Molecular Identification of some isolates of Fungi Isolated from Al-Mishkhab Rice Field research station

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Abstract This study was conducted at the Microbiology Laboratory of the Department of Environmental Science - College of Science - University of Kufa with the aim of isolating and diagnosing fungi isolated from the rice field at the Rice Research Station in Al-Mishkhab in Najaf Governorate. Fungi isolates were diagnosed using the Polymerase Chain Reaction (PCR) technique and nucleotide sequence determination for DNA products multiplied using the ITS1 and ITS4 prefixes. The results of analyzing the nucleotide sequence of the double DNA products of fungal isolates isolated in this study and using the BLAST program showed that some fungal isolates in this study refer to Pencillium citrinum (1), others belong to Alternaria alternata (2), Aspergillus rugulosus (3), F. chlamydosporum (4). Trichoderma harzianum(5) showed a difference in the sequence of some nitrogenous bases for DNA products. Aspergillus flavus (6),Penicillium Spinulosum (7). The results showed that two isolates of fungi isolates isolated in this study (Aspergillus rugulosus and Aspergillus flavus) were not registered formerly at the National Center for Biotechnology Information (NCBI), so they were registered under the numbers MT356126 and MT358845.

Keywords. Activated carbon, Nano Iron oxide, Dyes, Adsorption and UV-visible spectroscopy.

1. Introduction
Rice (Oryza sativa L.) is one of the major and important cereal crops in the world, ranking second after wheat in terms of cultivated area and production, which is fed by about half of the world's population and is the main resource for millions of people in the continent of Asia (1). Rice is grown in more than 113 countries and gives 27% of the energy needed for humans and 27% of protein in developed countries (2). Its nutritional importance comes from its high content of carbohydrates that are easily digestible. That a person needs in his food for energy, in addition to that the rice protein has a balanced content of essential amino acids, especially lysine, compared to other grains (3). Rice in Iraq comes second after wheat, and it is one of the strategic crops in our food security. The cultivated area in Iraq in the year 2000 was about119,500 hectares and total production 251650 tons (4). Iraq is one of the countries known to cultivate this crop since ancient times, especially the appropriate environmental conditions for its cultivation, due to the lack of attention to soil service operations and the crop, especially combating Agricultural pests, especially the weeds (5).

Soil is the main reservoir of all microorganisms, including fungi (6 and 7). Fungi are species that are scattered throughout all ecosystems and in numbers that may reach five times the number of plant species. About 95% of fungi species are still unknown and awaiting detection (8 and 9). Dependence on
morphological characteristics in classifying fungi may give accurate results sometimes, but many researchers do not count on these characteristics as they require sufficient experience in the field of classification, especially in fungi groups closely resembling to a large extent, in addition to their need for time. And a great effort, as well as being often inaccurate due to its influence by environmental factors that affect the size, shapes, and colors of spores and innate colonies (9, 10 and 11). PCR technology is one of the molecular techniques that depend on the selection and amplification of a specific region of the genome of the organism, and based on the differences in the DNA sequence of that region, and thus knowledge of the genetic relationships in terms of similarity. And the difference between the types of fungi that will support the phenotypic diagnosis of the studied fungus (12 and 13). This study aimed to isolate and diagnose seven isolates of fungi by using polymerase chain reaction (PCR) and determining the sequence of nitrogenous bases and identifying similarities and genetic differences between those isolates and with other internationally diagnosed isolates installed in the National Center for Biotechnology Information (NCBI).

