

## Six Species of *Penicillium* Associated with Blue Mold of Grape

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Grape fruits with blue mold symptoms were collected from house storages in different locations in Korea and were investigated for their association with *Penicillium* species. A total of 12 isolates of *Penicillium* were isolated from the collected fruits. Based on morphological and cultural characteristics and  $\beta$ -tubulin gene sequence data analysis, they were identified as *P. bialowiezense*, *P. citrinum*, *P. echinulatum*, *P. expansum*, *P. solitum* and unidentified *Penicillium* species. *P. solitum* was the predominant followed by *P. expansum*. *P. bialowiezense* and *P. echinulatum* were newly recorded in Korea.  $\beta$ -Tubulin gene sequences could be used to distinguish each species of *Penicillium* and the molecular groups were correlated well with the morphological species. The unidentified species was supposed to be a new species, not previously reported in literature.

**KEYWORDS:** Blue mold, Colony characteristics, Grape, Morphological characteristics, *Penicillium*,  $\beta$ -Tubulin gene

During the storage period, fruits of grape may undergo various attacks from insects and microorganisms like fungi, yeasts and bacteria. *Penicillium* species are a major cause of deterioration and decay amongst a wide range of post harvest plant products, particularly fruits such as grape, oriental pear and citrus.

*Penicillium* of Korea are insufficiently known. The published Korean literature on the genus is scattered and fragmentary and pertains to about 60 species (Anonymous, 2004; Cho *et al.*, 2005; Lee *et al.*, 2003). Most of them are soil-borne and only 10 species have been reported to be associated with storage diseases of plant products (Anonymous, 2004; Kim *et al.*, 2002; Oh *et al.*, 1999).

Identification of *Penicillium* is not easy. It is a large genus, and many common species look alike to the uninitiated. At the same time there is a great deal of variability within the species, therefore, unambiguous identification of the species requires molecular identification (Guerche *et al.*, 2004). Among the molecular tools available,  $\beta$ -tubulin gene has proven useful for identification of closely related *Penicillium* species (Kim *et al.*, 2006; Seifert and Louis-Seize, 2000; Samson *et al.*, 2004).

The objectives of this study were to clearly identify *Penicillium* isolates from blue mold of grapes, by using two approaches; the analysis of morphological and cultural characteristics and the analysis of their  $\beta$ -tubulin gene sequences.

### Materials and Methods

**Isolation.** Grape fruits with blue mold were collected from store houses in Daejeon, Naju and Suwon in Korea

during the period of December 2004 and February 2006. The conidia assumed to be *Penicillium* were picked up from blue molds of grapes and transferred to malt extract agar (MEA; malt extract 20 g, peptone 1.0 g, glucose 20 g, agar 20 g, distilled water 1 liter) and grown for 7 days at 25°C.

**Culture.** Isolates were inoculated onto Czapek yeast extract agar (CYA; K<sub>2</sub>HPO<sub>4</sub> 1.0 g, Czapek concentrate 10 mg, yeast extract 5 g, sucrose 30 g, agar 15 g, distilled water 1 liter) and malt extract agar (MEA) media in 3 points in a petridish. Colony appearance, exudates production, pigmentation and reverse coloration were assessed and colony diameters were measured and recorded after one week incubation at 25°C.

**Morphological observations.** *Penicillium* isolates were identified with the help of keys developed by Pitt (1979, 2000) and Frisvad and Samson (2004). Cultures were inoculated on CYA and 2% MEA media in three-points of 9 cm plastic Petri dishes. Petri dishes were incubated at 25°C in a dark condition. The cultures were examined after 7 days of incubation. All morphological data were examined on cultures grown on 2% MEA for 7 days at 25°C. The examination and measurements of conidiophores and conidia were made from slide preparations stained with 3% KOH. Differential interference contrast microscopy was used for the observation and 30 units of each morphological character were measured.

