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A N I N T E R N A T I O N A L J O U R N A L

Polymerase Chain Reaction (PCR) Identification of Terverticillate *Penicillium* Species Isolated from Agricultural Soils in Eskişehir Province

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ABSTRACT

In the present study, nine terverticillate Penicillium *isolates (*P. griseofulfum, P. puberulum, P. crustosum, P. aurantiogriseum, P. chrysogenum, P. primulinum, P. expansum, P. viridicatum, Eupenicillium egyptiacum*) from 56 soil samples were characterized genetically by a PCR method. The DNAs of the strains were isolated using the glass beads and vortexing extraction method and then used for PCR amplification with the internal transcribed spacer 1 (ITS1) and ITS4 universal fungal specific primers. The ITS regions of fungal ribosomal DNA (rDNA) were sequenced through the CEQ 8000 Genetic Analysis System. ITS-5.8S sequences obtained were compared with those deposited in the GenBank Database. The results indicated that the identification of* Penicillium *species with PCR based methods provided significant information about the solution to taxonomy and improve food safety and to protect the users from harmful contaminants such as mycotoxins, which must be controlled during the production of agricultural materials as well as during the processing of food and feed.*

Key words: *Penicillium*, identification methods, PCR, ITS

INTRODUCTION

Penicillium is a genus of Hyphomycetes, characterized by the production of conidia in chains from the verticils of the phialides. The most complex penicillus characteristically has three branch points. These penicillus types are defined as terverticillate (Pitt 2000). This group includes important food and feed spoilage moulds, pathogens of mature fruits and cereal grains. In addition to causing deterioration and quality loss, these moulds may contaminate agricultural products with potential mycotoxins (Logrieco et al. 1990).

The taxonomy of terverticillate penicillia has been considerably studied (Pitt 1979). However, there are still significant problems to identify them due to the isolates commonly having characteristics found in more than one species (Logrieco et al. 1990). Furthermore, 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 (Pitt 2000). To clarify the systematic and species concepts in the terverticillate penicillia, a major integrated multidisciplinary study was undertaken. This involved selecting and developing the taxonomic characters from morphology, physiology, biochemistry and scanning electron microscopy (Bridge et al. 1990). Studies were also carried out with molecular biological techniques (Mullaney and Klick 1990). Conventional identification of

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moulds is mainly performed by phenotypic criteria, including micro- and macro-morphology and requires considerable expertise. This procedure may take several days and is dependent on the growth and sporulation, as well as on individual interpretation. Even then, related species may be confused and rare species may remain unidentified (Ciardo et al. 2007). Though many biochemical and physiological techniques have provided information useful for the classification, the nucleotide sequence and the organization of DNA are the most likely to give a clear and sensitive discrimination between the organisms and also to indicate clearly their evolutionary and phylogenetic relationship (Croft et al. 1990).

Comparative studies of the nucleotide sequences of ribosomal RNA (rRNA) genes provide a means for analyzing the phylogenetic relationships over a wide range of taxonomic levels (White et al. 1990). The small subunit rRNA gene has been used extensively for phylogenetic analysis, and for the identification purposes at genus, or species level in bacteria and eukaryotes. The ITS region of the rRNA operon is located between the 18S and 28S rRNA genes and includes the two intervening regions, ITS1 and ITS2, and the highly conserved 5.8S rRNA gene (White et al. 1990). This work aimed to study the use of ITS sequences for the identification of the closely related terverticillate *Penicillium* species isolated from agricultural soils in Eskişehir province. In addition to molecular characterization of *Penicillium* species, the study also aimed to study the potential of *Penicillium* species as a risk factor because of their mycotoxin, spoilage and pathogenic properties in agricultural soils in Eskişehir province.

MATERIALS AND METHODS

Fungal strains

All terverticillate *Penicillium* strains (Table 1) used in this study were obtained from the agricultural soils in the Eskişehir province and identified using the traditional methods according to Pitt (1979) and Demirel et al. (2005). All the strains were stored in suitable conditions at the Culture Collections of KUKENS (WDCM101), the Centre for Research and Application of Culture Collections of Microorganisms. Cultures were maintained at 4ºC on potato dextrose agar (PDA).

Table 1 - List of the terverticillate *Penicillium* species in this study.

