Genetic variation of some isolates of *Cladosporium sphaerospermum* isolated from different environments

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Abstract. The study was carried out, in the laboratory of Plant Virology, Plant Protection Department, College of Agriculture, University of Kerbala, to isolate and identify six isolates of the fungus *Cladosporium sphaerospermum* isolated from different environments (seeds, air, plant residues, and soil) in Al-Najaf province, Iraq. The fungal isolates were identified using the polymerase chain reaction (PCR) and determining the nucleotide sequence products of DNA using ITS1 and ITS4 primer pair. Results of analysis of the nucleotide sequences using BLAST (Basic Local Alignment Search Tool) showed that all the identified isolates of the fungus belong to *C. sphaerospermum*. A comparison of the nucleotide sequences with those available in the National Centre for Biotechnology Information (NCBI) revealed that all the identified *C. sphaerospermum* sequences were previously registered in NCBI.

1. Introduction

Fungi are existing in all ecosystems and have different effects among them, as well as with the other organisms present in the environment. Among these fungi, *Cladosporium sphaerospermum* that is one of the most common fungi available in all external and internal environments. It was reported that *C. sphaerospermum* is highly spread by spores in the air and other environments at a rate of 2000-50000 spore/ m³ air [1; 2]. The fungus has the ability to grow heavily on building materials and the edges of wet windows as well as food including salt and chilled sugar [3]. *C. sphaerospermum* also causes skin and lung infections such as asthma, allergies, and respiratory inflammations [4].

It has been noted from previous studies that the reliance on morphological characteristics in the classification of fungi may sometimes give accurate results, but many researchers are not reliable as such characteristics need sufficient expertise from the taxonomist, especially in closely related fungal groups such as *Fusarium* spp. in addition to the need for considerable time and effort, in addition to being inaccurate because of the impact of environmental factors that affect the size, shapes and colors of spores and fungal colonies [5].

Polymerase chain reaction (PCR) is one of the molecular techniques based on the selection and amplification of a specific region of the genome of an organism based on the differences in DNA sequence of that region. This technique was used to diagnose several microorganisms including...
fungi. Due to the importance of accurate identification of the fungi, this study aimed to diagnose some isolates of *C. sphaerospermum* molecularly using polymerase chain reaction (PCR) and to identify the nucleotide sequences to determine the genetic similarities and differences among these isolates and the other *C. sphaerospermum* isolates previously registered in the National Center for Biotechnology Information (NCBI).

2. Materials and Methods

2.1. Fungal Isolation of *C. sphaerospermum*

Soil, air, seed, plant residues and water samples were collected from regions of Al-Najaf province (Al-Haidariyah, Al-Abbasiyah, Al-Mishkhab and Bahr Al-Najaf). Twenty samples were planted in Petri dishes containing Potato Dextrose Agar (PDA), supplemented with Chloramphenicol antibiotic at 200 mg/L and incubated at 2±25 °C for 4 days. The isolates of *C. sphaerospermum* were purified on the same medium (PDA) following the method of Hyphal Tip, *C. sphaerospermum* isolates were identified according to morphological features and by Polymerase (PCR)[6]. The same fungal isolates were also identification by using polymerase chain reaction (PCR) and nucleotide sequencing, according to the method described later.

2.2. DNA extraction, PCR amplification and DNA sequencing

Hyphae and spores of all pure fungus cultures were scraped from the surface of culture media using heat sterilized needle and 50-100 mg of fresh fungal culture were transferred to an Eppendorf tube. These fungal cultures were stored at -80°C. Total DNA extraction was followed by using genomic DNA extraction kit (Zymo Research company (U.S.A), Cat. No. D6005) as per manufacturer’s instructions. The quality and quantity of DNA extracted from each isolate was measured by a UV spectrophotometer (Thermo Scientific, Germany) and subsequently DNA was stored at -20°C until use.

ITS region of fungal isolates was amplified by using universal primers set of ITS1 (TCCGTTGTGAACCAAGCGG) and ITS4 (TCCTCCGC TTATGATATGC) [7]. Master mixture was prepared by adding 1 μl each primer (10 pmol), 2 μl 10X PCR buffer, 2 μl dNTPs (2 mM), 3 μl template DNA (30 ng/μl), 1 unit *Taq* polymerase enzyme and adjust the volume with nucleases free water. PCR amplification was done in the thermal cycler by providing a specific program of denaturation at 94 °C for 1 minute followed 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 minute and post extension at 72°C for 5 minutes. Amplified PCR products were analyzed on 1% (w/v) agarose gel containing 4μl of ethidium bromide (100μg/ml) in 0.5X Tris-borate EDTA (TBE) buffer. Amplified samples were mixed with 6X loading dye (bromophenol blue) and electrophoresis at 120V until the bromophenol migrated approximately two third the length of the gel. After electrophoresis, DNA bands were observed on gel documentation apparatus and photographs were taken by using VilberLourmat, Taiwan gel documentation system.