**DNA extraction and PCR amplification.** Isolates were grown on liquid shake culture of potato dextrose broth medium for 3–4 days at 25°C. Mycelia were collected from the cultures by filtration and transferred to 1.5 ml

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tubes. These samples were frozen at  $-70^{\circ}\text{C}$  before use. DNA was extracted by the method of Cubero *et al.* (1999). For the amplification of  $\beta$ -tubulin gene, primers Bt2a (5'-GGTAACCAAATCGGFGCTGCTTTC-3') and Bt2b (5'-ACCCTCAGTGTAGTGACCCCTGGC-3') (Glass and Donaldson, 1995) were used. PCR mixture contained 0.5 pmol of each primer, 0.2 mM of dNTP's, 10 mM Tris-HCl, 50 mM KCl, 1.5 MgCl<sub>2</sub>, 2.5 U *Taq* polymerase and 15 ng of template DNA. PCR amplification was carried out in a *i*-cycler (BIO-RAD, USA) for 30 cycles of  $94^{\circ}\text{C}$  for 40 s denaturing,  $55^{\circ}\text{C}$  for 40 s annealing and  $72^{\circ}\text{C}$  for 1 min extension. Initial denaturing at  $94^{\circ}\text{C}$  was extended to 3 min and the final extension was at  $72^{\circ}\text{C}$  for 15 min.

The PCR products were purified by using a Wizard PCR prep. kit (Promega, Madison, WI, USA). Purified double stranded PCR fragments were directly sequenced with BigDye terminator cycle sequencing kits (Applied Biosystems, Forster City, CA, USA) by following 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 310 Genetic Analyzer (Applied Biosystems, Forster City, CA, USA).

**Sequencing and phylogenetic analysis.** The sequences were compared with  $\beta$ -tubulin gene sequence available in the GenBank by the BLAST search analysis. Sequences generated from materials in this study and retrieved from GenBank were initially aligned using the program CLUSTAL X (Thompson *et al.*, 1997) and then the alignment was refined manually using the PHYDIT program version 3.0 (Chun, 1995; available at <http://plaza.sun.ac.kr/~jchun/phydit>).

Ambiguously aligned regions were excluded from the subsequent analyses. A neighbor-joining tree was reconstructed with Kimura's 2-parameter distance model (Kimura, 1980) using the PHYDIT 3.57c package (Felsenstein, 1995). The bootstrap analysis using 1000 replications were performed to assess the relative stability of the branches.

## Results and Discussion

Grape fruits with blue mold were collected from different store houses in Korea. Twelve isolates were selected as *Penicillium* on the basis of their morphological structure. According to cultural and morphological characteristics, the *Penicillium* species were divided into 6 groups. On the basis of previous description by Pitt (1979, 2000) and Frisvad and Samson (2004), each group was identified as *P. bialowiezense*, *P. citrinum*, *P. echinulatum*, *P. expansum*, *P. solitum* and unidentified *Penicillium* sp. Taxonomic descriptions, photos of colonies and drawings of fungal structures of each species are given below.

***Penicillium bialowiezense* K. Zaleski Figs. 1(A, B), 3(A)**  
Bul. Int. Acad. Pol. Sci. Lett., Ser. B 1927: 462, 1927

Colonies on CYA 20–28 mm diam, readily sulcate, surface texture vutinous; conidiogenesis moderate to heavy, dark green to grey green; exudate often present as pale to reddish brown droplets; reverse beige to yellowish cream.

Colonies on MEA 15–23 mm diam, pale or slightly sulcate; surface texture velutinous, floccose in center; conidiogenesis light to moderate, pale green to pale yellow green; exudates absent; reverse pale yellow to yellow orange.

Conidiophores long, appressed, stipes 250–450  $\mu\text{m}$  long, smooth-walled, terverticillate; rami 20–25  $\mu\text{m}$  long; metulae cylindrical but apically inflated, 10–15  $\mu\text{m}$  long, phialides cylindrical with gradually tapering collula, 6–10  $\times$  2.5–3  $\mu\text{m}$ ; conidia subglobose to ellipsoidal, finely roughened, 2.5–3.8  $\times$  2–3  $\mu\text{m}$ .

Isolate examined: CNU 060156.

Notes: Colony characteristics and micromorphology of the fungus agreed well with the description of *P. bialowiezense* (Frisvad and Samson, 2004). The species is most closely related to *P. bravicompectum*. *P. bialowiezense* has shorter and less wide stipes than *P. bravicompectum* which differentiate it from *P. bravicompectum*. The fungus has been reported from forest soil, wheat bread, grapes etc. in Denmark, Poland, Italy, Slovenia, Chile, Wyoming, USA, Canada, Saudi Arabia etc. (Frisvad and Samson, 2004).