Species	Isolate number
Penicillium griseofulvum Dierckx	T2
Penicillium puberulum Bainier	T3
Penicillium crustosum Thom	T4
Penicillium aurantiogriseum Dierckx	T5
Penicillium chrysogenum Thom	T7
Penicillium primulinum Pitt	T8
Eupenicillium egyptiacum (J.F.H. Beyma) Stolk & D.B. Scott	T9
Penicillium expansum Link	T10
Penicillium viridicatum Westling	T11

DNA extraction

Genomic DNA was isolated from the cultures grown on PDA for seven days. About 50-100 mg of mycelia were scraped from the agar surface and placed in a 1.5 mL micro-centrifuge tube containing 400 µL lysis buffer (2% Tripton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA). Lysis of the was achieved by the addition of 500 mg acid-washed 0.4-0.6 mm diameter glass beads, 400 µL phenol/chloroform/iso amyl alcohol (Phe/Chl/IAA) (25:24:1), and continuous vortexing for 30 min at the highest intensity setting (Van Burik et al. 1998) using a vortex (IKA Vortex GENIUS 3). The sample was then centrifuged at 13,000 rpm for 5 min. The aqueous (top) layer was transferred into a new tube and re-extracted with an equal volume of Phe/Chl/IAA (25:24:1) two times. At the re-extraction stage, following the addition of Phe/Chl/IAA (25:24:1), the sample was gently mixed for 5 min at mixer (Multi KS-60 BIOSAN Programmable Rotator-mixer 90º), and then centrifuged at 13,000 rpm for 5 min. The top aqueous layer was transferred to a clean 1.5 mL micro-centrifuge tube and the DNA was precipitated with the addition of 0.1 volume 3 M sodium acetate, followed by two volumes cold 98% ethanol. After incubation at – 20ºC for 20-30 min, the DNA was pelleted by centrifugation at 13,000 rpm for 5 min. The ethanol was decanted and the pellet was re-suspended in 200 µL distillated water. DNA was precipitated by the addition of 0.1 volume 0.3 M sodium acetate, followed by two volume cold 98% ethanol. After incubation at – 20ºC for 20-30 min, the DNA was pelleted by centrifugation at 13,000 rpm for 10 min, the ethanol was decanted, and the pellet was air dried in a laminar flow hood. DNA pellet was re-suspended in 50 µL $T_{10}E_1$ (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)) and its concentrations were

estimated visually in 1% agarose gels containing 5 µg/mL ethidium bromides by comparing band intensity with known quantities of DNA high range markers.

Polymerase chain reaction (PCR)

The nuclear ribosomal ITS1-5.8S-ITS2 region was amplified with the primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´). Reactions were performed in 25 µL volumes containing 1 µL genomic DNA, 2.5 µL 2.5 µM ITS1, 2.5 µL 2.5 µM ITS4, 2.5 µL 10X *Taq* buffer +KCl – $MgCl_2$ (Fermentas), 2.5 µL 25 mM MgCl (Fermentas), 2 µl 2.5 mM dNTPmix, 0.25 µL 5 U/ µL *Taq* DNA polymerase (Fermentas), and 11.75 µL sterile deionized water. DNA amplification was performed in a thermocycler with an initial denaturation step for 2 min at 95ºC, followed by 35 cycles of denaturation for 1 min at 95ºC, anneling for 45 sec at 55ºC, and extension for 1 min 30 sec at 72ºC. A final extension at 72ºC was performed for 5 min. To confirm the amplification of solely the ITS, 5 µL of PCR product together with marker (GeneRuler DNA Lader 50 bp Fermentas) was resolved by gel electrophoresis on 1% agarose gel containing 5 µg/mL ethidium bromide in 1X TAE buffer. The gel samples were photographed in the Gel Documentation System (Uvitec M02 4611).

Sequencing

Template DNA for sequencing was prepared as follows: agarose gel blocks containing DNA fragment were cut out and purified with Promega Wizard® SV Gel and the PCR Clean-Up System. PCR products for sequencing were obtained with the GenomeLab DTCS Quick Start Kit and sequences were determined with the primers ITS1 and ITS4. Cycle sequencing products were purified with Dynabeads® Sequencing Clean-Up to remove unincorporated dye-labeled nucleotides. Direct sequencing was performed with Beckman Coulter CEQ 8000 Genetic Analysis System.

Phylogenetic analysis

The sequences were blasted with the Genbank sequences to ensure the identifications; the closest Blast results are reported below each taxon (Table 2). New sequences from these groups were aligned along with the similar GenBank sequences in software ClustalW in the MEGA5 programme (Tamura et al. 2011). Ambiguously aligned regions and sequence ends were manually excluded. The Tajima-Nei model in Neighbor-joining method in MEGA5 programme was used for the analysis of phylogenetic relationship with 1000 bootstrap and *Penicillium aurantiogriseum* (DQ249210) was used as an outgroup in the ITS analysis.

