

First Report of *Penicillium brasilianum* and *P. daleae* Isolated from Soil in Korea

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(Received December 3, 2004)

In this study, a total of 300 isolates of *Penicillium* and related teleomorphic genera were collected from soils of 17 locations in Korea from April to May, 2004. Ninety four isolates were identified as the species of *Penicillium* subgenus *Furcatum* based on cultural and morphological characteristics and β -tubulin gene sequences. Among the species, Korean isolates of *P. brasilianum* Bat. and *P. daleae* K. M. Zalesky were phylogenetically identical to the reference species based on DNA sequence of the β -tubulin gene. Here we described and illustrated *P. brasilianum* and *P. daleae* that are new in Korea.

KEYWORDS: Cultural and morphological characteristics, β -Tubulin gene, *P. brasilianum*, *P. daleae*

Penicillium is a taxonomically difficult genus of great importance in fields as diverse as food spoilage, biotechnology, plant pathology and medicine. In nature, Penicillia are almost everywhere and many species of *Penicillium* are soil fungi. Four subgenus and 225 species of *Penicillium* were published in the world (Pitt *et al.*, 2000). The published Korean literature on this genus is scattered and fragmentary and pertains to some individual species (Kim *et al.*, 1989; Lee, 1992; Lee *et al.*, 2003). There have been no comprehensive studies on *Penicillium* in Korea.

Traditional identification of *Penicillium* species has mainly been depended on morphological, physiological and biochemical methods (Pitt, 1979; Samson and Van Reenen-Hoekstra, 1998). In recent years, molecular approaches have been used increasingly in identification and phylogenetic classification of filamentous fungi and their application has led to the reconsideration of several genera (Bruns *et al.*, 1991). Analysis of ribosomal DNA sequences has become a common tool in modern systematics, and has been used to establish phylogenetic relationships within many species of fungi. Some protein-coding genes have been used to study the phylogenetic analysis of fungi at the species level. Protein-coding genes have advantages at molecular markers over ribosomal genes in that they offer a large number of unlinked sources of phylogenetic information (Geiser *et al.*, 1998). In the genus *Penicillium*, the region spanning of the nuclear ribosomal internal transcribed spacer (ITS 1, ITS2 and 5.8S rDNA) has been investigated to clarify the subdivisions within the genus and to evaluate phylogenetic relationship of some species (Peterson, 2000; Skouboe *et al.*, 2000). The ribosomal DNA gene has too few informative differences to reveal the phylogeny of *Penicillium*. Among protein-coding genes,

β -tubulin gene has proven useful for studying phylogenetic relationships between close taxonomic relatives (Glass, 1995). The sequencing analysis of β -tubulin gene has been used for identification of closely related *Penicillium* species (Seifert and Louis-Seize, 2000; Samson and Frisvad, 2004). The β -tubulin gene sequences provided well support for the species based on morphological characters.

During our studies on the genus *Penicillium* from Korean soil, we encountered many species of *Penicillium* previously unreported from Korea. In this report, descriptions on morphology and β -tubulin gene sequences of two species belonging to *Penicillium* subgenus *Furcatum* from soil are given.

Materials and Methods

Collection of soil samples. Soil samples were collected from forest in Korea from April to May, 2004. Samples were placed in polyethylene bags and were stored at 4°C.

Isolation of fungi. The soil dilution plate method was used to isolate *Penicillium* species and related teleomorphs inhabiting the soil. Approximately 10 g of soil from each sampling position was added to 90 ml of distilled water in Scott flush 250 ml and then was shaken at 200 rpm for 30 min. One ml of the soil suspension was spread on each of two replicate Petri dishes containing DG18 (dichloran 18% glycerol) agar plates (Dichloran Glycerol 25.8 g, Glycerol 180.3 g, Trace metal solution 0.82 ml, Chloramphenicol solution 0.82 ml, Distilled water 1 l). The plates were kept in darkness at 25°C for 5~7 days. The conidia assumed to be *Penicillium* were picked up from colonies and transferred to MEA slants. Furthermore, nine isolates of *P. brasilianum*, *P. daleae* and