***Penicillium citrinum* Thom Figs. 1(C, D), 3(A)**  
Bull. Bur. Anim. Ind. US Dep. Agric. 118: 61, 1910

Colonies on CYA 26–32 mm diam, readily sulcate, surface texture floccose in center and vutinous at margins; conidiogenous light to moderate, greyish green; exudates clear, pale yellow or pale brown to reddish brown; reverse yellow, yellow brown to reddish brown.

Colonies on MEA 20–22 mm diam, plane or radially sulcate; surface texture floccose; conidiogenesis moderate to heavy, grey blue at the margin, elsewhere dull green; exudates absent; reverse pale brown to yellow brown.

Conidiophores arising from subsurface or surface hyphae, stipes 100–300  $\mu\text{m}$  long, smooth walled, biverticillate with 3–5 divergent metulae in a whorl; metulae usually uniform, 15–20  $\times$  2–5  $\mu\text{m}$ , bearing 6–10 phialids; phialids ampulliform, 7–10  $\times$  2–2.5  $\mu\text{m}$ ; conidia globose to subglobose, 2.5–3.0  $\mu\text{m}$  diam, smooth-walled or finely rough-walled, produced in long columns.

Isolates examined: CNU 04245, CNU 04246.

Notes: The principle distinguishing character of this species is the penicillus, which consists of 3–5 divergent and usually vesiculate metulae, bearing long well defined columns of conidia (Pitt, 1979). Colonies on CYA at  $25^{\circ}\text{C}$  were dominated by copious clear to yeallow or brown exudates at center; on MEA, growth was slow and dense, with heavy conidiogenesis (Pitt, 1979). This fungus has

been reported from soils in Korea (Kim, 1979; Kim and Lee, 1980; Min *et al.*, 1980).

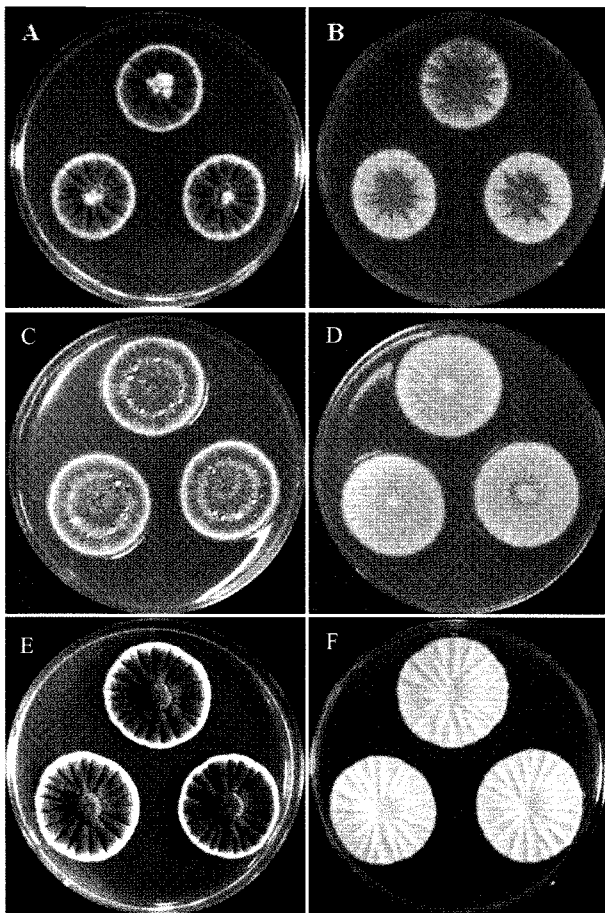
***Penicillium echinulatum* Fassatiouva Figs. 1(E, F), 3(C)**  
Acta Univ. Carol. Biol. 12: 326, 1977

Colonies on CYA 32~40 mm diam, deeply radially sulcate, dense, texture velutinous to weakly fasciculate, floccose in center; conidiogenesis heavy, dull green to dark green; exudate absent or clear droplets; reverse light bluish grey or cream coloured.

Colonies on MEA 20~30 mm diam, lightly radially sulcate, texture velutinous to granular, occasionally crustose centrally; conidiogenesis moderate to heavy, dull green to dark green; exudates clear; reverse yellow orange to orange brown.

