Phylogenetic Analysis, Morphology and Pathogenicity of Penicillium spp. associated with Blue Mold of Apple in Korea

Hyun-Kyu Sang1, Young-Phil Choi1, Seung Hun Yu1*

ABSTRACT

Blue mold is the most important postharvest disease of apples in Korea. Apple fruits with blue mold symptoms were collected from storages in different locations in Korea and were investigated for their association with Penicillium species. A total of sixty five isolates of Penicillium were sampled from the collected apples. Based on DNA sequence analysis of β-tublin gene and ITS and lsu rDNA (ID region) and morphological characteristics, they were identified as P. crustosum, P. expansum, P. italicum, P. solitum and P. sp.. P. sp. which is closely related to P. hirsutum is a new species, not reported before. P. expansum (35%) was predominant species followed by P. crustosum. The phylogenetic tree inferred from combined β-tublin and ID region sequence showed good correlation with species that are defined by morphological characteristics. In pathogenicity test, apples were wound-inoculated with conidial suspension and incubated at 20–22°C. The most severe and destructive species was P. expansum. The species caused a decayed area 42–50mm in diameter after 8–10 days. Decayed area caused by P. crustosum and P. sp. was 26–32mm and 20–26mm, respectively. This is the first record of P. crustosum, P. italicum and P. sp. from apple in Korea.

Key words: Apple, Penicillium species, β-tubulin, ITS and lsu rDNA, Morphological characteristics, Pathogenicity

Ⅰ. Introduction

Apples are often stored for 6-10 months after harvest and are subject to be attacked by many post harvest pathogens. Despite modern storage facilities, severe loss by pathogenic causes still occurs. Most of these losses were attributed to Penicillium, Botrytis, Monilinia and Alternaria (Jones and Aldwinckle, 1997). Blue mold, also known as soft rot, caused by Penicillium species is the most important postharvest disease of apple in Korea.

Identification of Penicillium is not easy. It is a large genus, and many common species look alike to the uninitiated. At the same time a great variability even within the species exists, resulting in the unambiguous identification and requiring molecular identification (Guérche et al., 2004). Among the molecular tools available, internal transcribed spacer [ITS (Berbee et al., 1995; Skouboe et al., 1996, 1999, 2000; Peterson, 2000)] and partial β-tubulin gene sequences (Seifert and Louis-Seize, 2000; Peterson and Sigler, 2002; Samson et al., 2004) have been used for identification of the Penicillium species. Skouboe et al. (1996, 1999, 2000) analyzed sequences of the ITS region, including the 5.8S region, of several terverticillate Penicillia and reported few sequence differences among the species. Peterson (2000) also found a few differences between terverticillate Penicillium species in the ribosomal DNA region. Seifert and Louis-Seize (2000) as well as Samson et al. (2004) used partial β-tublin sequences to demonstrate a more resolved phylogeny of Penicillium subgenus Penicillium including terverticillate Penicillia.

During our studies on etiology of post harvest diseases of agricultural products in Korea, several species of Penicillium were isolated from soft rot and blue mold of apples. They were identified based on sequence analysis of the ITS and large subunit ribosomal DNA (lsu-rDNA) (ID region) and β-tubulin gene, and cultural and morphological characteristics. In this paper, we report on five terverticillate Penicillium species associated with blue mold of apple.
II. Materials and Methods

1. Isolation

Apple fruits with blue mold were collected from store houses in Daejoen, Daegu, Suwon, Yeongju and Yesan in Korea during 2006. The conidia assumed to be *Penicillium* were picked up from blue molds of Apples 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. A total of sixty five *Penicillium* isolates were sampled from the collected apples and 10 representative isolates were used in this study (Table 1).

2. Culture

Isolates were three point inoculated onto Czapek yeast extract agar (CYA; K2HPO4 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) in a Petridish. Colony appearance, exudates production, pigmentation and reverse coloration were assessed and colony diameters were measured and recorded after 7 days incubation at 25°C.

3. Morphological observation

*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.

4. DNA extraction

The isolates were cultured in potato dextrose broth for 3–4 days at 25°C with shaking. 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 as described previously (Cubero et al., 1999).