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Table 1. List of isolates used in this study

Code	Species	Isolate no.	Source	Location
1	<i>P. brasilianum</i>	KACC 41628	soil	Korea
2	<i>P. brasilianum</i>	KACC 41629	soil	Korea
3	<i>P. brasilianum</i>	CBS 253.55	–	Brazil
4	<i>P. brasilianum</i>	CBS 430.65	soil	Japan
5	<i>P. daleae</i>	KACC 41627	soil	Korea
6	<i>P. daleae</i>	CBS 211.28	soil	Poland
7	<i>P. daleae</i>	CBS 228.60	soil	France
8	<i>P. griseopurpureum</i>	CBS 406.65	soil	UK
9	<i>P. ochrochloron</i>	CBS 357.48	copper sulphate solution	USA
10	<i>P. oxalicum</i>	CBS 300.97	soil	Slovenia
11	<i>P. rolfsii</i> var. <i>rolfsii</i>	CBS 368.48	fruit of <i>Ananas sativus</i>	USA
12	<i>P. simplicissimum</i>	CBS 275.83	Secale cereale	Spain

related species obtained from Centraalbureau voor Schimmcultures (CBS) were included to broaden the comparison (Table 1).

Genomic DNA extraction, PCR amplification and sequencing. *Penicillium* isolates were grown in liquid shake culture in ME broth medium for 3–4 days at 25°C. Mycelia were collected from the cultures by filtration and transferred to 1.5 ml tubes. These samples were frozen at –70°C. DNA was extracted by method of Lee and Taylor (1990). For the amplification of the β -tubulin gene, primers Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2b (5'-ACCCTCAGTGTAGTGACCCCTGGC-3') (Glass and Donaldson, 1995) were used. PCR mixture contained 0.8 pmol of each primer, 0.2 mM of dNTP's, 10 mM Tris-HCl, 50 mM KCl, 1.5 MgCl₂, 5U *Taq* polymerase and 15 ng of template DNA. PCR cycling conditions were as follows: an initial denaturation step of 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. A final elongation step of 72°C for 7 min was performed.

The PCR product was purified by using a Wizard PCR prep kit (Promega, Madison, WI, U.S.A.). Purified double stranded PCR fragments were directly sequenced with BigDye terminator cycle sequencing kits (Applied Biosystems, Forster City, CA, U.S.A.) following to the manufacturer's instructions. Same primer sets with PCR amplification were used to sequence both DNA strands. The gel electrophoresis and data collection were performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Forster City, CA, U.S.A.). The sequences were proofread, edited and merged into comparable sequences using DNASTar program (Lasergene 5.05). The phylogenetic analysis and distance values were calculated using MEGA 2.1 (Kumar *et al.*, 2001).

Cultural conditions. For comparative study of cultural characteristics, a conidial suspension was prepared by adding a small portion of conidia grown on MEA for 7

days into 1 ml semisolid detergent agar, consisting of 0.2% agar and 0.05% Tween 80 (Pitt, 1988). This suspension was inoculated at three points onto 9 cm Petri dishes containing standard media, Czapek yeast extract agar (CYA) (Samson, 2000), malt extract agar (MEA) (Samson, 2000), 25% glycerol nitrate agar (G25N) (Pitt, 1979) and Czapek agar (CZA) (Samson, 2000). The media contained an additional trace element solution (Frisvad & Filtenborg, 1983). The inoculated CYA plate were incubated in the dark at 25°C and 37°C, while MEA, G25N and CZA plates were incubated at 25°C only. Morphological characteristics was done exclusively from colonies grown on MEA for 7 days and were observed under a differential interference contrast microscope at 1000× magnification.