Conidiophores borne from subsurface hyphae, singly or in fascicles, appressed, stipes 250~500 × 3.0~4.0 μm long, rough-walled, terverticillate; rami cylindrical 12~20 × 3.5~4.0 μm long; metulae cylindrical, 10~15 × 2~3 μm; phialides ampulliform tapering to a distinct column, 8~11 × 2~3 μm; conidia globose to subglobose, rough-walled, 3.5~4.5 μm.

Isolate examined: CNU 04225.



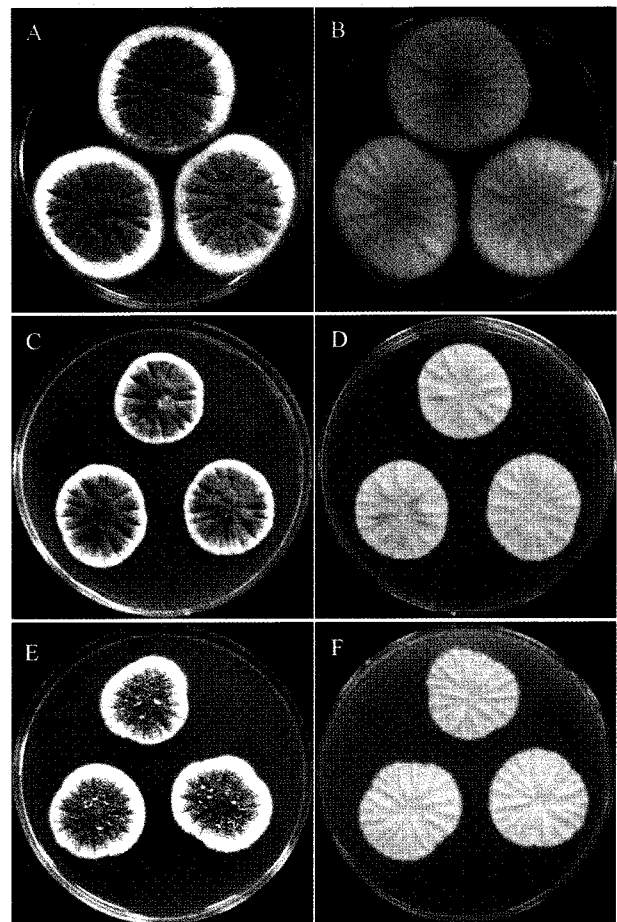
**Fig. 1.** *Penicillium* colonies on CYA after 7 days of incubation: (A, B) *P. bialowiezense*; (C, D) *P. citrinum*; (E, F) *P. echinulatum*: (A, C, E) obverse; (B, D, F) reverse.

Notes: Colony characteristics and micromorphology of the fungus agreed well with the description of *P. echinulatum* (Frisvad and Samson, 2004). The species is closely related to *P. solitum* and *P. discolor*, but differs from *P. solitum* by producing rough-walled conidia and from *P. discolor* by inability to produce a diffusible red pigment (Frisvad and Samson, 2004). The fungus has been reported from food (margarine, chesse and other lipid containing substrate) in Japan, Denmark, Sweden, Norway, the Netherlands, Germany, France, Russia, USA, Wyoming, Thailand, South Africa and Canada (Frisvad and Samson, 2004). This is the first record of *P. echinulatum* from grape in Korea.

***Penicillium expansum* Link Figs. 2(A, B), 3(D)**  
Obs. Mycol. 1: 16, 1809

Colonies on CYA 35~50 mm diam, radially sulcate, moderately deep, texture floccose to weakly fasciculate; conidiogenesis moderate, blue green to moderate bluish green; exudate clear to pale orange brown; reverse light to vivid yellow.

Colonies on MEA 25~35 mm diam, plane, texture velu-



**Fig. 2.** *Penicillium* colonies on CYA after 7 days of incubation: (A, B) *P. expansum*; (C, D) *P. solitum*; (E, F) unidentified *Penicillium* sp.: (A, C, E) obverse; (B, D, F) reverse.

tinose or coremial, sometimes becoming crustose; conidiogenesis heavy, yellow green to blue green; reverse pale to orange brown.