Table 2 - Newly generated ITS sequences with their closest GenBank sequences (according to Blast searches).

Species	Collection	GenBank accession number	Closest Blast hit (% identity/% coverage)
Penicillium griseofulvum Dierckx	T2	KF293650	*Penicillium griseofulvum* JX091409 (99/100)
Penicillium puberulum Bainier	T3	KF293652	*Penicillium tricolor* JN942703 (99/97)
Penicillium crustosum Thom	T4	KF293653	*Penicillium chrysogenum* KC492574 (99/96)
Penicillium aurantiogriseum Dierckx	T5	KF293654	*Penicillium chrysogenum* KC492574 (99/96)
Penicillium chrysogenum Thom	T7	KF293651	*Penicillium* sp. JX982473 (98/96)
Penicillium primulinum Pitt	T9	KF293655	*Penicillium* sp. JX997060.1 (99/96)
Eupenicillium egyptiacum (J.F.H. Beyma) Stolk & D.B. Scott	T8	KF293657	*Penicillium chrysogenum* JQ724529 (94/96)
Penicillium expansum Link	T10	KF293658	*Penicillium brevicompactum* JQ781814 (82/76)
Penicillium viridicatum Westling	T11	KF293656	*Penicillium polonicum* KC692223 (99/96)

RESULTS AND DISCUSSION

Using the universal fungal primers (ITS1/ITS4), PCR products (570 bp) were obtained from all of the species. Figure 1 showed that the sizes obtained for the full ITS region amplified different terverticillate *Penicillium* species.

The phylogenetic trees were obtained by comparison to all the sequences in the Genbank nucleotide sequence database that had ITS1-5.8S rRNA-ITS2 sequences (Fig. 2). When each of these sequences was investigated with BLAST N, different maximum identification values were obtained, with the highest being 99%, and the lowest 82% (Table 2).

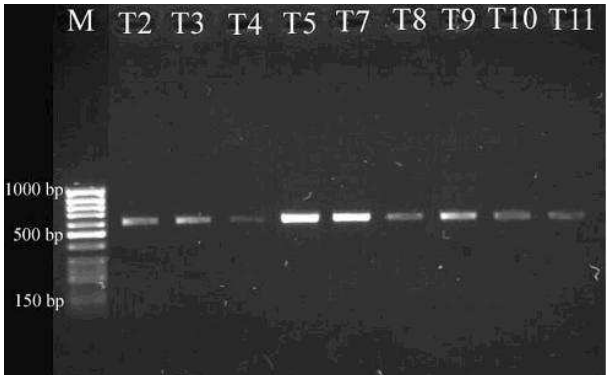

Figure 1 - Full ITS PCR products amplified from different terverticillate *Penicillium* species with ITS1/ITS4 primers. M, molecular-weight markers (50 bp GeneRuler DNA Lader, Fermentas).

The rDNA genes, generally used in the taxonomic and identification studies, were confirmed in the present study. ITS primers 1 and 4 were used in this study to amplify the entire 5.8S rDNA gene, both ITS1 and ITS2. The most similar ITS sequences up to 82% similarity values were determined by BLAST N comparison of ITS to other ITS sequences from penicillia in the GenBank database. In this comparison, the T2 strain that was identified as *P. griseofulvum* by the traditional identification methods showed a similarity to *P. griseofulvum* strains with 99% maximum identification. The T3 strain identified as *P. puberulum* showed a similarity to *P. tricolor* strains with a 99% maximum identification with *P. tricolor*. Thus, useful results were obtained about this strain with sequences of the ITS region. The T4, T5 and T8 strains identified as *P. crustosum, P. aurantiogriseum* and *Eupenicillium egyptiacum,* respectively had a 94-99% similarity to *P. chrysogenum*. The T9 strain identified as *P. primulinum* had 99% similarity to *Penicillium* sp. strains. The T7, T10 and T11 strains identified as *P. chrysogenum, P. expansum* and *P. viridicatum* had 98, 82 and 99% similarities to *Penicillium* sp., *P. brevicompactum* and *P. polonicum*, respectively.

