The First Report of *Penicillium georgiense* in Malaysia

**Teh Li Yee and Latiffah Zakaria***

School of Biological Sciences, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia

**Abstract** *Penicillium georgiense* was isolated from sandy beach soil from Batu Ferringhi beach, Penang Island, Malaysia. The identification was based on morphological characteristics and phylogenetic analysis of internal transcribed spacer regions and β-tubulin sequences. To the best of our knowledge, this is the first report of *P. georgiense* in Malaysia.

**Keywords** Beach soil, β-Tubulin, ITS regions, Morphology, *Penicillium georgiense*

*Penicillium georgiense* was first isolated from peanut-field soils in Georgia, USA, and was reported to show specificity for conidial heads of *Aspergillus* section *Nigri* [1, 2]. Previously, the only published data on the occurrence of *P. georgiense* in soil was the original report by Peterson and Horn [1]. During a biodiversity survey of microfungi in sandy beach soil, *P. georgiense* was isolated and the species identified based on morphological characteristics as well as internal transcribed spacer (ITS) regions and β-tubulin gene sequences. Therefore, this is the first report of the occurrence of this species in sandy beach soil in Malaysia.

Eight *P. georgiense* isolates were recovered from sandy beach soil in Batu Ferringhi (N 5°28', E 100°15'), Penang Island, Malaysia. Isolation was performed on malt extract agar (MEA; Himedia, Mumbai, India). Soil samples were taken from approximately 10-15 cm depth, air dried, and stored in paper bags at 4°C until use. Soil was weighed and 0.3 g was evenly distributed on three plates of MEA. The plates were incubated for five to seven days at 25°C until the growth of fungal colonies was observed. Individual colonies were picked with a sterile inoculation needle and transferred onto new MEA plates. *P. georgiense* isolates were obtained using the direct isolation technique [3]. All eight isolates were deposited in the culture collection Plant Pathology Lab, School of Biological Sciences, Universiti Sains Malaysia.

The macromorphology of all the isolates was observed on Czapek yeast extract agar (CYA; Himedia), MEA, and yeast extract sucrose agar (YES). Three-point inoculation was carried out in 9 cm petri dishes using a dense conidial suspension, followed by incubation in the dark at 25°C for 7 days. The micromorphology was studied on MEA and compared with the description of Peterson and Horn [1]. For microscopic observation, lactic acid (60%) without color dye and lactophenol blue were used as the mounting medium. Twenty-five conidia were randomly measured from each isolate. Morphological features of the isolates are shown in Table 1 and Figs. 1 and 2.

Generally, morphological characteristics of the *P. georgiense* isolates were in agreement with the description by Peterson and Horn [1] although some variations were observed between isolate A3S1-14 and the other seven *P. georgiense* isolates (Table 1). For the seven isolates of *P. georgiense*, the colonies on CYA were 13-19 mm in diameter after seven days, and consisted of velvety, white mycelium with olive green conidial masses, and an absence of exudates (Fig. 1A and 1D). On MEA, the colonies were 18-25 mm in diameter after seven days, velvety, and consisted of dark green conidial masses with white mycelium in the center, and an absence of exudates (Fig. 1B and 1E). On YES, the colonies were 13-17 mm diameter after seven days, and consisted of velvety, dark green conidial masses with white mycelium in the center, and an absence of exudates (Fig. 1C and 1F).

The conidiophores of the seven isolates of *P. georgiense* were biverticillate, with smooth-walled stipes, bearing short conidial chains (Fig. 1G and 1H). Metulae measured 8.5-11 x 2-3.5 μm, whilst phialides were cylindrical and measured...
Penicillium raistrickii reported in indicate intraspecific genetic variation, similar to that characteristics of all eight isolates were very similar (Table 1). The conidial size of isolate A3S1-14 was also larger than the seven other isolates, but the microscopic and 2F). The conidial size of isolate A3S1-14 was also yellow soluble pigments, and absence of exudates (Fig. 2C). On MEA, the colonies were 26~29 mm in diameter after seven days, radially sulcate, yellow soluble pigments produced, exudates absent (Fig. 2A and 2D). For the amplification of ITS regions, the ITS1 and ITS4 primers were used [5], while β-tubulin was amplified using Bt2a and Bt2b primers [6]. PCR conditions and PCR cycles for amplification were adapted from White et al. [5] for ITS, and from Glass and Donaldson [6] for β-tubulin, with some modifications. The annealing temperature used for both ITS and β-tubulin was 58°C.