5. PCR amplification and sequencing

For amplification of the ITS and lsu-rDNA (ID) region, primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and D2R (5'-AAC CAG GCA CAA AGT TCT GC-3') (White et al., 1990; Peterson, 1993) were used. Amplification of the β-tubulin gene was performed using primers Bt2a (5'-GGT AAC CAA ATC GGT GCT GCT TTC-3') and Bt2b (5'-ACC CTC AGT GTA GTG ACC CTT GGC-3') (Glass and Donaldson, 1995). The PCR mixture contained 0.5 pmol of each primer, 0.2 mM of dNTP’s, 2.5 U Taq polymerase, and 15 ng of template DNA. PCR conditions for ID region were an initial denaturation step of 96°C for 7 min followed by 30 cycles of 96°C for 30 sec, 51°C for 30 sec and 72°C for 150 sec. A final extension step of 72°C for 10 min was performed. PCR conditions for β-tubulin gene were an initial denaturation step of 94°C for 5 min followed by 25 cycles of 94°C.

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**Table 1.** Origin and GeneBank accession numbers of *Penicillium* species isolated in this study.

<table>
<thead>
<tr>
<th><em>Penicillium</em> Species</th>
<th>Isolate no.</th>
<th>Source</th>
<th>Location of origin</th>
<th>Genebank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. crustosum</em></td>
<td>CNU 6043</td>
<td>Apple</td>
<td>Yesan, Korea</td>
<td>HQ225711</td>
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<td><em>P. crustosum</em></td>
<td>CNU 6045</td>
<td>Apple</td>
<td>Yeongju, Korea</td>
<td>HQ225710</td>
</tr>
<tr>
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<td>Apple</td>
<td>Yeongju, Korea</td>
<td>HQ225712</td>
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<tr>
<td><em>P. expansum</em></td>
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<td>Apple</td>
<td>Daejoen, Korea</td>
<td>HQ225714</td>
</tr>
<tr>
<td><em>P. expansum</em></td>
<td>CNU 7003</td>
<td>Apple</td>
<td>Daejoen, Korea</td>
<td>HQ225715</td>
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<tr>
<td><em>P. italicum</em></td>
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<td>Yesan, Korea</td>
<td>HQ225716</td>
</tr>
<tr>
<td><em>P. solitum</em></td>
<td>CNU 4096</td>
<td>Apple</td>
<td>Daegu, Korea</td>
<td>HQ213935</td>
</tr>
<tr>
<td><em>P. solitum</em></td>
<td>CNU 4196</td>
<td>Apple</td>
<td>Daegu, Korea</td>
<td>HQ213934</td>
</tr>
<tr>
<td><em>P. solitum</em></td>
<td>CNU 6086</td>
<td>Apple</td>
<td>Yesan, Korea</td>
<td>HQ213936</td>
</tr>
<tr>
<td><em>P. sp.</em></td>
<td>CNU 6080</td>
<td>Apple</td>
<td>Yesan, Korea</td>
<td>HQ225717</td>
</tr>
</tbody>
</table>

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유승헌: 사과푸른곰팡이병에 관여하는 *Penicillium*의 계통분석, 형태 및 병원성
for 1 min, 56°C for 1 min, and 72°C for 1 min. A final elongation step of 72°C was performed for 10 min. The PCR product was purified using a Wizard PCR prep kit (Promega, Madison, WI, USA). Purified doublestranded PCR fragments were directly sequenced with a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s instructions. The same primer sets as were used in PCR amplification were used to sequence both DNA strands. Gel electrophoresis and data collection were performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A).

6. Phylogenetic analysis

The sequences were proofread, edited, and merged into comparable sequences using the PHYDIT program version 3.2 (Chun, 1995). Sequences generated from materials in this study and retrieved from GenBank were initially aligned using the CLUSTAL X program (Thompson et al., 1997), and then alignment was refined manually using the PHYDIT program version 3.2. Maximum parsimony trees were calculated using PAUP* (Swofford, 2002) and were estimated using heuristic searches consisting of random addition order and tree bisection-reconnection (TBR) branch swapping. Bootstrap analysis using 1000 replications was performed to assess the relative stability of the branches.

7. Pathogenicity tests

Inoculation experiment for the five species of Penicillium were carried out with apples. Five species of Penicillium were grown on malt extract agar (MEA) for 7 days at 25°C. Conidia were scraped from the cultures on MEA plates using a sterile loop with sterile distilled water and the spore concentration was adjusted to 1×10⁵ spore/ml with a hemocytometer. Apples were wound-inoculated with 20 µl of conidial suspension and were placed on moistened paper towels in plastic boxes. The boxes were sealed with Parafilm and incubated to induce symptoms at 20-22°C for 10 days. Two isolates of each species were tested separately as well as combined together.