2. Material and Methods

Samples were collected from the Rice Research Station in Al-Mishkhab in Najaf, where samples of soil (5-10 cm deep) were collected from the root area of the rice. Potato dextrose Agar Media Used for Isolation of Fungi. P.D.A was prepared according to Himedia company that used for fungal isolation, dissolved 39 gm from P.D.A in 1 litter of water in conical flask the medium was then sterilized by autoclave temperature 121°C and pressure 15 psi/ for 15 minutes, then cooled the medium slightly and add the antibiotic (Chloramphenicol) 25 mg to inhibition bacteria growth in cultured specimens. Soils dilution series method used to Isolation and Diagnosis of Fungi: (14) was used for the purpose of isolating fungi for all sites soils samples, 1g of soil and put in tube and then add 10 ml distilled water to the tube shaking the solution inside the tube, then take 1 ml of the suspension from each tube with a sterile pipette and added to series a sterile of test tubes containing 9ml of distilled water until the fourth dilution 10-4, 1 ml of fourth dilution( 10-4 )were taken separately and placed in plastic Petri dishes with three replicates for each dilution add 15 ml of the PDA medium, the prepared plates were then stirred to ensure that the diluted soil particles spread within the medium, left the Petri dishes until the PDA hardened and then incubated at 27 °C temperature for 5 days to grow the microbial colonies properly and then purification and diagnosis morphologically and microscopic features.

2.1. Molecular diagnosis of fungi isolates

Deoxyribonucleic acid (DNA) recovery DNA was extracted for fungal isolates, according to the method described by the American company Zymo Research, using the kit (Cat. No. D6005) prepared by the aforementioned company.

2.2. Polymerase chain reaction (PCR)

Polymerase reaction test was conducted using the (Maxime PCR PreMix (i-Taq), Cat. No. 25026) kit supplied by the Korean-born company iNtRoN. Perform a chain polymerase reaction with a total volume of 20 Microliter and containing 1 Microliter of each front initiator (TCCGTA GGTGAACCTGCGG: ITS1) and posterior (TCCTCCGCTTA TTGATATGC: TS4) and (1) microliter of the extracted DNA. All of the above components are placed in the tube supplied by the manufacturer and complete the volume with (Nuclease-free water) to (20) microliter.

The DNA of fungi isolates was doubled using the following PCR steps and conditions: Initial denaturation of DNA for 5 minutes at a temperature of 98 °C followed by 35 cycles consisting of a final denaturation process. For 40 seconds at a temperature of 94°C, primer annealing for 40 seconds at a temperature of 55°C and then an initial elongation of the PCR-amplified product for 1 minute at a temperature of 72°C. Finally, the final polymerase reaction (PCR) is terminated by a final elongation step at 72 °C (17 °C). 10 Microliter of double-stranded DNA was added by polymerase chain reaction (PCR) to each Well hole from the previously prepared acarose gel layer. Also, 5 Microliter of the DNA ladder marker (1Kbp DNA ladder marker) was added to the hole on the left side of the added samples.
for the purpose of determining the size of the double DNA. The power supply electrodes connected to the electrical current and ran at 150 mAh for one hour. After completing the sample migration process, the agarose gel layer containing the DNA bundles was examined under UV transillumination, I took pictures of it.

2.3. DNA sequence analysis for fungi
Multiplex nucleic acid products (PCR amplicons) were sent from fungi isolates by polymerase chain reaction (PCR) with ITS1 and ITS4 to Korean Macrogen for the purpose of defining the nucleotide sequence and for the front and back directions of the double nucleic acid products. All nitrogen base sequences using the BLAST (Basic Local Alignment Search Tool) to compare them with the data provided in the National Center for Biotechnology Information, NCBI) of the same fungi and globally diagnosed.

3. Results and Discussion
Seven isolates of fungi were isolated from the rice field at Al-Mishkhab rice research station. These isolates were diagnosed based on the general characteristics mentioned (6) from which the Haifa fungus branch in a right angle. As shown in the figure (1).

![Fungi isolated in this study](image)

Figure 1. Fungi isolated in this study
Figure 2. A phylogenetic tree showing the genetic relationship among the isolates of Pencillium citrinum isolated in this study and other isolates of the same fungus and Registered formerly at the (NCBI).

