Mycobiology

Research Note

A New Record of *Penicillium raphiae* Isolated from Agricultural Soil of Ulleung Island, Korea

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Abstract A fungal isolate EML-NCP01 was recovered from agricultural soil in Ulleung Island, Korea. Phylogenetic analysis of internal transcribed spacer and β-tubulin genes identified the isolate as the *Penicillium* species *P. raphiae*. Morphologically, the EML-NCP01 isolate was identical to the previous description of *P. raphiae*. The species presented here has not been reported in Korea.

Keywords Morphology, *Penicillium raphiae*, Phylogenetic analysis, Soil fungi, Ulleung Island

A large number of species constitute the genus *Penicillium*: these species occupy a wide spectrum of habitats, including soil environments such as forest soil, beach soil, cultivated soil and desert soil [1, 2]. Sufficient researches on distribution and classification system on *Penicillium* have not been conducted in Korea. Approximately, 62 species were reported, and most of them are known to be soil borne [1]. Identification of *Penicillium* species by traditional taxonomic methods is known to be difficult. This may be due to lack of information on the teleomorphic states and the similarities of morphological criteria between anamorphic states [3]. However, Pitt and Hocking [4] considered that the identification of *Penicillium* to the species level could be accomplished when strains were grown under standardized coditions. Recently, phylogenetic analysis with multigene sequences has been widely used in the identification of *Penicillium* species, and in the study of their intraspecific and interspecific relationships [5, 6]. A biodiversity study of fungal communities in samples of cultivated soil from Ulleung Island, Korea in 2013 recovered a number of *Penicillium* isolates, including a species not previously encountered in Korea. On the basis of molecular and morphological characteristics, the species was identified as *P. raphiae*.

Soil samples were collected from different locations of Ulleung Island, North Gyeongsang Province, Korea during July 2013. Each soil sample was collected from cultivable land and taken at a 10~15 cm depth. Samples were maintained in sterile falcon tubes and stored at 4°C until use. *Penicillium* isolates were obtained using the soil dilution plate method [7]. Soil was diluted (100 μg/L) using distilled water and spread on dichloran rose bengal chloramphenicol agar and then incubated at 25°C for 3~7 days. Individual colonies of filamentous fungi were picked up and assigned isolate numbers. Pure cultures were transferred to potato dextrose agar (PDA; Difco, Detroit, MI, USA) slant tubes and deposited in the Culture Collection of Chonnam National University Fungal Herbarium. Two cultures, EML-NCP01 and EML-NCP02 were examined.

Genomic DNA was extracted from mycelia grown on PDA plates using the method of Park *et al.* [8] with minor modifications. For the amplification of the internal transcribed spacer (ITS) region and β-tubulin (BT2) gene of the *Penicillium* species (isolate EML-NCP01), primers ITS5 and ITS4 [9], and primers Bt2a and Bt2b [10] were used, respectively. The resulting sequences and relevant sequences available in the GenBank database were initially aligned with the CLUSTAL_X program [11]. Maximum parsimony analysis of the sequences was carried out using the MEGA program. Phylogenetic tree was constructed (Fig. 1) and evaluated by 1000 bootstrap replications. The ITS sequence of the isolate EML-NCP01 showed 99.00% sequence similarity with the type strain *P. raphiae* CBS 126234[T].

Mycobiology 2014 September, **42**(3): 282-285
http://dx.doi.org/10.5941/MYCO.2014.42.3.282
pISSN 1229-8093 • eISSN 2092-9323

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Received May 14, 2014
Revised July 5, 2014
Accepted September 6, 2014

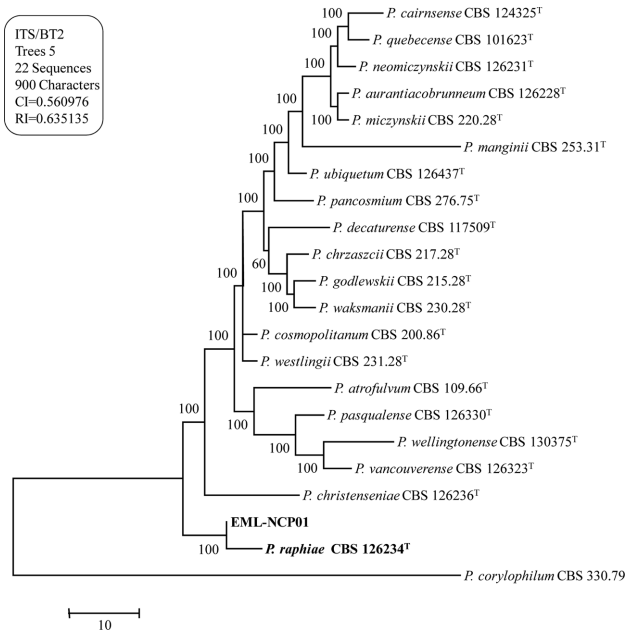

Fig. 1. Maximum parsimony (MP) tree of EML-NCP01 using MEGA based on combined analysis of internal transcribed spacer and β-tubulin gene sequences. Bootstrap percentages are presented at the nodes. Values less than 70% supported in the maximum parsimony analysis are not shown. The bar indicates the number of substitutions per site.