Conidiophores borne from subsurface or aerial hyphae, single or in fascicles, appressed, stipes usually smooth-walled, occasionally very finely rough-walled, terverticillate, less commonly biverticillate; rami cylindrical,  $15\sim 25 \times 3\sim 4 \mu\text{m}$ ; metulae more or less cylindrical,  $10\sim 17 \times 3\sim 4 \mu\text{m}$ ; phialids ampulliform,  $9\sim 12 \times 2\sim 4 \mu\text{m}$ ; conidia ellipsoidal to subglobose, smooth-walled,  $3\sim 3.5 \times 2.5\sim 3.0 \mu\text{m}$  diam.

Isolates examined: CNU 04209, CNU 04210.

Notes: Colony characteristics and micromorphology of the fungus agreed well with the description of *P. expansum* (Frisvad and Samson, 2004). The species is similar to *P. crustosum* and *P. viridicatum* in colony morphology and colouration. The fungus, however, has ellipsoidal conidia and usually smooth stipes. The fungus has been reported from apples, pears and other pomaceous fruits, cherries, peaches etc. in Denmark, United Kingdom, Sweden, Norway and many other countries (Frisvad and Samson, 2004).

***Penicillium solitum* Wrestling** Figs. 2(C, D), 3(E)  
Ark. Bot. 11: 52, 1911

Colonies on CYA 20~30 mm diam, radily sulcate, surface texture vultinous, less commonly fasciculate; conidiogenesis very heavy, dark bluish green to dark green; exudate often present, clear to light yellow droplets on the surface; reverse pale or cream to light beige.

Colonies on MEA 15~27 mm diam, radially sulcate; surface texture velutinous; conidiogenesis heavy, pale bluish green to greyish blue green; exudates rarely present, clear to light yellow; reverse light to moderate orange yellow.

Conidiophores arising from subsurface hyphae, stipes 200~400  $\mu\text{m}$  long, rough-walled, terverticillate; rami cylindrical 10~18  $\mu\text{m}$  long; metulae cylindrical, 10~15  $\mu\text{m}$  long; phialides ampuliform,  $9\sim 11 \times 2\sim 3 \mu\text{m}$ ; conidia globose to subglobose, less commonly broadly ellipsoidal, smooth to finely rough-walled, 3~4.5  $\mu\text{m}$ .

Isolates examined: CNU 04211, CNU 04213, CNU 04214.

Notes: Colony characteristics and micromorphology of the fungus agreed well with the description of *P. solitum* (Frisvad and Samson, 2004). The species is most closely related to *P. echinulatum* and *P. discolor*, but differs from *P. echinulatum* by producing smooth-walled conidia and from *P. discolor* by inability to produce a diffusible red pigment (Frisvad and Samson, 2004). The fungus has been reported from apples, oriental pear, grape, citrus, processed meat in Japan, Denmark, Greenland, United Kingdom, Sweden, Norway, the Netherlands, Germany, France, Russia, USA and Canada (Frisvad and Samson, 2004).