Results and Discussion

Sequence analysis of β -tubulin gene. The partial β -tubulin gene from 7 different species of *Penicillium* including KACC isolates from soil in Korea and CBS isolates were amplified. Amplification of the β -tubulin gene with primers Bt2a and Bt2b yields fragment of approximately 500 bp. In a distance analysis with neighbor-joining method, sequences of KACC 41627 isolate were 98.5–99.4% identical to those of *P. daleae*, CBS 211.28 and CBS 228.60, with a bootstrap value of 99% (Fig. 2 and Table 2). KACC 41628, KACC 41629 and *P. brasilianum*, CBS 253.55 and CBS 430.65, were belonged to the same group. Sequence similarity among them was 98.1–100%, which was supported by a bootstrap values of 100% (Fig. 2 and Table 2). Sequence similarity among *P. brasilianum*, *P. daleae* and related species ranged from 70.0–95.7%.

The β -tubulin gene has been widely used for phylogenetic analysis in fungi. The amount of variation is suitable for studying phylogenetic relationships among closely related species (O'Donnell and Cigelnik, 1995). In this study, the phylogenetic tree inferred from the sequences of

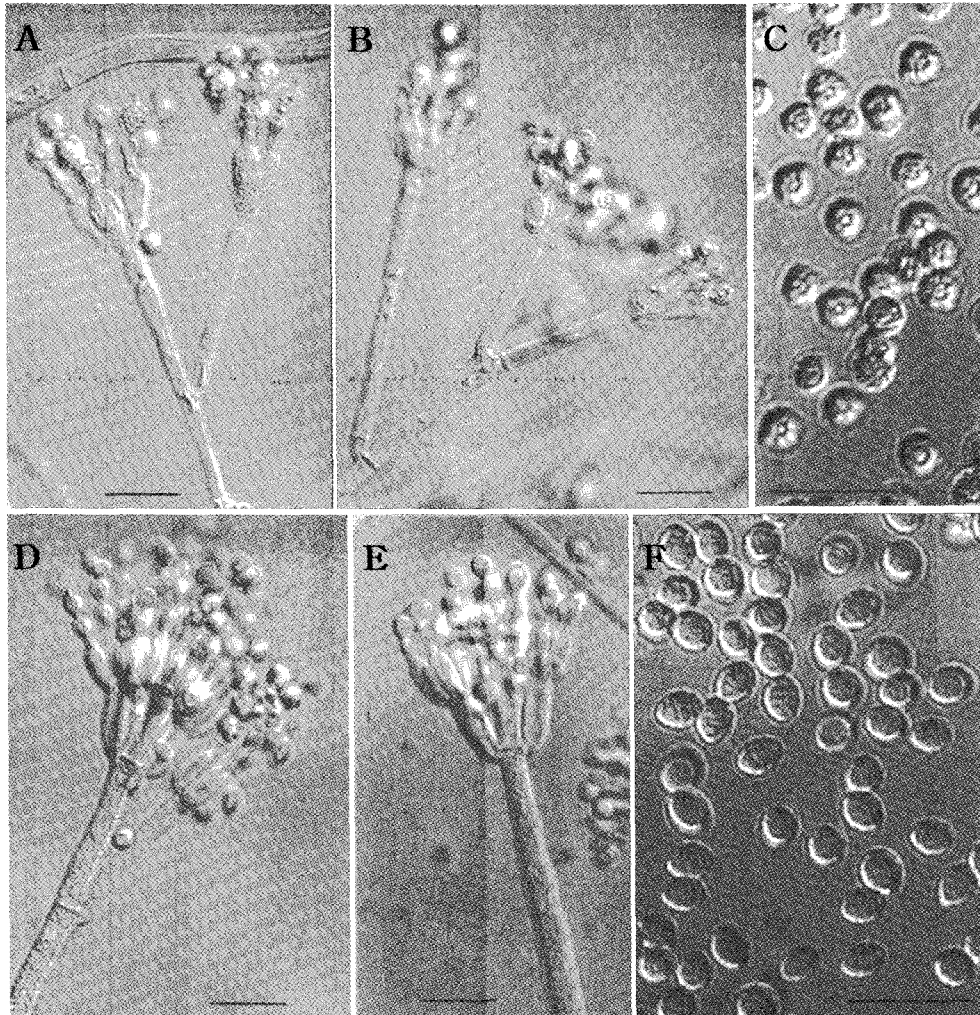


Fig. 1. *Penicillium daleae* (KACC 41627) (A-C): (A, B) Penicilli, (C) Conidia. *Penicillium brasilianum* (KACC 41628) (D-F): (D, E) Penicilli, (F) Conidia. Bar = 10 μ m.

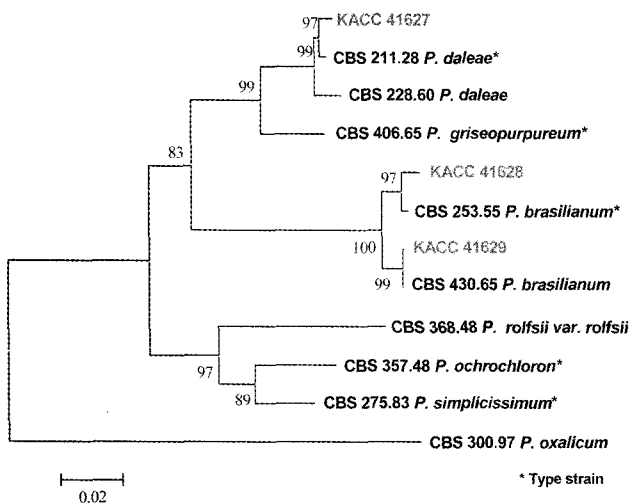


Fig. 2. Neighbor-joining tree based on phylogenetic analysis of β -tubulin gene sequences. The number below each branch indicate bootstrap values of distance. The bootstrap value was obtained after a bootstrap test 1000 replications.

β -tubulin gene correlated well with the species that were defined by cultural and morphological characteristics.