Furthermore, the BT2 gene sequence of the isolate was identical to its type strain [12]. The phylogenetic tree of the combined sequences and their relatives formed a *P. westlingii* clade under the citrina section with a high bootstrap value (Fig. 1). The results confirmed that the isolate EML-NCP01 was the species of *Penicillium raphiae*.

To confirm the molecular species identification, the morphology of the isolate EML-NCP01 was observed under light microscope (DFC 290; Leica, Wetzlar, Germany) and scanning electron microscope (SEM, FE-SEM S4700; Hitachi, Tokyo, Japan). For SEM, the isolate was fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2). After critical-point drying, they were coated with nanoparticle gold labels. Cultural characteristics were observed on Czapek yeast extract agar (CYA), yeast extract sucrose agar (YES), malt extract agar (MEA). Three-point inoculation was carried out 9 cm plastic Petri dishes using a dense conidial suspension and incubated in the dark at 25°C for 7 days. Conidial morphology was measured and compared with the previous description [12]. Morphology of the present isolate agreed with the previous description of *P. raphiae*. Morphological structures of the isolate EML-NCP01 are shown in Table 1 and Fig. 2. Morphological characteristics of the isolate were identical to *P. raphiae*, described by Houbraken *et al.* [12].

Penicillium raphiae Houbraken, Frisvad & Samson, 2011 (Table 1, Figs. 2, 3).

Etymology: This species was isolated from a soil sample collected from cultivated agricultural land on Ulleung Island, Korea.

Description:

Colony: Colony diameter on CYA was approximately 28~33 mm at 25°C after 7 days of inoculation; Velvety, exudates absent, pigment absent, margin slightly irregular, reverse light brown. On YES, the colony diameter was approximately 29~32 mm. Good sporulation, pigment absent, reverse brown, and margin irregular. On MEA, the colony diameter was 20~26 mm at 25°C. Velvety, moderate to good sporulation, margin slightly irregular, conidia light to

Table 1. Comparison of the cultural and morphological characteristics of the present isolate EML-NCP01 with *Penicillium raphiae* characteristics described previously

Characteristics	Present isolate	*P. raphiae*[a]
Colony		
CYA	Velvety, exudates absent, pigment absent, margin slightly irregular, reverse light brown	Velvety, exudates absent, mycelium inconspicuous, pigment absent, margin slightly irregular, reverse crème to light brown
YES	Good sporulation, pigment absent, reverse brown, margin irregular	Good sporulation, soluble pigment absent, reverse (light) brown
MEA	Velvety, moderate to good sporulation, margin slightly irregular, conidia light to blue-green	Velvety, good sporulation, margin slightly irregular, conidia light-blue-green
Size (diameter)	28~33 mm on CYA 29~32 mm on YES 20~26 mm on MEA	32~36 mm on CYA 31~35 mm on YES 21~25 mm on MEA
Sclerotia	Absent	Absent
Stipe	150~450 μm long, smooth or finely rough walled, 2.0~3.0 μm wide	300~500 μm long, smooth or finely rough walled, 2.0~3.0 μm wide
Phialide	Ampulliform, 7~9.5 × 2.0~3.0 μm	Ampulliform, 7~9 × 2.0~3.0 μm
Conidia	Smooth or finely rough walled, broadly ellipsoidal, 1.6~3.0 × 1.5~3.0 μm	Smooth or finely rough walled, broadly ellipsoidal, 1.8~2.5 × 2.0~2.5 μm

CYA, Czapek yeast extract agar; YES, yeast extract sucrose agar; MEA, malt extract agar.
[a]From description by Houbraken *et al.* [12].