Table 1. Comparison among the similarity percentage of P. citrinum isolates isolated in this study from rice crop with the other isolates of the same fungus Registered formerly at NCBI.

| Fungi          | strain name or Isolate | Country | GenBank Accession Number | Sequence similarity (%) |
|----------------|------------------------|---------|--------------------------|-------------------------|
| P. citrinum *  | -                      | Iraq    | -                        | 100                     |
| P. citrinum    | SCSIO z049             | China   | KX258808.1                | 98                      |
| P. citrinum    | EIODSF016              | India   | KJ173539.1                | 99                      |
| P. citrinum    | ND23                   | Zimbabwe| MG659617.1                | 99                      |
| P. citrinum    | HNMF093                | China   | MH725581.1                | 99                      |
| P. citrinum    | MUSEF105               | China   | KT310999.1                | 98                      |
| P. citrinum    | F7-1C                  | China   | KE999813.1                | 98                      |
| P. citrinum    | DQ-25                  | China   | KY022750.1                | 98                      |
| P. citrinum    | SCGAF0092              | China   | JN851020.1                | 98                      |
| P. citrinum    | NZD-mf197              | China   | KM277958.1                | 98                      |
| P. citrinum    | SCAU080                | China   | MF040209.1                | 99                      |
| P. citrinum    | -                      | Saudi Arabia | KU743898.1                | 99                      |
| P. citrinum    | SCSGAF0167             | China   | JN851046.1                | 99                      |
| P. citrinum    | NZD-mf108              | China   | KM278038.1                | 99                      |
| P. citrinum    | NZD-mf121              | China   | KM278026.1                | 98                      |
| P. citrinum    | NZD-mf124              | China   | KM278023.1                | 99                      |
| P. citrinum    | SCAU156                | China   | MF135519.1                | 98                      |

Isolation of P. citrinum * fungus isolated in this study.

The isolate of P. citrinum showed a genetically similar symmetry of 99% with each of the previously diagnosed P. citrinum isolates in India (KJ173539.1) and Saudi Arabia (KU743898.1) and China (KM278023.1, KM278038.1, JN851046.1, MF040209.1, MH725581.1) and Zimbabwe (MG659617.1). The similarity between the isolate of fungi isolated in this study and the rest of the other isolates was 98% (Table 1).
Figure 3. A phylogenetic tree showing the genetic relationship among the isolate of Alternaria alternata isolated in this study and other isolates of the same fungus Registered formerly in (NCBI).

Table 2. Comparison among the similarity percentage of A. alternata isolates isolated in this study from rice crop with the other isolates of the same fungus Registered formerly at NCBI.

| Fungi    | strain name or Isolate | Country | GenBank, Accession Number | Sequence similarity (%) |
|----------|------------------------|---------|---------------------------|-------------------------|
| A. alternata* | -                      | Iraq    | -                         | 100                     |
| A. alternata | F11                    | China   | HQ380765.1                | 100                     |
| A. alternata | D33-2                  | China   | KY365562.1                | 100                     |
| A. alternata | 2S.331                 | Turkey  | KY439023.1                | 99                      |
| A. alternata | GRSH10                 | Iran    | KY788027.1                | 100                     |
| A. alternata | TGF29-MRL              | Pakistan| KM977758.1                | 100                     |
| A. alternata | CG1                    | Iran    | KR632488.1                | 99                      |
| A. alternata | TGF50-MRL              | Pakistan| KM977774.1                | 100                     |

Isolation of A. alternata* fungi isolated in this study.

The results showed that by comparing the sequence of nitrogenous bases of double DNA from fungi isolation A. alternata With data available at the (NCBI) the similarity percentages are 100% in China isolates (HQ380765.1, KY365562.1) also in Pakistan (KM977758.1, KM977774.1), Iran (KY788027.1) in addition to the rest of the mentioned isolates in Turkey (KY439023.1) and Iran (KR632488.1) showed great genetic similarity, reaching 99%.
Figure 4. Difference in the sequence of some nitrogenous bases of the DNA multiplier by polymerase chain reaction (PCR) from the isolate of A. rugulosus isolated in this study and the isolate of the closest V010G4 fungus similar to it and recorded in the (NCBI) under the entry number MK450692.1

Figure 5. A phylogenetic tree showing the genetic relationship among the isolate of A. rugulosus isolate in this study and other isolates of the same fungus Registered formerly at (NCBI) is shown.