PCR-amplified products were gel-purified using the *FavorPrep PCR purification kit* (Cat. No. FAGCK 001, Favorgen, Taiwan) and sent along with the primer pairs (ITS1 and ITS4) to the Macrogen DNA sequencing service in Korea. PCR products were directly sequenced in both directions. Nucleotide sequences were aligned and compared with the sequences of the other fungal isolates available at the NCBI database using the Basic Local Alignment Search Tool (BLAST) [5]. Phylogenetic analysis of all fungal nucleotide sequences were compared by using MEGA 6.

3. Results and Discussion

3.1. Isolation of *C. sphaerospermum* isolates

Six isolates of *C. sphaerospermum* were isolated from samples collected from soil, air, seed, plant residues, and water (Table 1). These isolates were initially identified based on the characteristics mentioned by Bench [6]. The hyphae of *C. sphaerospermum* are olivaceous-brown in colour, septate, and thick walled. The colonies of the *C. sphaerospermum* fungus are flattened and velvety in
texture. The fungal conidiophores are septate, branched, and dark. The appearance of the conidiophores are tree-like and unlike other related species, the conidiophores of *C. sphaerospermum* without swollen nodes at the branching points. Conidia of this fungus are characteristically globose to ellipsoid and formed in branching chains in which the youngest conidia are situated at the top.

**Table 1.** *C. sphaerospermum* isolates obtained in this study.

| Number | Isolates of fungus       | Region    | Isolated from |
|--------|--------------------------|-----------|---------------|
| 1      | *C. sphaerospermum*      | Al-Najaf  | Seed          |
| 2      | *C. sphaerospermum*      | Al-Najaf  | Air           |
| 3      | *C. sphaerospermum*      | Al-Najaf  | Plant residues|
| 4      | *C. sphaerospermum*      | Al-Najaf  | Air           |
| 5      | *C. sphaerospermum*      | Al-Najaf  | Plant residues|
| 6      | *C. sphaerospermum*      | Al-Najaf  | Soil          |

3.2. **Molecular identification of fungus isolates**

PCR amplification of DNA extracted from these isolates showed the possibility of amplifying 500 bp PCR products using the universal ITS1-ITS4 primers (Fig. 1). The PCR product (ITS1, 5.8S rDNA and ITS4) amplified from each fungal isolate was sequenced with both directions and the generated nucleotide sequences were subjected to a BLAST search. Confirming the morphological identification, all obtained sequences belonged to *C. sphaerospermum*.

![Figure 1](image)

**Figure 1.** PCR products amplified from the isolates of *C. sphaerospermum* isolated from seeds (1), air (2 and 4), residues (3, 5), and soils (6) in this study. M: 1Kbp DNA ladder marker. NC: negative treatment (without adding DNA to the other PCR components).

The results of nucleotide sequence alignments showed that most isolates of *C. sphaerospermum* isolated in this study had distinct genetic differences among them in some positions of the nucleotide sequences (Fig. 2).
Figure 2. A graphical view of the similarity and difference in the ITS1, 5.8S rDNA and ITS4 sequences generated from the *C. sphaerospermum* isolates obtained from seeds (1), air (2 and 4), residues (3, 5) and soils (6) in this study. Identical nitrogenous bases are represented in dots. Numbers on the right side of the figure represent nucleotide sequences obtained from PCR products amplified from the different *C. sphaerospermum* isolates.

From the comparison of nucleotide sequences, it was found that the *C. sphaerospermum* isolates 4, 5, and 6 had the highest similarity ratio that was (100%) in the nucleotide sequences generated from the PCR products. Whereas, the other *C. sphaerospermum* isolates (1, 2, and 3) showed clear genetic variations among them in some positions of nucleotide sequences with a similarity percentage ranged between 98%-99% in nucleotide sequences (Table 2).

