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Isolation and Molecular Identification of Soil Inhabitant *Penicillia*

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ABSTRACT

Penicillium is one of the most abundant components of soil fungal flora around the world. Several studies have been conducted on Penicillium identification to date,but most of them were associated with classical methods and morphological studies. In this investigation, more than fifty of soil samples from different provinces of Iran were collected and examined with soil suspension culture. Among various fungi, only Penicillium was selected. The pure cultures were prepared on PDA slants. Total DNA of isolates was extracted after culture in PDB media and was amplified with ribosomal region primers, ITS4 and ITS5,then were sequenced. Results of obtained sequences were compared with sequences exist in GenBank and these species were identified: Penicillium aculeatum, P. aurantiogriseum, P. chrysogenum, P. citrinum, P. commune, P.crustosum, P.pinophilum, and P. simplicissimum. Molecular identification of soil inhabitant Penicillia is a rapid method for accurate assessment of these fungal populations.

Key words: *Penicillium*, soil inhabitant, ITS, PCR, Molecular identification.

INTRODUCTION

The genus *Penicillium* is one of the largest and widely distributed of all fungal genera described todate. In the period from the publication of the first taxonomic study on *Penicillium* in 1930 to the latestclassification in 2004, 225 new *Penicillium* species have been described(Alexopoulos et al., 1996). The results of previouslypublished taxonomic studies, in which only morphological characteristics were used to identify fungalspecies, yielded different classification proposals because of strain variation(Onions AHS et al., 1984). Strain variationin a fungal species is common due to differences in the environmental conditions of their habitat.Since the species concept is based primarily on morphology, the exact identification of some fungalspecies is not always possible,and further investigationis then necessary in order to identify the fungus.

Taxonomic studies that supplemented traditional morphological character sets included many studies of extrolites(Frisvad et al., 2000; Frisvad and Samson, 2004; Stevenson and Lochhead, 1953); Isosymes(Cruickshank and Pitt, 1987) or combinations of phenotypic characters (Bridge and Hawksworth, 1984), usually analyzed using similarity or clustering methods.

In recent years, molecular techniques exploiting variations in nuclear and mitochondrial rDNA have been used extensively for phylogenetic and systematic studies in fungi, including *Penicillium*. Different regions of the rDNA diverge at different rates, allowing their sequences to be exploited at different taxonomic levels (Bruns et al., 1991; Skouboe P et al., 1999; Weisburg et al., 1991). The non-coding rDNAregions, especially the ITS 1 and ITS 2 region

but also to a lesser extent the IGS region, are generally more variable than the rRNA genes and have been most widely used for taxonomic studies near the species level(Baldwin et al., 1995; Bruns et al., 1991; Stevenson and Lochhead, 1953).

ITS in the genus *Penicillium*the noncodingregion of the rDNA unit has been particularly investigated to clarify the subdivisions within the genus(LoBuglio and Taylor, 1995; Peterson, 2000) and to investigate the genetic structure of some species.

In the present study, we have used sequence data from the rDNA ITS regions to characterize more than 50*Penicillium* isolates in order to identify species-specific differences.

MATERIALS AND METHODS

Fungal isolates

Fungi used in this study were isolated from agricultural soils of different provinces in Iran. List of some places which are used for collecting *Penicillium* isolates, is in table1.

2.2. Isolation from soil

Soil suspensions which were prepared from different soil samples were cultured on Rose Bengal medium (KH2Po4, MgSo4, K2HPo4, Pepton, Dextrose, Yeast extract, Agar and DW). Rose Bengal (an inhibitor of fungal growth) and Streptomycin added to the media after autoclave. Samples were kept at 28° C in incubator. After 3 days plates were investigated and between different fungi which were grown, only *Penicillia* were selected and transferred to WA medium. After 3 days, a single mycelial tip was isolated from plate cultures and transferred to PDA to obtain a pure culture. The plates incubated 3-5 days at 28°C and then were kept in refrigerator (4° C) up to the time of morphological and molecular assays.

2.3. DNA Extraction, PCR amplification, sequencing and analysis

A modified CTAB DNA extraction protocol was used to acquire DNA from the *Penicillium* isolates. The isolates were grown in 30 ml of liquid medium (potato dextrose broth (Merck). Agar blocks (approximately 0.5 cm^2) of each isolate were used to inoculate liquid medium. The cultures were incubated for three to four days at 28 °C in a rotary shaker (120 rpm). The mycelia mat were centrifuged and washed once in sterile water and twice in 500 mM NaCl2 and 50 mM EDTA pH 8.0. The mycelia were blotted dry and macerated in liquid nitrogen with 800 µl of DNA extraction buffer [1.0% CTAB, 0.5 M NaCl, 69 mM EDTA pH 8.0, 34 mMTris pH 8.0]. 2-mercaptoethanol (0.009%) was added and the solution was incubated at 65 °C for 30-40 min. After incubation and adding 600 µl of Isoamyl alcohol /chloroform (1:24) the solution was centrifuged for 10 min at 12000 rpm. Then the supernatant was transferred to the new 1.5 ml tubes, DNA was precipitated in 0.6 volumes of isopropanol, centrifuged at 13000 rpm for15 min. The Pellet washed with 70% ethanol, dried under flow hood, eventually resuspended in 70 µl of DEPC treated water and stored at 4 °C till to use in consequent operations (Rapley R., 2000).