Table 3. Comparison among the similarity percentage of A. rugulosus isolates isolated in this study from Rice Field with the other isolates of the same fungus Registered formerly at NCBI.

| Fungi strain name or Isolate | Country | Genbank accession number | Sequence similarity (%) |
|-----------------------------|---------|--------------------------|-------------------------|
| A. rugulosus *              | -       | -                        | 100                     |
| A. rugulosus ZSF2           | India   | KT844551.1                | 99                      |
| A. rugulosus DTO 325-A7     | USA     | KU866657.1                | 98                      |
| A. rugulosus CBS 200.75     | India   | KU866664.1                | 99                      |
| A. rugulosus FOEV2          | India   | MN294702.1                | 99                      |
| A. rugulosus NRRL 206       | USA     | NR-131290.1               | 100                     |
| A. rugulosus R53-DOWN       | Jordan  | MN258576.1                | 96                      |
| A. rugulosus APBSWTPF114    | India   | MG569669.1                | 99                      |

Isolation of A. rugulosus * fungi isolated in this study.

The isolation of the fungus A. rugulosus showed a 100% pure genetic match with the fungus isolation in USA (NR-131290.1), while in the other isolation from USA it was 98% and in the fungi isolates in India it reached 99% (KT844551.1, KU866657.1, MN294702.1), while in Jordan (MN258576.1) the lowest similarity with respect to data in the table was 96%. Table (3)
Figure 6. A phylogenetic tree showing the genetic relationship among the isolate of F. clamydosporum isolated in this study and other isolates belonging to the same fungus and Registered formerly at (NCBI).

Table 4. Comparison among the similarity percentage of F. clamydosporum isolates isolated in this study from Rice Field with the other isolates of the same fungus Registered formerly at NCBI.

| Fungi                | strain name or Isolate | Country | Genbank accession number | Sequence similarity (%) |
|----------------------|------------------------|---------|--------------------------|-------------------------|
| F. chlamydosporum*   | DFRP4                  | India   | MN871799.1               | 100                     |
| F. chlamydosporum    | Ng30                   | Egypt   | MH141316.1               | 100                     |
| F. chlamydosporum    | DFRP8                  | India   | MN871803.1               | 100                     |
| F. chlamydosporum    | NZD-mf112              | China   | KM278035.1               | 100                     |
| F. chlamydosporum    | MAOS2                  | Egypt   | MN480498.1               | 99                      |
| F. chlamydosporum    | LrSF22                 | China   | MG543702.1               | 100                     |
| F. chlamydosporum    | KUSF2102               | India   | MF136407.1               | 100                     |
| F. chlamydosporum    | QJP1                   | China   | KY290716.1               | 99                      |
| F. chlamydosporum    | DS09                   | China   | KY432359.1               | 100                     |
| F. chlamydosporum    | FC8                    | Iraq    | MT196807.1               | 100                     |
| F. chlamydosporum    | 13                     | Iraq    | MT102263.1               | 99                      |
| F. chlamydosporum    | CHJ3                   | China   | KF999821.1               | 99                      |
| F. chlamydosporum    | ZB11060983             | China   | KX783375.1               | 100                     |
| F. chlamydosporum    | DOBPGPF V37           | India   | MH173818.1               | 99                      |
| F. chlamydosporum    | ZB11263544             | China   | KX783372.1               | 99                      |
| F. chlamydosporum    | FC1                    | Iraq    | MT196795.1               | 100                     |
| F. chlamydosporum    | C                      | Switzerland | KT634074.1      | 99                      |
It was also noticed by comparing the sequence of nitrogenous bases of the DNA double from the isolation of the fungus *F. chlamydosporum* with the data available at (NCBI) that the percentages for similarity are 100% in the isolates of Iraq (MT102263.1, MT196795.1, MT196807.1), Also in China (KX783373.1), Iraq (MT196807.1), and Egypt (MH141316.1) were 100% as well as for the rest of the isolates mentioned in Table (4) showed a great genetic similarity, reaching 99%.