Table 2. Similarity rates among the nucleotide sequences amplified by PCR from the isolates of *C. sphaerospermum* isolated from seeds (1), air (2 and 4), residues (3, 5) and soil (6) in this study.

|                      | -   | 1   | 2   | 3   | 4   | 5   | 6   |
|----------------------|-----|-----|-----|-----|-----|-----|-----|
| 1                     | -   | 98  |     | 98  |     |     |     |
| 2                     | -   | 99  | 98  |     |     |     |     |
| 3                     | -   | 99  | 99  | 99  |     |     |     |
| 4                     | -   | 100 | 99  | 99  | 99  |     |     |
| 5                     | -   | 100 | 99  | 99  | 99  | 99  |     |
| 6                     | 5   | 4   | 3   | 2   | 1   |     |     |

As shown in the constructed phylogenetic tree (Figure 3), all isolates of *C. sphaerospermum* were appeared in three main clades: the first clade have the isolates 4, 5 and 6; however, the second clade included the isolates 2 and 3. whereas the isolate of *C. sphaerospermum* (1) was appeared in a clade separated from the other isolates of *C. sphaerospermum* because of the highest genetic variability comparing with the other *C. sphaerospermum* isolates (2, 3, 4, 5, and 6).
**Figure 3.** Phylogenetic tree generated using the neighbor-joining method based on the comparison of the ITS1, 5.8S rDNA and ITS4 sequences generated from *C. sphaerospermum* isolated in this study from seeds (1), air (2 and 4), residues (3, 5), and soils (6).

A BLAST search using the nucleotide sequences obtained from the *C. sphaerospermum* isolates 1, 2 and 3 isolated from seeds, air, and plant residues, respectively, showed a similarity percentage of 100% with many *C. sphaerospermum* isolates previously identified and registered in NCBI (Tables 3, 4, and 5).

**Table 3.** Comparison of similarity ratios of the nucleotide sequence of *C. sphaerospermum* (1) isolated in this study from seeds with the other *C. sphaerospermum* isolates previously registered NCBI.

| Isolate or strain name | Origin   | The most similar sequences in GenBank database | Sequence similarity (%) |
|-----------------------|----------|-----------------------------------------------|-------------------------|
| *-*                   | Iraq     |                                               | 100                     |
| F130 18S              | South Korea | KY952177.1                                        | 100                     |
| DTO:307-I3            | Netherlands | MF473272.1                                        | 100                     |
| DTO:306-D8            | Netherlands | MF473268.1                                        | 100                     |
| DTO:090-H9            | Netherlands | MF473262.1                                        | 100                     |
| CPC:22379             | Netherlands | MF473261.1                                        | 100                     |
| NIHHS321 18S          | Korea     | KY554990.1                                        | 100                     |
| NIHHS309 18S          | Korea     | KY554981.1                                        | 100                     |
| NIHHS509              | Korea     | KY929280.1                                        | 100                     |
| SA24                  | China     | KY944992.1                                        | 100                     |
| Isolate or strain name | Origin       | The most similar sequences in GenBank database |                      |                  |
|-----------------------|-------------|-----------------------------------------------|---------------------|-----------------|
|                       |             | GenBank Accession Number                      | Sequence similarity (%) |
| *-*                   | Iraq        | -                                             | 100                 |
| S-1-5                 | China       | MG787259.1                                    | 100                 |
| GF-MEA-1              | Slovakia    | MH101394.1                                    | 100                 |

*C. sphaerospermum (2) isolated in this study.

**Table 4.** Comparison of similarity ratios of the nucleotide sequence of *C. sphaerospermum* (2) isolated in this study from air with the other *C. sphaerospermum* isolates previously registered NCBI.
Table 5. Comparison of similarity ratios of the nucleotide sequence of *C. sphaerospermum* (3) isolated in this study from plant residues with the other *C. sphaerospermum* isolates previously registered NCBI.

| Isolate or strain name | Origin     | Gen Bank Accession Number | Sequence similarity (%) |
|------------------------|------------|---------------------------|-------------------------|
| *-                      | Iraq       | -                         | 100                     |
| CT3A                   | United Kingdom | P794134.1               | 100                     |
| IFM 64740              | Chiba, Japan      | LC317551.1               | 100                     |
| IFM 63693              | Chiba, Japan      | LC317550.1               | 100                     |
| IFM 63510              | Chiba        | LC317549.1               | 100                     |
| SCAU103                | Chiba       | MF061765.1               | 100                     |
| GRSH50                 | Iran        | KY788067.1               | 100                     |
| GRSH47                 | Iran        | KY788064.1               | 100                     |
| S-1-5                  | China       | MG787259.1               | 99                      |
| DN03                   | China, Shanghai | KY781384.1              | 99                      |

*C. sphaerospermum* (3) isolated in this study

From comparison, results also indicated that the highest genetic similarity (100%) for ITS-generated sequence of *C. sphaerospermum* isolated in the study from air, plant residues, and soil that was observed with some isolates of *C. sphaerospermum* identified in Iraq (MG669153.1 and KY046239.1), Saudi Arabia (LN482434.1 and G798764.1), and Malaysia (JX966567.1 and JX966564.1). The lowest genetic similarity was found the *C. sphaerospermum* isolate identified in India (Accession No.: F467882.1) that had a genetic similarity of 99% and published in NCBI (Table 6).