The ITS regions and β-tubulin gene sequences of all eight P. georgiense isolates were deposited in GenBank with accession Nos. KC329462~KC329469 for ITS, and KC344981~KC344988 for β-tubulin. The sequences of P. georgiense isolates were compared with sequences in GenBank by using the Basic Local Alignment Search Tool (BLAST). The results of the BLAST search showed 98% similarity for ITS regions and 95~96% similarity for β-tubulin with sequences of P. georgiense isolates examined by Peterson and Horn [1].

Phylogenetic relationships of the DNA sequences were identification was carried out. Cultures were grown for DNA extraction in potato dextrose broth using universal bottles and incubated at 25°C. The mycelium was harvested by filtration after 16~48 hr, frozen and lyophilized, and then crushed using liquid nitrogen. Genomic DNA was extracted using Invisorb Spin Plant Mini Kit (STRATEC Molecular GmbH) according to the manufacturer’s instructions. For the amplification of ITS regions, the ITS1 and ITS4 primers were used [5], while β-tubulin was amplified using Bt2a and Bt2b primers [6]. PCR conditions and PCR cycles for amplification were adapted from White et al. [5] for ITS, and from Glass and Donaldson [6] for β-tubulin, with some modifications. The annealing temperature used for both ITS and β-tubulin was 58°C.

The ITS regions and β-tubulin gene sequences of all eight P. georgiense isolates were deposited in GenBank with accession Nos. KC329462~KC329469 for ITS, and KC344981~KC344988 for β-tubulin. The sequences of P. georgiense isolates were compared with sequences in GenBank by using the Basic Local Alignment Search Tool (BLAST). The results of the BLAST search showed 98% similarity for ITS regions and 95~96% similarity for β-tubulin with sequences of P. georgiense isolates examined by Peterson and Horn [1].

Phylogenetic relationships of the DNA sequences were

Table 1. Comparison of cultural and morphological characteristics between the isolates of the present study and Penicillium georgiense isolates described by Petersen and Horn [1]

| Source of description [1] | CYA | MEA | YES | Diameter | Conidiophore | Metulae | Phialid | Conidia |
|---------------------------|-----|-----|-----|----------|--------------|---------|---------|---------|
| Pelliculay [1] | White with olive green conidial masses, velvety, exudates absent | Dark green conidial masses with white mycelium in the center, velvety, exudates absent | Dark green conidial masses with white mycelium in the center, velvety, exudates absent | 13~19 mm on CYA | Biverticillate, bearing short conidial chains, smooth-walled | 8.5~11 × 2~3 μm | 9~11 × 2~3 μm | Born in short chains, globose to subglobose, smooth-walled, 2.5~3.5 × 2~3 μm |
| Peterson and Horn [1] | Dark green conidial masses with white mycelium in the center, velvety, exudates absent | Dark green conidial masses with white mycelium in the center, velvety, exudates absent | Dark green conidial masses with white mycelium in the center, velvety, exudates absent | 26~29 mm on MEA | Biverticillate, bearing short conidial chains, smooth-walled | 12.5~18 × 2.5~3 μm | 12~15~(17.5) × 2.5~3 μm | Born in short chains, globose to subglobose, smooth-walled, 3~3.5 × 2~3 μm |
| C. | Dark green conidial masses with white mycelium in the center, velvety, exudates absent | Dark green conidial masses with white mycelium at the margin, wrinkled, velvety, yellow soluble pigments produced, exudates absent | Near celandine green with velvety, exudates absent | 21~29 mm on CYA | Biverticillate, bearing short conidial chains, smooth-walled | 6~10 × 2.5~3 μm | 8~10 × 2.5~3 μm | Born in short chains, ellipsoidal to subglobose, smooth-walled, 3~4 (4.5) × 2.5~3 μm |

N/A indicates not available in the previous description [1].