**Table 5.** Comparison among the similarity percentage of *A. flavus* isolates isolated in this study from Rice Field with the other isolates of the same fungus registered formerly at NCBI.

| Fungi       | strain name or Isolate | Country | Genbank accession number | Sequence similarity (%) |
|-------------|------------------------|---------|--------------------------|-------------------------|
| A. flavus   | L-1298/2013            | Iraq    | MN533889.1                | 99                      |
| A. flavus   | A50R                   | China   | MN095121.1                | 99                      |
| A. flavus   | A45R                   | India   | MN095118.1                | 99                      |
| A. flavus   | A42R                   | India   | MN095117.1                | 99                      |
| A. flavus   | CMXY29346              | China   | MG991664.1                | 99                      |
| A. flavus   | CMXY27481              | China   | MG991658.1                | 99                      |
| A. flavus   | CMXY25420              | China   | MG991651.1                | 99                      |
| A. flavus   | CMXY24985              | China   | MG991649.1                | 99                      |
| A. flavus   | CMXY22879              | China   | MG991643.1                | 99                      |
| A. flavus   | MC-5-L                 | India   | KU527785.1                | 100                     |
| A. flavus   | WZ-309                 | China   | MN856426.1                | 99                      |
| A. flavus   | WZ-286                 | China   | MN856403.1                | 99                      |
| A. flavus   | L-3607/2012            | India   | MN533855.1                | 99                      |
| A. flavus   | A26R                   | China   | MN095167.1                | 99                      |
Isolation of Aspergillus flavus * isolated in this study.

Isolation of A. flavus showed a 100% genetic similarity with each of the previously diagnosed A. flavus isolates in Iraq (MH237632.1) and India (MG759550.1, KU527785.1). The genetic similarity between isolates of fungi isolated in this study and other isolates, 99% Table (5).

Table 6. Comparison among the similarity percentage of P. spinulosum isolates isolated in this study from rice crop with the other isolates of the same fungus Registered formerly at NCBI.

| Fungi          | strain name or Isolate | Country      | Genbank accession number | Sequence similarity (%) |
|----------------|------------------------|--------------|--------------------------|-------------------------|
| P. spinulosum * | 0.0223                 | USA          | KT067756.1               | 99                      |
| P. spinulosum   | A4S3_12                | Malaysia     | JX501409.1               | 99                      |
| P. spinulosum   | L-1298/2013            | India        | MN533889.1               | 99                      |
| P. spinulosum   | A4                     | Iraq         | MH237625.1               | 99                      |
| P. spinulosum   | A3                     | Iraq         | MH237624.1               | 99                      |
| P. spinulosum   | A17                    | Iraq         | MH237632.1               | 100                     |
| P. spinulosum   | AF2                    | Pakistan     | MN893385.1               | 99                      |
| P. spinulosum   | BT27                   | Turkey       | MH137934.1               | 99                      |
| P. spinulosum   | A17                    | Iraq         | MH237632.1               | 99                      |
| P. spinulosum   | A3                     | Iraq         | MH237624.1               | 99                      |
| P. spinulosum   | A4                     | Iraq         | MH237625.1               | 99                      |
| P. spinulosum   | L-1298/2013            | India        | MN533889.1               | 99                      |
| P. spinulosum   | A4S3_12                | Malaysia     | JX501409.1               | 99                      |
| P. spinulosum   | L-1298/2013            | India        | MN533889.1               | 99                      |
| P. spinulosum   | A4                     | Iraq         | MH237625.1               | 99                      |
| P. spinulosum   | A3                     | Iraq         | MH237624.1               | 99                      |
| P. spinulosum   | A4                     | Iraq         | MH237625.1               | 99                      |
| P. spinulosum   | L-1298/2013            | India        | MN533889.1               | 99                      |
| P. spinulosum   | A4S3_12                | Malaysia     | JX501409.1               | 99                      |