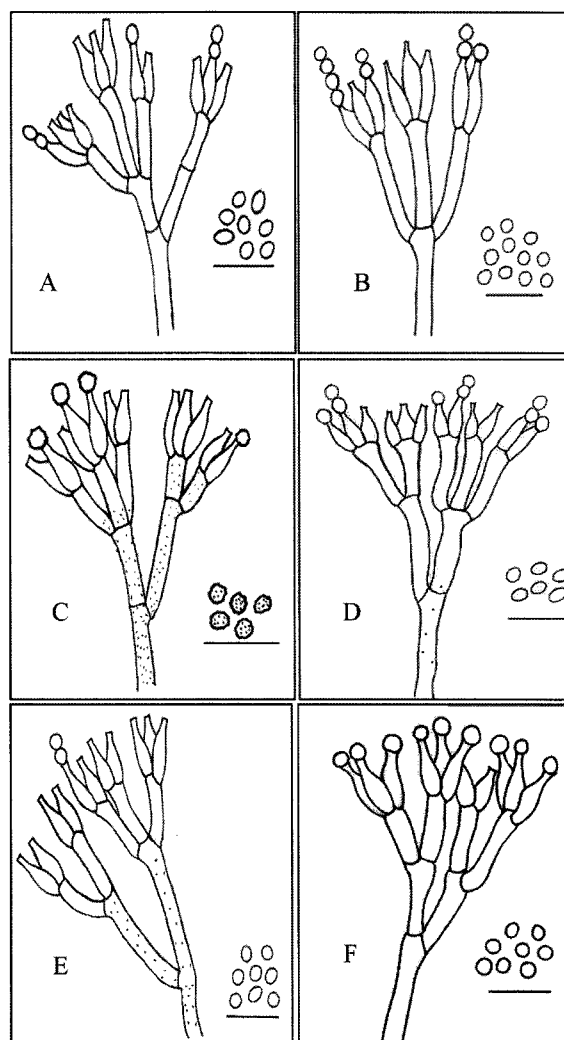


Fig. 3. Conidiophores and conidia of (A) *P. bialowiezense*, (B) *P. citrinum*, (C) *P. echinulatum*, (D) *P. expansum*, (E) *P. solitum* and (F) unidentified *Penicillium* sp. Scale bar = 12  $\mu\text{m}$ .

***Penicillium* taxon** Figs. 2(E, F), 3(F)

Colonies on CYA 20~30 mm diam, radily sulcate, surface texture vultinous; conidiogenesis moderate to heavy, green to dull green; exudate present as clear or colourless droplets on surface; reverse pale greenish yellow to greyish yellow green.

Colonies on MEA 15~25 mm diam, plane or lightly sulcate; surface texture velutinous; conidiogenesis light to moderate, pale green; exudates absent, reverse light yellow to vivid yellow.

Conidiophores arising from submerged or aerial hyphae, stipes 150~300  $\mu\text{m}$  long, smooth or rough-walled, usually terverticillate, sometime biverticillate; rami cylindrical 15~25  $\mu\text{m}$  long; metulae cylindrical, 8~14  $\mu\text{m}$  long, phialids ampuliform, flask-shaped with short necks, 10~13 mm; conidia globose to subglobose, smooth-walled, 2.5~4.0  $\mu\text{m}$  diam.

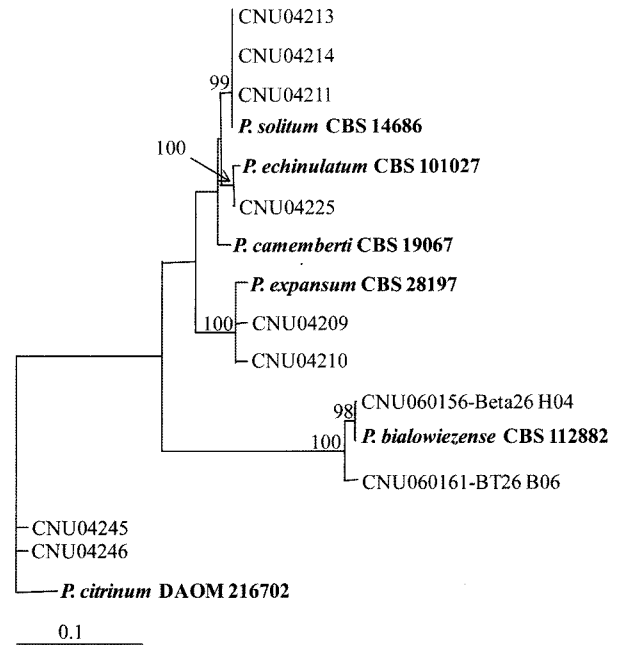
Isolate examined: CNU 060161.

Notes: This taxon can be distinguished from *P. bialowiezense* by its globose conidia.

**Sequence analysis of  $\beta$ -tubulin gene.** The partial  $\beta$ -tubulin gene from 12 isolates of *Penicillium* from blue mold fruits of grape was amplified. Amplification of  $\beta$ -tubulin gene with primers Bt2a and Bt2b yields fragments of approximately 500 bp. BLAST database searches were performed with partial  $\beta$ -tubulin gene as queries to reveal relationship to published sequences.