CYA, Czapek yeast extract agar; MEA, malt extract agar; YES, yeast extract sucrose agar; N/A, not available.

Source of description [1].
Fig. 1. Morphological characteristics of seven isolates of *Penicillium georgiense* (A2S2-D35, A2S4-D60, A3S1-37, A3S2-6, A3S2-20, A3S3-31, and A3S6-47). Colonies grown on Czapek yeast extract agar (A, D), malt extract agar (B, E), and yeast extract sucrose agar (C, F) after seven days at 25°C. Conidiophores (G, H) and conidia (I) (scale bars: G–I = 10 μm).

Fig. 2. Morphological characteristics of *Penicillium georgiense* isolate A3S1-14. Colonies grown on Czapek yeast extract agar (A, D), malt extract agar (B, E), and yeast extract sucrose agar (C, F) after seven days at 25°C.
analyzed using Molecular Evolutionary Genetic Analysis (MEGA5) software [7]. The combined dataset of both ITS and β-tubulin were analyzed using the maximum likelihood (ML) method, constructed using the Kimura two-parameter substitution model [8]. The tree was inferred using the ML heuristics search option with nearest-neighbour-interchange. Bootstrap analysis was performed with 1,000 replications in order to determine the support for each clade. Penicillium georgiense is in section Aspergillioides, and therefore other species from the same section, as well as from different sections, were included in the phylogenetic analysis. Five Penicillium species included in the analysis were P. glabrum (section Aspergillioides), P. citrinum (section Citrina), P. gerundense (section Charlesia), and P. charlesi (section Charlesia) from subgenus Aspergillioides, and an outgroup (P. brevissimum) from section Ramigena.

The phylogenetic analysis resulted in three highly supported subclades. Seven isolates were grouped together in the same clade and one isolate (A3S1-14) was grouped with the P. georgiense type strain NRRL 35508 (Fig. 3). The other two types of strain (NRRL 35507 and NRRL 35509) formed separate subclades. The results indicated that the P. georgiense isolates from beach soil might represent different phylogenetic strains of P. georgiense, which may be either different phylogenetic species or genetically differing intraspecific lineages. The results of the present study are similar to a study by Scott et al. [9] on P. chrysogenum, in which phylogenetic analysis identified three lineages, representing distinct phylogenetic species. Although the isolates of P. georgiense in the present study might be different phylogenetic strains from the strain reported by Petersen and Horn [1, 2], this study, to the best of our knowledge, is the first recorded occurrence of P. georgiense in Malaysia.

ACKNOWLEDGEMENTS

This study was supported by USM-RU-PGRS grant (1001/PBIOLOGI/834057) and the School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia.

REFERENCES

1. Peterson SW, Horn BW. Penicillium parvulum and Penicillium georgiense, sp. nov., isolated from the conidial heads of Aspergillus species. Mycologia 2009;101:71-83.
2. Horn BW, Peterson SW. Host specificity of Eupenicillium ochrosalmoneum, E. cinnamopurpureum and two Penicillium species associated with the conidial heads of Aspergillus. Mycologia 2008;100:12-9.
3. Watanabe T. Pictorial atlas of soil and seed fungi: morphologies of cultured fungi and key to species. 2nd ed. Boca Raton: CRC Press; 2002.
4. Davolos D, Pietrangeli B, Persiani AM, Maggi O. Penicillium simile sp. nov. revealed by morphological and phylogenetic analysis. Int J Syst Evol Microbiol 2012;62(Pt 2):451-8.
5. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. San Diego: Academic Press; 1990. p. 315-22.
6. Glass NL, Donaldson GC. Development of primer sets designed for use with the PCR to amplify conserved genes from
filamentous ascomycetes. Appl Environ Microbiol 1995;61:1323-30.
7. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731-9.
8. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 1980;16:111-20.
9. Scott J, Untereiner WA, Wong B, Straus NA, Malloch D. Genotypic variation in *Penicillium chrysogenum* from indoor environments. Mycologia 2004;96:1095-105.