III. Results and Discussion

1. Phylogenetic analysis

PCR amplification of the ITS and lsu-rDNA (ID) region for all isolates generated 1120 bp fragment and alignment of the sequence resulted in a data set of 1100 characters. Based on 17 parsimony-informative characters, one most parsimonious tree was located with tree length 39 steps (CI = 0.7692, RI = 0.8861) (Fig. 1). In the maximum parsimony analysis, all ten isolates of Pecnicillium were identified as P. crustosum, P. expansum, P. italicum, P. solitum and P. sp.. Three isolates (CNU 6043, CNU 6045, CNU 6046) and reference strains of P. crustosum (NRRL 35441, NRRL 968) were clustered together in a group, which was supported by a bootstrap value of 77%. The ID region sequences of isolates CNU 6043, CNU 6045, CNU 6046 were identical to that of P. crustosum NRRL 968. The sequences from two isolates (CNU 7002, CNU 7003) and reference strains of P. expansum (NRRL 2304, NRRL 6069) were identical. CNU 6089 isolate and reference strain of P. italicum (KACC 43474) were clustered together in a group, although the sequence of CNU 6089 isolate was differed from the sequence of P. italicum KACC 43474 at one nucleotide position. The sequences from three isolates (CNU 4096, CNU 4196, CNU 6086) were 100% identical to that of reference strains of P. solitum (CBS 14786, NRRL 1151). Isolate CNU 6080 was closely related with P. hirsutum, however, the sequence from isolate CNU 6080 was differed from the sequence of P. hirsutum CBS 41910 at five nucleotide positions. The ID region sequences of all the isolates were deposited in the Genebank (Table 1).

PCR amplification of the β-tubulin gene for all isolates generated 480 bp fragments and alignment resulted in a 393 characters. Based on 69 parsimony-informative characters, 11 most parsimonious trees were located with tree length 188 steps (CI = 0.7394, RI = 0.8435) (Fig. 2). In the maximum parsimony analysis, all ten isolates of Pecnicillium were identified as five species of Penicillium, mentioned before. Three isolates (CNU 6043, CNU 6045, CNU 6046) and reference strains of P. crustosum (CBS 47184, CBS 101025) were clustered together in a group, which was supported by a bootstrap value of 99%. The sequences of three isolates (CNU 6043, CNU 6045, CNU 6046) were 100% identical to the sequence of P. crustosum CBS 101025. The sequences of two isolates (CNU 7002, CNU 7003) and P. expansum CBS 32548 were identical and differed at three nucleotide positions from P. expansum CBS 48184. Sequence of CNU 6089 isolate was 98.5% identical to that of P. italicum CBS 33948 and 97% identical to that of P.
Fig. 1. One parsimony tree inferred from the sequence of ID region. Numbers at the nodes indicate bootstrap values from a test of 1000 replications. The scale bar indicates the number of nucleotide substitutions. GenBank accession numbers of referenced isolates are showed in the parentheses.

italicum CBS 27858, respectively. Three isolates (CNU 4096, CNU 4196, CNU 6086) and reference strains of P. solitum (CBS 14786, CBS 14686) were placed in the same group. The sequence of CNU 6086 was 100% identical to that of P. solitum CBS 14786 and the sequence from CNU 4096 was 99.8% identical to that of P. solitum CBS 14686. The sequence from CNU 6080 isolate was differed from P. hirsutum CBS 110100 at only three nucleotide positions. The β-tubulin sequences of all the isolates were deposited in the Genebank (Table 1).

Phylogenetic analysis based on combined sequence of ID region and β-tubulin yield 5 most parsimonious tree (steps = 208, CI = 0.7404, RI = 0.0812). Parsimony analysis of the combined dataset revealed a tree with similar topology as that revealed in analysis with the ID region and β-tubulin data. In this study, the phylogenetic trees inferred from the sequences of ID region and β-tubulin gene correlated well with the species that were defined by cultural and morphological characteristics except the isolate CNU 6080.

2. Taxonomic description

Penicillium crustosum Thom Figs. 4(A, B), 6(A)
Fig. 2. One of parsimony trees inferred from the sequence of β-tubulin. Numbers at the nodes indicate bootstrap values from a test of 1000 replications. The scale bar indicates the number of nucleotide substitutions. GenBank accession numbers of referenced isolates are showed in the parentheses.

Penicillia: 399, (1930).