Figure 8. A phylogenetic tree showing the genetic relationship among the isolate of P. spinulosum isolated in this study and other isolates belonging to the same fungus and Registered formerly at (NCBI).
The results shown in Table (6) show that the highest 100% genetic similarity ratio to isolate *P. spinulosum* was with isolates *P. spinulosum* isolated from India (KF031019.1, KF514384.1, KM063185.1 respectively) and South Africa (MK841437.1) 99% were in China (JQ717356.1, MH484030.1) and Zimbabwe (MG659685.1).

**Figure 9.** A phylogenetic tree showing the genetic relationship among the isolate of *T. harzianum* isolated in this study and other isolates belonging to the same fungus were registered formerly at (NCBI).

**Table 7.** Comparison among the similarity percentage of *T. harzianum* isolates isolated in this study from rice crop with the other isolates of the same fungus Registered formerly at NCBI.

| Fungi       | strain name or Isolate | Country | Genbank accession number | Sequence similarity (%) |
|-------------|------------------------|---------|--------------------------|-------------------------|
| T. harzianum* | -                      | Iraq    | -                        | 100                     |
| T. harzianum   | CEN202               | Brazil  | KC576652.1                | 97                      |
| T. harzianum   | CEN192               | Brazil  | KC576646.1                | 96                      |
| T. harzianum   | CEN170               | Brazil  | KC576645.1                | 98                      |
| T. harzianum   | CEN857               | Brazil  | KC576741.1                | 98                      |
| T. harzianum   | CEN854               | Brazil  | KC561083.1                | 98                      |
| T. harzianum   | CEN855               | Brazil  | KC576745.1                | 98                      |
| T. harzianum   | CEN268               | Brazil  | KC576690.1                | 98                      |
| T. harzianum   | NECC21233            | China   | MN585667.1                | 98                      |
| T. harzianum   | CEN438               | Brazil  | KC576702.1                | 97                      |
Isolation of Trichoderma harzianum fungi isolated in this study.

By comparing the sequence of nitrogenous bases of the product of T. harzianum with the data available at the National Center for Biotechnology Information (NCBI), the highest genetic similarity was found with isolation of T. harzianum isolated from India (KU215923.1). The similarity between the isolate of the fungi isolated in this study and the rest of the other isolates was 96-98% Table (7).

4. Conclusion
From this study it is concluded that there is a great diversity in the species of fungi in the rice field in the rice research station in Al-Mishkhab. Also, it follows from the results of this study that all isolates of the seven fungi were genetically different among them. And the results proved that two isolates among these isolates are not registered previously in the above mentioned center, so it was registered under the entry numbers MT356126.1 and MT358845. In this study, PCR technology was used in the diagnosis of isolates from the fungus Pencillium citrinum and Fusarium spp. And Aspergillus spp. And others to get rid of diagnostic problems based on morphological characters. Despite the benefit of phenotypic diagnosis in confining the fungi under investigation in smaller groups before proceeding to using other methods of diagnosis, there are many problems that accompany the phenotypic diagnosis of fungi spp., as well as requiring significant time and effort (7, 8, 15). Also, there are some other factors that affect these phenotypic traits, including the type and nature of growth medium, humidity, and lighting that from the 8th can affect the color, shapes, and sizes of spores and fungal growing colonies. Some researchers found that there was an error in the phenotypic classification of many fungi diagnosed in previous studies, including species belonging to the fungus (fusarium spp).

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