Table 6. Comparison of similarity ratios of the nucleotide sequence of *C. sphaerospermum* (4) isolated in this study from air with the other *C. sphaerospermum* isolates previously registered NCBI.

| Isolate or strain name | Origin      | Gen Bank Accession Number | Sequence similarity (%) |
|------------------------|-------------|---------------------------|-------------------------|
| *-                      | Iraq        | -                         | 100                     |
| CCTU1122 18S           | Iran        | KY046240.1                | 100                     |
| CCTU1120 18S           | Iran        | KY046239.1                | 100                     |
| TUHT38                 | Saudi Arabia | LN482434.1              | 100                     |
| UM 333 18S             | Malaysia    | JX966574.1                | 100                     |
* C. sphaerospermum (4) isolated in this study.

**Table 7.** Comparison between the similarity rate of the nitrogen bases sequence of *C. sphaerospermum* (5) isolated in this study from plant residues with other isolates belonging to the same fungus which is registered globally in NCBI site.

| Isolate or strain name | Origin  | The most similar sequences in Gen Bank database |
|-----------------------|---------|-----------------------------------------------|
|                       |         | Gen Bank Accession Number | Sequence similarity (%) |
| *-                     | Iraq    | -                             | 100                      |
| CCTU1120 18S           | Iran    | KY046239.1                    | 100                      |
| CCTU1122 18S           | Iran    | Y046240.1                     | 100                      |
| CCTU1123 18S           | Iran    | Y046238.1                     | 100                      |

* C. sphaerospermum (5) isolated in this study.

**Table 8.** Comparison of similarity ratios of the nucleotide sequence of *C. sphaerospermum* (6) isolated in this study from soil with the other *C. sphaerospermum* isolates previously registered NCBI.

| Isolate or strain name | Origin | The most similar sequences in Gen Bank database |
|-----------------------|--------|-----------------------------------------------|
|                       |        | Gen Bank Accession Number | Sequence similarity (%) |
| *-                     | Iraq   | -                             | 100                      |
| 6018 18S               | Brazil | KX363452.1                    | 100                      |
| CCTU1124 18S           | Iran   | KY046243.1                    | 100                      |
| CCTU1125 18S           | Iran   | KY046242.1                    | 100                      |
| CCTU1121 18S           | Iran   | KY046241.1                    | 100                      |
| CCTU1122 18S           | Iran   | KY046240.1                    | 100                      |
| CCTU1120 18S           | Iran   | KY046239.1                    | 100                      |
| CCTU1123 18S           | Iran   | KY046238.1                    | 100                      |
| UM 333 18S             | Malaysia | JX966574.1                    | 100                      |
| UM 165 18S             | Malaysia | JX966566.1                    | 100                      |
* C. sphaerospermum (6) isolated in this study.

PCR technique was used in this study to identify drenisolaees of C. sphaerospermum. It was proven by comparing the nucleotide sequences of PCR products amplified from these isolates that all of these fungal isolates are previously registered in the NCBI. In previous studies, PCR has widely used as a rapid and accurate technique in the diagnosis of many microorganisms, including plant fungi such as Fusarium spp., R. solani, cladosporium cladosporiodes and R. solani to eliminate the restrictions of identification based on different morphological characters [8]. Differences in the Internal Transcribed Spacer (ITS) regions of the ribosomal DNA (rDNA), repeat units are well-investigated sequences that are existing in multiple copies and can be isolated and amplified by PCR. PCR amplification of ITS region has been provided high efficiency in diagnosing many fungi such as Fusarium spp., Cladosporium spp. and Fusarium verticillioides [8].

4. Conclusion
The six fungal isolates were identified using the polymerase chain reaction (PCR) and determining the nucleotide sequence products of DNA using ITS1 and ITS4 primer pair. Results of analysis of the nucleotide sequences using BLAST (Basic Local Alignment Search Tool) showed that all the identified isolates of the fungus belong to C. sphaerospermum. A comparison of the nucleotide sequences with those available in the National Centre for Biotechnology Information (NCBI) revealed that all the identified C. sphaerospermum sequences were previously registered in NCBI.

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