*P. solitum* CBS 110100 (AY674330)

*P. expensum* CBS 32548 (AY674400)

*P. italicum* CBS 33948 (AY674398)

*P. atramentosum* CBS 29148 (AY674402)

*P. cruentum* CBS 14786 (AY674355)

*P. echinulatum* CBS 101027 (AY674342)

*P. crustosum* CBS 47184 (AY674352)

*P. crustosum* CBS 101025 (AY674351)

*P. camemberti* CBS 11207

*P. commune* CBS 27967 (AY674361)

*P. freii* CBS 112292 (AY674292)

*P. viridicatum* CBS 109826 (AY674294)

*P. aurantiogriseum* CBS 79295 (AY674298)

*P. polonicum* CBS 69077 (AY674307)

*P. expansum* CBS 48184 (AY674399)

*P. marinus* CBS 109549 (AY674391)

*P.刷卡* CBS 27858 (AY674397)

*P. ulaiense* CBS 31497 (AY674407)

*P. glucosidum* CBS 30797 (AY674394)

*P. griseofulvum* CBS 18527 (AY674432)

 Colonies on CYA 35-45 mm in diam, plane or radially sulcate, surface texture velutinous to fasciculate, becoming...
Fig. 3. One of parsimony trees obtained from a combined ID region and β–tubulin sequences alignment of the Penicillium species. Numbers at the nodes indicate bootstrap values from a test of 1000 replications. The scale bar indicates the number of nucleotide substitutions.

crustose; exudates clear droplets on surface; conidiogenesis moderate to heavy, dull green to grey green; reverse cream to yellow brown.

Colonies on MEA 25-35 mm diam, plane, texture velvutinous, occasionally becoming crustose; exudates absent; conidiogenesis heavy, forming masses of conidia with dry powdery appearance, dull green to grey green; reverse pale brown.

Conidiophores borne from surface hyphae, appressed, stipes 200-400 μm long, rough-walled, terverticillate; rami cylindrical, 15-27 μm long; metulae cylindrical, 10-15 μm long; phialides ampulliform, 9-10 × 2.5-3.0 μm, with short to moderately long collula; conidia globose to subglobose, smooth to finely rough-walled, 3-5 μm.

Isolates examined: CNU 6043, CNU 6045, CNU 6046.

Notes: Colony characteristics and micromorphology of the fungus agreed well with the description of *P. crustosum* (Frisvad and Samson, 2004). The species is closely related to *P. echinulatum* and *P. expansum*. However, the fungus has produced more abundant number of conidia on MEA...
in age than *P. echinulatum* and *P. expansum*. The fungus has been reported from apple and oriental pear fruit in Korea, Denmark, Norway, Germany, Slovenia, Greece, Bulgaria and many other countries. (Frisvad and Samson, 2004). Sixteen out of 65 isolates were identified as *P. crustosum* in this study.

*Penicillium expansum* Link Figs. 4(C, D), 6(B) 
Obs. Mycol. 1: 16, 1809 

Colonies on CYA 22-30 mm in diam, radially sulcate, surface texture floccose to fasciculate; exudate clear to pale brown droplets on surface; conidiogenesis moderate, blue green to green; reverse cream to yellow.

Colonies on MEA 25-35 mm diam, plane, surface texture velutinous, occasionally becoming crustose; exude clear droplets; conidiogenesis heavy, pale 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 finely rough-walled, terverticillate, less commonly biverticillate; rami cylindrical, 15-25 × 3-5 μm; metulae more or less cylindrical, 10-15 × 3-5 μm; phialides ampulliform, 8-13 × 2.5-3.5 μm; conidia ellipsoidal to subglobose, smooth-walled, 3.0-3.5 × 2.5-3.0 μm, borne in long irregular chains.

Isolates examined: CNU 7002, CNU 7003 
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. However, the fungus 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). *P. expansum* has also been reported from onions and garlics in Korea (Yu et al., 1997). This is the predominant species from apples. Twenty three out of 65 isolates (35%) were identified as *P. expansum* in this study.

*Penicillium italicum* Wehmer Figs. 4(E, F), 6(C) 
Hedwigia 33: 211, 1894 

Colonies on CYA 33-43 mm diam, plane or radially sulcate, surface texture velutinous to fasciculate; exudate absent or few clear droplets; conidiogenesis heavy, grey green; reverse brown to red brown.