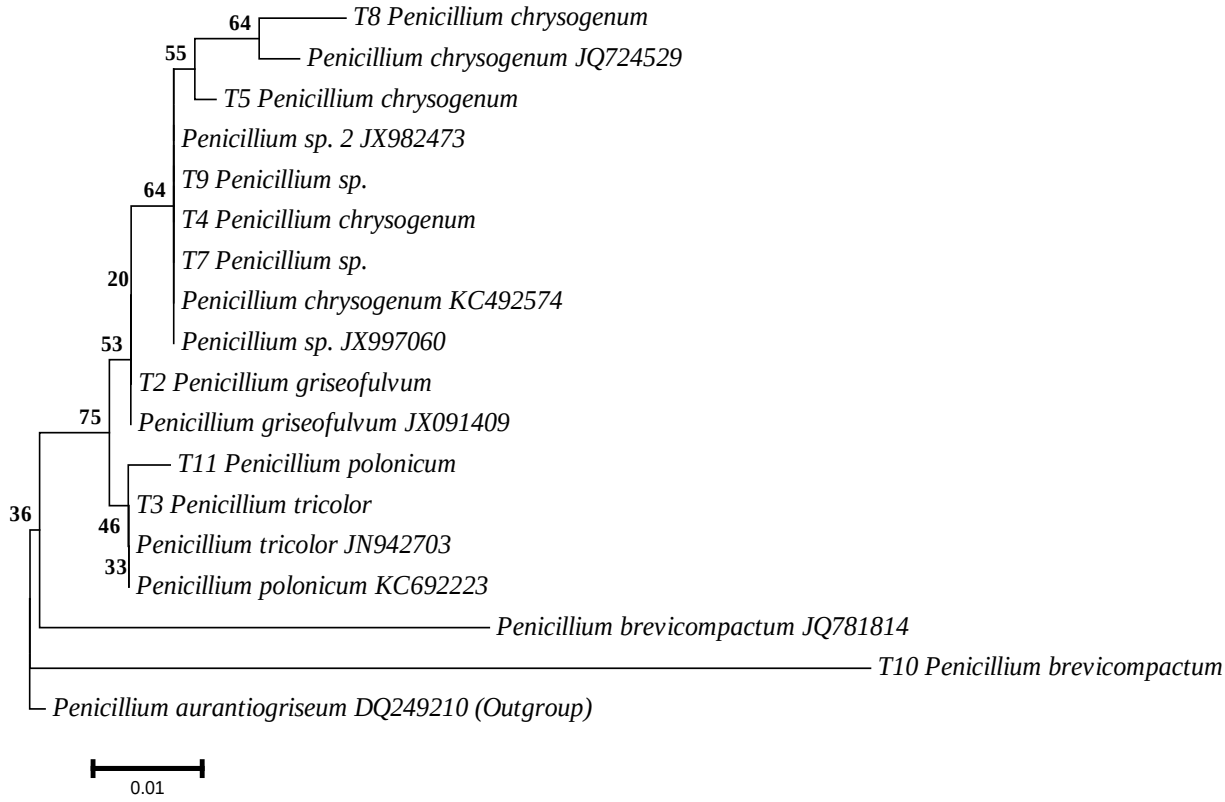

Figure 2 - Best-scoring Neighbour joining tree calculated using MEGA5.0 based on ITS sequences showing the relationships of the newly generated sequences in this study with previously known taxa in NCBI GenBank. The tree is rooted with *Penicillium aurantiogriseum* (DQ249210) (Bootstrap 1000).

Phylogenetic tree analyses showed that the studied terverticillate penicillia were prominently divided to four clades. One of them was included as *P. chrysogenum*, *Penicillium* species and closely related isolates as T4, 5, 7, 8 and 9. Furthermore, T5 and T8 were distinctly differentiated from other clade members in the phylogenetic tree. The isolate T2 and *P. griseofulvum* occurred as another clade in phylogenetic tree. This species has been distinguished from *P. chrysogenum* with short phialides and strongly divergent branching. *P. tricolor* and *P. polonicum* were clustered into the same clade; besides, these species were member of *Penicillium* section *Viridicata* series *Viridicata*. The last clade belonged to *P. brevicompactum*. This species was distinguished from others with appressed all elements.

The studied terverticillate penicillia species had some distinguishable characters but the discrimination of these characters was very difficult by microscopic investigation. On the other hand, PCR identification techniques provided highly useful information about the molecular identification and distinguishing of fungi, especially closely related fungi.

CONCLUSIONS

This study resulted reliable information using universal fungal primers. In addition, information regarding the closely related terverticillate *Penicillium* species were clarified. Through the use of molecular techniques correct descriptions and individual selections were achieved.

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