Descriptions of species. Among the strains examined, the following two species of *Penicillium* new to Korea were identified based on the morphological and molecular phylogenetic analyses.

Penicillium brasilianum Bat.

Batista & Maia, Anais da Sociedade de Biologia de Pernambuco **15**(1): 160, 1957

Colonies on CYA (7 days, 25°C) 25~30 mm diam., radially sulcate, velutinous or somewhat floccose; mycelium white; conidiogenesis moderate to heavy, greyish green to dull green, sometimes yellowish grey; small amount of exudate produced by some isolates; soluble pigment absent; reverse yellow to reddish brown.

Colonies on MEA (7 days, 25°C) 35~45 mm diam., plane, surface texture velutinous or occasionally some-

Table 2. DNA similarity matrix for β -tubulin gene sequences of *P. brasilianum*, *P. daleae* and their related species

	1	2	3	4	5	6	7	8	9	10	11
1											
2	99.4										
3	98.5	98.8									
4	88.5	88.6	88.8								
5	88.5	88.0	88.5	98.1							
6	88.7	88.6	89.0	99.2	98.5						
7	88.5	88.2	88.8	98.1	100.0	98.5					
8	89.5	89.7	89.3	86.3	87.2	86.6	87.2				
9	87.7	88.1	87.7	85.8	87.0	86.6	87.0	91.6			
10	78.9	78.9	80.8	77.8	77.7	77.0	78.1	80.9	79.5		
11	90.4	90.6	89.9	86.8	87.4	87.2	87.4	95.4	92.0	81.3	
12	95.3	95.7	94.8	89.3	89.5	89.9	89.9	89.5	86.7	81.8	89.7

1, *P. daleae* KACC 41627; 2, *P. daleae* CBS 211.28; 3, *P. daleae* CBS 228.60; 4, *P. brasilianum* KACC 41628; 5, *P. brasilianum* KACC 41629; 6, *P. brasilianum* CBS 253.55; 7, *P. brasilianum* CBS 430.65; 8, *P. ochrochloron* CBS 357.48; 9, *P. rolfsii* var. *rolfsii* CBS 368.48; 10, *P. oxalicum* CBS 300.97; 11, *P. simplicissimum* CBS 275.83; 12, *P. griseopurpureum* CBS 406.65.

what floccose; mycelium white; conidiogenesis moderate to heavy, blue green, exudate and soluble pigment absent; reverse yellow to yellow brown.