In a distance analysis with neighbor-joining method, sequence of isolates CNU 04211, CNU 04213 and CNU 04214 was 100% identical to that of *P. solitum* CBS 14686, with a bootstrap value of 99% (Fig. 4, Table 1). Sequence of CNU 04225 isolate was 100% identical to that of *P. echinulatum* CBS 19067, with a bootstrap value of 100%. Isolates CNU 04209 and CNU 04210 and *P. expansum* CBS 28197 were belonged to the same group. Sequence similarity among them was 100%, which was supported by a bootstrap value of 100%.  $\beta$ -tubulin sequence of CNU 060156 and CNU 060161 were 100% and 98% identical to *P. bialowiezense* CBS 112882, respectively. Sequence of isolates CNU 04245 and CNU 04246 was 99.5% identical to that of *P. citrinum* DAOM 216702 (Table 1). Sequence of CNU 04206 isolate matched well with that of *P. camemberti* CBS 19067 (Fig. 3, Table 1).

The  $\beta$ -tubulin gene sequence has been used extensively to characterize a wide range of fungi, specially



**Fig. 4.** Neighbor-joining tree based on phylogenetic analysis of  $\beta$ -tubulin gene sequences. The number below each branch indicates bootstrap values of distance. The bootstrap values were obtained after a bootstrap test of 1000 replications. References, which were taken from GenBank database, are in bold type.

*Penicillium*. The amount of variation is suitable for studying phylogenetic relationship among closely related species of *Penicillium* (Samson *et al.*, 2004) and filamentous

**Table 1.** DNA similarity matrix for  $\hat{\alpha}$ -tubulin gene sequences of *P. bialowiezense*, *P. citrinum*, *P. echinulatum*, *P. expansum*, *P. solitum* and unidentified *Penicillium* species and their related species

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1																
2	80.5															
3	80.5	100														
4	80.5	100	100													
5	80.3	94.4	94.4	94.4												
6	80.9	94.7	94.7	94.7	99.5											
7	80.9	94.7	94.7	94.7	96.8	97.1										
8	80.9	94.7	94.7	94.7	96.8	97.1	100									
9	99.5	80.9	80.9	80.9	80.7	81.2	80.9	80.9								
10	99.5	80.9	80.9	80.9	80.7	81.2	80.9	80.9	100							
11	81.3	93.7	93.7	93.7	97.6	98.2	97.9	97.9	81.7	81.7						
12	81.3	93.7	93.7	93.7	97.6	98.2	97.9	97.9	81.7	81.7	100					
13	81.3	93.7	93.7	93.7	97.6	98.2	97.9	97.9	81.7	81.7	100	100				
14	81.3	93.7	93.7	93.7	97.6	98.2	97.9	97.9	81.7	81.7	100	100	100			
15	74	81.6	81.6	81.6	81.0	81.3	81.0	81.0	74	74	81.6	81.6	81.6	81.6		
16	74.4	81.6	81.6	81.6	81.1	81.4	81.1	81.1	74.4	74.4	81.6	81.6	81.6	81.6	98.1	
17	74.4	81.7	81.7	81.7	81.1	81.4	81.1	81.1	74.4	74.4	81.7	81.7	81.7	81.7	98.1	100

1. *P. citrinum* DAOM216702 (GenBank database); 2. *P. expansum* CBS28197 (GenBank database); 3. *P. expansum* CNU04209; 4. *P. expansum* CNU04210; 5. *P. echinulatum* 101027(GenBank database); 6. *P. echinulatum* CNU04225; 7. *P. camemberti* CBS19067 (GenBank database); 8. *P. sp.* CNU04206; 9. *P. citrinum* CNU04245; 10. *P. citrinum* CNU04246; 11. *P. solitum* CBS14686 (GenBank database); 12. *P. solitum* CNU04211; 13. *P. solitum* CNU04213; 14. *P. solitum* CNU04214; 15. *P. bialowiezense* CNU060161; 16. *P. bialowiezense* CNU060156; 17. *P. bialowiezense* CBS112882 (GenBank database).

fungi (Glass and Donaldson, 1995).

In this study, the phylogenetic tree inferred from the sequences of  $\beta$ -tubulin gene correlated well with the species that were defined by cultural and morphological characteristics except CNU 04206 and *P. camemberti*. The  $\beta$ -tubulin gene sequence evidenced that isolate CNU 04206 was identical to the GenBank sequence of *P. camemberti* CBS 19067 (Table 1), but morphological and cultural characteristics of our isolates did not agree with the description of *P. camemberti* (Frisvad and Samson, 2004). The species was not identified and was supposed to a new species, not previously reported in literature.

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