Colonies on MEA 30-40 mm diam, plane, texture velutinous; exudate absent; conidiogenesis moderate to heavy, pale blue green to grey green; reverse brown orange to red brown.
Conidiophores arised from subsurface hyphae, stipes 100-290 μm long, smooth-walled, terverticillate; rami cylindrical, 15-25 μm long; metulae cylindrical 12-20 μm long; phialides cylindrical, with short but distinct neck, 10-15 × 2-5 μm; conidia cyldrical, but often ellipsoidal to subglobose, smooth-walled, 3-5 × 2.5-4.0 μm. Some isolate produce sclerotia, up to 200-500 μm diam (Raper & Thom, 1949).

Isolate examined: CNU 6089

Notes: The colony characteristics and micromorphology of the fungus agreed well with the description of Penicillium italicum. (Frisvad and Samson, 2004) The fungus is similar to P. digitatum in microscopic morphology. However, the fungus can be distinguished from P. digitatum by its smaller conidia. The fungus has been reported from apple and citrus fruit in Korea, Japan, Italy, Spain, Portugal, Turkey and many countries (Frisvad and Samson, 2004). It has also been reported from onions and garlics in Korea (Yu et al.,1997).

Penicillium solitum Westling Figs. 5(A, B), 6(D)
Ark. Bot. 11: 52, 1911

Colonies on CYA 25-35 mm diam, radially sulcate, surface texture velutinous, less fasciculate; exudate present, clear to light yellow droplets; conidiogenesis heavy, blue green to dark green; reverse cream to light beige.

Colonies on MEA 15-25 mm diam, radially sulcate, texture velutinous; exudate rarely present, clear to light yellow; conidiogenesis heavy, blue green to grey green; reverse orange yellow.

Conidiophores arised from subsurface hyphae, stipes 200-400 μm long, rough-walled, terverticillate; rami cylindrical 10-20 μm long; metulae cylindrical, 10-14 μm long; phialides ampulliform tapering to a distinct collulum, 9-12 × 2.5-3.2 μm; conidia globose to subglobose, less commonly broadly ellipsoidal, smooth to finely rough-walled, 3-4.5 μm.

Isolates CNU 4096, CNU 4196, CNU 6086
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).

*Penicillium* taxon Figs. 5(C, D), 6(E)

Colonies on CYA 25-40 mm diam, radially sulcate or less commonly plane, surface texture velutinous to fasciculate; exudates present, clear to pale yellow; conidiogenesis moderate, green or dull green; reverse cream to yellow cream.

Colonies on MEA 25-37 mm diam, plane or very rarely sulcate, surface texture velutinous to fasciculate; exudates present, pale yellow to red brown; conidiogenesis moderate, green; reverse brown.

Conidiophores arised from subsurface hyphae, stipes 150-500 μm long, rough-walled, terverticillate; rami cylindrical 15-25 μm long; metulae cylindrical 8-15 μm long; phialides ampulliform tapering to a short collulum, 8-12 × 2.5-3.5 μm; conidia globose to subglobose, smooth-walled or very finely rough-walled, 3-3.5 μm

Isolate examined: CNU 6080

Notes: This taxon can be distinguished from *P. hirsutum* by not producing yellow synnemata.

3. Pathogenicity

Since there was no significant difference in the incidence or severity of the decayed symptoms among two isolates of each species inoculated separately or combined together on apples, only results from apples inoculated with combined isolates are shown (Fig. 7). The most severe and destructive species was *P. expansum*. The species caused a decay area 42-50 mm in diameter after 8-10 days. Decayed area caused by *P. crustosum* and *P. sp.* was 26-32 mm and 20-26mm, respectively (Fig. 7). *P. italicum* and *P. solitum* also produced moderate to mild symptoms 7-10 days after inoculation. Each species of *Penicillium* was re-isolated from the blue mold of inoculated fruits of apple. It was reported that *P. expansum*, *P. solitum* were associated with blue mold of apple in Korea (Yu, 2006), however, *P. crustosum*, *P. italicum* and *P. sp.* were the first record from apple in Korea.

IV. Conclusion

The occurrence of blue mould disease in stored apples results in severe loss of fresh apples in Korea. Five *Penicillium* species including *P. crustosum*, *P. expansum*, *P. italicum*, *P. solitum* and *P. sp.* were found to cause blue mould disease. *P. sp.*, closely related with *P. hirsutum* was revealed as a new species. The most highly pathogenic species was *P. expansum*, *P. crustosum*, *P. italicum* and *P. sp.* has not been reported so far and this is the first record.

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References