Colonies on CZA (7 days, 25°C) 36–40 mm diam., plane or lightly sulcate, sometimes centrally depressed, velutinous to floccose; mycelium white; conidiogenesis moderate or commonly heavy, green to grey green; exudate absent or numerous small drops, soluble pigment not produced; reverse typically yellow.

Colonies on G25N (7 days, 25°C) 15–18 mm diam., plane, floccose, mycelium white; conidiogenesis absent or light, blue; exudate and soluble pigment absent; reverse beige.

Colonies on CYA (7 days, 37°C) 15–20 mm diam., radially sulcate to strongly convolute, sometimes centrally depressed, white to yellowish mycelium; conidiogenesis light, grey green; exudate and soluble pigment absent; reverse pale or brownish.

Conidiophores borne from surface hyphae. Stipes 75–360 × 2.8–3.0 μ m, rough walled, Metulae 9.5–20 × 3.5–4 μ m. Rami when present divergent, usually rough walled, 10–32 × 3.5 μ m. Phialides ampulliform, 7.5–10 × 2.5–3.0 μ m. Conidia most commonly ellipsoidal, 3.5–4.5 × 2.7–3.0 μ m, striate walled.

Isolates examined : KACC 41628, KACC 41629.

Note: Morphological characteristics of two isolates agreed well with the description of *P. brasilianum* (Pitt, 1979). This is the first record of *P. brasilianum* in Korea.

Penicillium daleae K.M. Zaleski

Bull. int. Acad. pol. Sci. Lett., Sér. B, 1927: 495, 1927.

Colonies on CYA (7 days, 25°C) 30–34 mm diam., radially sulcate, sometimes convolute, centrally depressed, moderately deep to deep, surface texture velutinous to floccose, mycelium white; conidiogenesis light to moderate, greyish olive; clear brown exudate sometimes present;

reverse beige to reddish brown.

Colonies on MEA (7 days, 25°C) 25–35 mm diam., plane or lightly radially sulcate, velutinous; mycelium white; conidiogenesis very light to heavy, coloured similarly to that on CYA; exudate absent or numerous small drops; yellow to reddish brown pigment typically produced; reverse yellow brown.

CZA (7 days, 25°C) 20–26 mm diam., plane or radially sulcate with centrally umbonate, surface texture velutinous to floccose; mycelium white; conidiogenesis moderate, coloured similarly to colonies on MEA; yellow exudate and reddish soluble pigment sometimes produced; reverse beige to brown.

Colonies on G25N (7 days, 25°C) 10–12 mm diam., plane, velutinous to lightly floccose; mycelium white; conidiogenesis light, grey; exudate and soluble pigment absent; reverse beige to grey green.

Colonies on CYA (7 days, 37°C) no growth.

Conidiophores borne from surface, Stipes 35–170 × 2.3–2.7 μ m, with thin and smooth walls; Metulae usually 15–25 × 2.4–2.8 μ m; Phialides in verticils of 4–6, ampulliform, 6–7(–12) × 2.4–3.0 μ m, with narrow collula; Conidia spheroidal, 2.6–3.2 × (2–)2.5–3 μ m, conspicuously spinose.

Isolates examined : KACC 41627.

Note: Morphological characteristics of isolate agreed well with the description of *P. daleae* (Pitt, 1979). This is the first record of *P. daleae* in Korea.

Acknowledgement

This study was supported by Post Doctoral Course Program of National Institute of Agricultural Biotechnology, Rural Development Administration, Republic of Korea.

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