The ITS region of the rDNA was amplified using ITS4 (5'- TCCTCCGCTTATTGATATGC) and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG) primers (White et al., 1990). The PCR reaction was performed in 25 µl total volumes containing 2.5 µl of 10x PCR buffer, 1 µl of MgCl2, 1µl of each primer (Final concentration 10 pmol/µl), 1µl DNTP, 0.2 µl Taq, 2 µl of DNA template and 16.45 µl of PCR Grade water. The amplification was performed in PCR thermal cycler. The cycle parameters were as follows: an initial denaturation at 94°C for 4 minutes, followed by 30 cycles consisting of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. Final extension was at 72°C for10 minutes. Following the PCR reaction, the amplified products were resolved in a 1.5% agarose gel, stained with ethidium- bromide 1 mg/ml for 15min. Electrophoresis was run at 75 V for 1 hour. The DNA bands were visualized using Kodak Gel logic 200 Gel documentation System.

PCR products sent for sequencing to Bioneer biotech.(South Korea). The obtained sequences compared with the other related sequences with the same region of *Penicillium* genus in GenBank (NCBI).

RESULTS AND DISCUSSION

One of the aims of this research was identifying of different species of *Penicillium*in various geographical region of Iran, specially the strain of those species that are used as biocontrol agents. Relying on morphology and ability to grow on selective culture media as the method to identify *Penicillium* species is time consuming and laborious. In addition, considerable expertise is needed to clearly differentiate between closely related species.

Several efforts have been made in the rapid and short term identification of *Penicillium* and without having to refer to the classical method, and many indicators have also been investigated. Among these, the Epizyme enzyme was proposed as one of the markers that have not been very appreciated because of problems with it.

The sequences of the coding region of the ribosomal DNA have been used as a reliable marker for population genetic studies of various fungi. At least two hundred copies of ribosomal genes are in mammalian genomes. They contain areas of 28s, 18s and 5/8 s, respectively. The Internal Transcribed spacer(ITS), is become popular as a general indicator for the study and comparison between the fungal species.18s and 28s regions are used for identification of genus and higher phylogenetic levels even families, but to compare the species and within genus the ITS and IGS indicators are further exploited. The information of these regions of the fungi is available in databases, especially NCBI.

Nowadays, DNA sequencing has been evolved rapidly, since introduction of basic techniques that were developed by Coulson and Sanger, and with help of fluorescence markers have become a semi-succession process. On this basis cost of sequencing is relatively reduced and the technique has become available for everyone and millions parts of the genome sequencing of organisms have been performed and recorded in databases. The complete genome of strategic plants and some organisms have been sequenced and are used in numerous advanced studies. Fungi aren't also excluded from this issue and all of the advanced studies, related to fungi are associated with molecular studies. In this study, the ribosomal DNA region containing the ITS 1, ITS 2 regions and 5.8S rRNA gene was amplified from 50 *Penicillium* isolates, using ITS4 and ITS5 primers (White et al., 1990). The PCR product was in all cases a unique fragment of approximately 600 bp.Final results concluded to identification of eight species from more than fifty isolates of *Penicillium* . The identified species were : *Penicillium aculeatum, P. aurantiogriseum, P. chrysogenum, P. citrinum, P. commune, P.crustosum, P.pinophilum,* and *P . simplicissimum*.

Table1 Some of *Penicillium* isolates and their collection place of

Isolate code	Place of collection	Identified species
C62	Aghghala- Wheat field	*P. crustosum*
A22	Tabriz- Wheat field	*P. crustosum*
D73	Salman shahr	*P. crustosum*
C42	Aghghala- Wheat field	*P. crustosum*
D32	Salman shahr- Rice	*P. crustosum*
C1	Gorgan- Corn field	*P. commune*
C20	Behshahr	*P. commune*
D28	Salman shahr- Rice	*P. commune*
D1	Salman shahr- Rice	*P. aurantiogriseum*
E9	Sari	*P. piaophilum*
B6/A	Amol- Kamardeh	*P. piaophilum*
E1	Noshahr	*P. piaophilum*
C13	Gorgan- Wheat field	*P. simplissimum*
A32	Tabriz	*P. verruculosum*
A34	Ghazvin	*Talaromyces helicus*

CONCLUSION

Identification of fungal genera and species is a critical step for using the isolates in complementary experiments. Classic methods using diagnostic dichotomic keys and species discussions are time consuming methods and final judgment for species determination is difficult . For rapid identification of soil *Penicillia* and other *Penicillium* species molecular markers specially ITS act effectively and correctly.

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