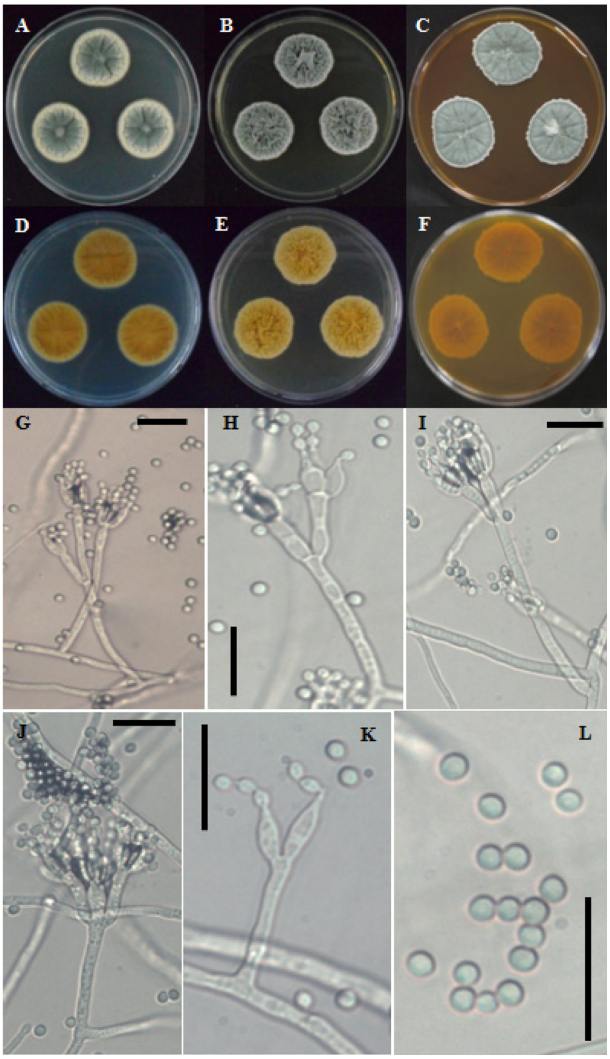

Fig. 2. Morphology of *Penicillium raphiae* EML-NCP01. A~F, Czapek yeast extract agar, yeast extract sucrose agar, and malt extract agar (A~C, obverse; D~F, reverse); G~K, Conidiophores; L, Conidia (scale bar: G~L = 10 μm).

blue-green. No growth at 37°C. Sclerotia: not observed. Conidiophores: biverticillate and occasionally with additional branch; stipes: up to 150~450 μm long, smooth or finely rough walled, 2.0~3.0 μm wide; metulae: in compact terminal whorls of 3~7, almost equal length, 11~17 × 2.0~4.5 μm. Phialides: ampulliform, 7~9.5 × 2.0~3.0 μm. Conidia: smooth or finely rough walled, broadly ellipsoidal, 1.6~3.0 × 1.5~3.0 μm.

Specimen examined: From agricultural soils; isolates EML-NCP01 and EML-NCP02 (Ulleung Island, July 2013).

Distribution: The species was first isolated and identified from soil in a primary forest under *Raphia* palm in Costa Rica. The species was isolated from agricultural lands on Ulleung Island in Korea in late summer.

Note: Colony morphology, cultural characteristics and molecular data analysis of the fungus agreed well with the description of *P. raphiae* CBS 126234^{T} [12]. This species is similar to *P. atrofulvum* and *P. paxilli*. However, the colony growth on MEA after 7 days was slower than *P. atrofulvum* (28~38 mm) and *P. paxilli* (28~35 mm). The conidial size of the present fungus was shorter than other *Penicillium* species previously mentioned. *P. atrofulvum* produced dark sclerotia while *P. raphiae* did not produce any sclerotia [12].

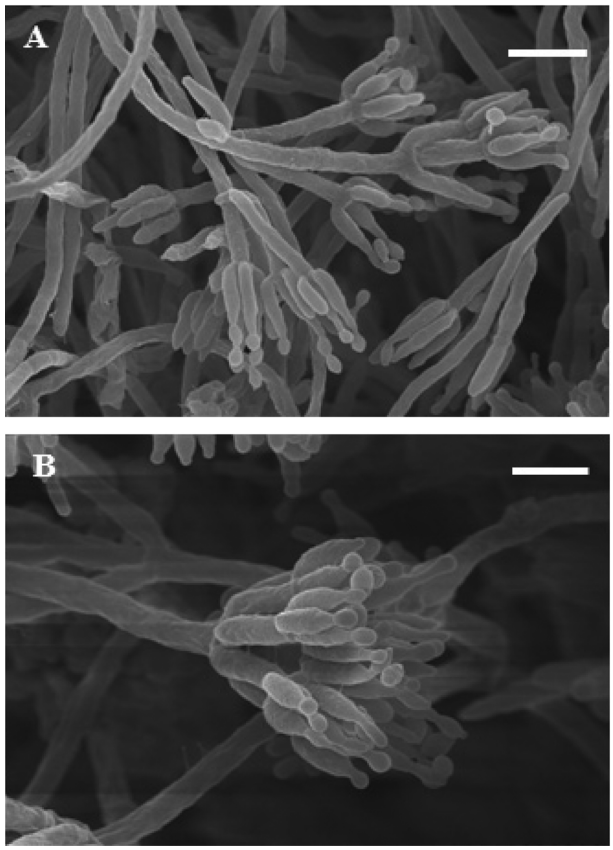

Fig. 3. Scanning electron micrograph pictures of conidiophores attached with conidia of *Penicillium raphiae* EML-NCP01 (scale bar: A = 10 μm, B = 5 μm).

ACKNOWLEDGEMENTS

This work was in part supported by a grant from the National Institute of Biological Resources (NIBR), funded by the Ministry of Environment (MOE) of the Republic of Korea, and by the Korea Foundation for the Advancement of Science & Creativity (KOFAC).

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