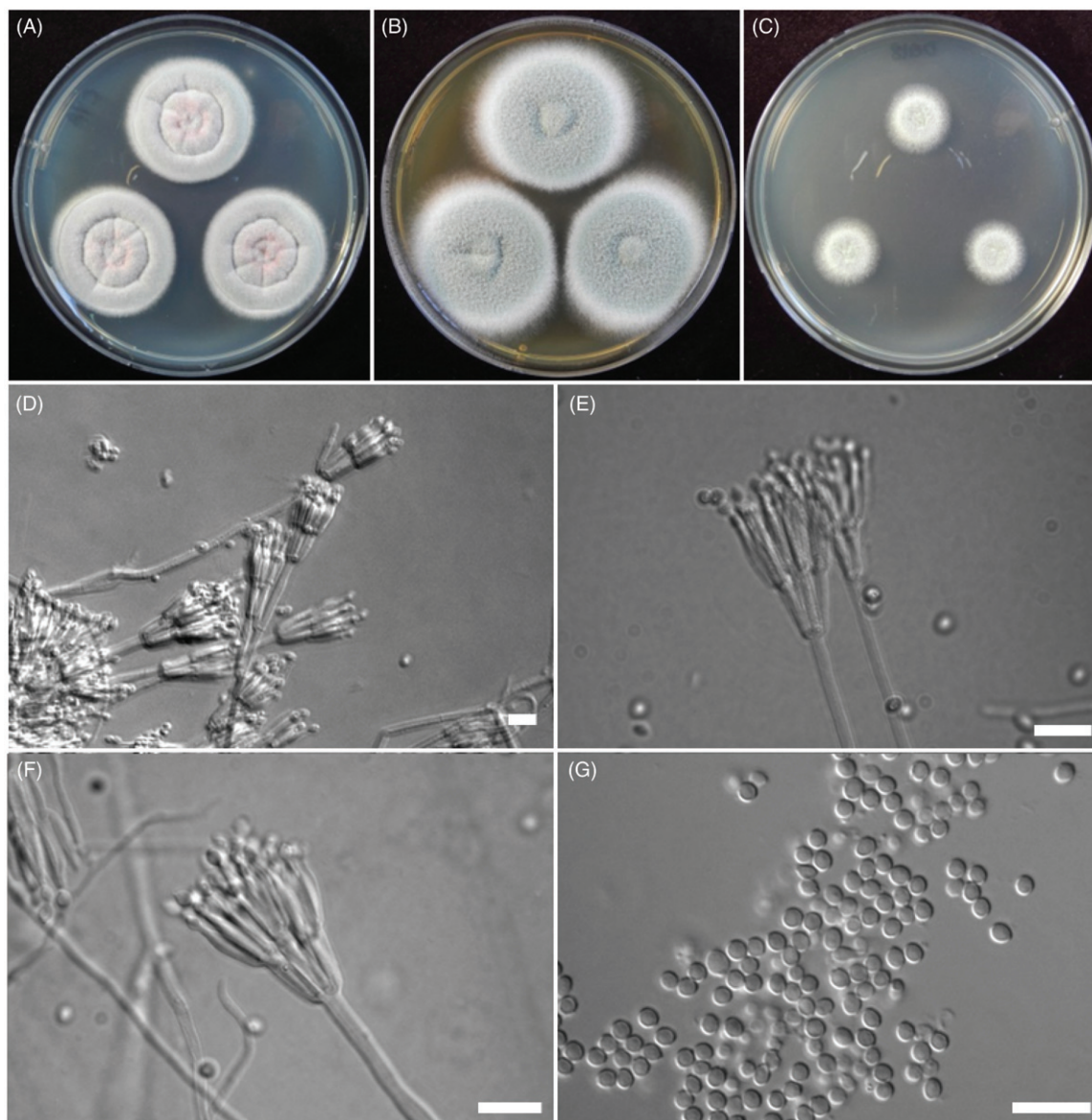


Figure 5. Morphological characteristics of *Talaromyces versatilis* KACC 48328. (A–C) Colonies on CYA (A), MEA (B), and DG18 (C) after 7 days growth, (D, E, F) penicilli, (G) conidia (scale bar = 10 μm).

Disclosure statement

No potential conflict of interest was reported by the authors.

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