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Isolation and Molecular Identification of *Fusarium* Species from Some Cereal Grains and Their Products in Egypt

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Abstract

18 strains of *Fusarium* species were isolated from cereal grains and their products (wheat, rice, barely, flour, corn flour and starch) collected from different locations in Cairo and El-Gharbia governorates, Egypt, using sample dilution and direct culture methods. The isolated *Fusarium* strains were identified morphologically as: 4 isolates *F. oxysporum*, 2 isolates from each of *F. solani, F. equiseti, F. fujikuroi, F. chlamydosporum and F. brachygibbosum* and 1 isolate of each of *F. verticillioides, F. subglutinans, F. anthophylum* and *F. moniliforme*. The identification of *Fusarium* species was confirmed genetically. Molecular identification based on ITS1 and ITS4 regions indicated PCR fragments ranged from 500 to 600 bp. PCR fragments of conserved ribosomal ITS regions were sequenced, aligned and deposited in NCBI GenBank with the following accession numbers. *F. fujikuroi* (MG211161), *F. fujikuroi* (MG211162), *F. verticillioides* (XM 018895258.1), *F. sporotrichioides* (MG211163), *F. solani* (MG211159), *F. solani* (MG211160) and *F. equiseti* (KM246255.1).

Keywords: *Fusarium, Feed, Toxins, ITS1, ITS4.*

1. Introduction

Fusarium is considered one of the most important fungal genera, where it includes many species which are plant pathogens responsible for a broad range of plant diseases [1]. Other species of *Fusarium* were distinguished as contaminants of human foods [2], animal feeds [3] and producing mycotoxins such as *F. acuminatum*, *F. equiseti*, and *F. poae* which can produce trichothecene [4]. Also, some *Fusaria* cause opportunistic infections to human and farm animals. Accordingly, different *Fusarium* species are considered the most harmful fungi worldwide [5], [6].

The development of fungal-specific primers for amplification of the internal transcribed spacer [ITS] region of rRNA genes introduces the possibility of molecular characterization and identification of different fungi [7] and has been recommended as the universal fungal barcode sequence [8]. It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). Because of its higher degree of variation than other genic regions of rDNA (for small and large subunit rRNA), variations among individual rDNA repeats can be observed within both the ITS and IGS regions. In addition, standard ITS1 and ITS4 primers are used by most laboratories [9].

Due to the importance of different *Fusarium* species as food and feed contaminants, the present study was aimed to isolate different *Fusarium* species from some cereal grains and their products in different locations in Cairo and El-Gharbia governorates, Egypt, then identifying them morphologically and genetically.

2. Materials and Methods

2.1 Samples Collection

Fifty samples of different cereal grains and their products (wheat, rice, barely, flour, corn flour and starch) were collected from farms, markets and mills in several locations in Cairo and El-Gharbia, Egypt. Locations in El-Gharbia governorate include El-Mahalla-El-kubra, Mahalla Abo-Ali and Kafr Al-Zayat, while the selected regions in Cairo were Al-Hussein, El-Ghoria, El-Azhar, El-Ataba). Samples were collected in polyethylene bags then stored at 4°C. The isolation process was performed within 72 h after sample collection to avoid contamination due to storage.

2.2 Isolation and Morphological Identification of *Fusarium* species

For isolation of fungi including *Fusarium* from the collected samples, dilution plate technique was used [10]. Dilutions from different tested samples were prepared by adding 10 g of each sample (air dried and sieved) to 90 ml sterile saline, mixed well and the mixture settled for 15 min. Then serial dilutions were prepared.

Plates containing modified Czapek's dox agar

medium (10 g glucose instead of sucrose) were prepared with adding aureomycin hydrochloride as antibacterial agent. The previously prepared dilutions were spread separately on the plate surfaces using sterilized spreader. The plates were incubated at 27°C for 7 days. The experiments were performed in triplicate. After incubation period, plates containing separated colonies of fungi were selected. Every plate appeared containing multiple types of fungi including different *Fusaria*. To isolate a pure culture from each *Fusarium* isolate, sub-culturing on PDA and modified Czapek's dox agar plates was performed. Each pure culture of *Fusarium* isolates was identified morphologically using the criteria provided in *Fusarium* identification key [11] and *Fusarium* Laboratory Manual [12].

2.3 Molecular identification

Pure cultures of different tested *Fusarium* species were prepared separately on broth PDA medium for 3 days at 25°C. DNA was extracted and purified according to protocol of Thermo Scientific Gene JET Plant Genomic DNA Purification Mini Kits #K0791, #K0792. Then, the purified DNA was stored at 4°C. The DNA was quantified by nano-drop method. To amplify the Internal Transcribed Spacer region [13], two oligonucleotides primers (ITS1: 5'-TCTGTAGGTGAACCTGCGG-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3') were constructed [9].

The 25 µl PCR mastermix contained 1 µl forward primer (ITS1), 1 µl reverse primer (ITS4), 0.25 µl *Taq* polymerase, 2.5 µl $MgCl_2$, 2.5 µl green buffer, 1 µl dNTPs and 2 µl DNA. PCR was performed on Thermal Mastercycler. Multi Gene, Gradienta. The PCR program was initial denaturation at 95°C for 2 min, denaturation at 95°C for 30 sec, annealing at 55°C for 1 min, extension at 72°C for 1 min (35 cycles), and final extension at 72°C for 10 min. After PCR amplification, DNA samples were run on 1.5% agarose gel in 0.5 M Tris Borate EDTA (TBE) buffer (pH 8) at room temperature at a constant voltage of 80 V for 60 minutes through an ethidium bromide (0.5 $\mu l.ml^{-1}$) stained agarose. The gel was analyzed and photographed using gel documentation system (Alpha-chem. Imager, USA). PCR products purification was carried out according to PCR clean up system, promgea, wizard R SV gel protocol. DNA sequencing was carried out by Macrogen Inc., Korea.

2.4 ITS data analysis:

The ITS nucleotide sequences for each isolate were compared to those in the public domain databases NCBI (National Center for Biotechnology Information: www.ncbi.nih.gov) using Basic Local Alignment Search Tool for Nucleotide Sequences (BLASTN). ITS sequences of *Fusarium* isolates were aligned with the consensus region using Clustal-W program.

3. Results and Discussions

3.1 Identification of isolated fungi

Some fungal species belong to *Aspergillus*, *Fusarium*, *Rhizopus* and *Trichoderma* were recognized in the plates after inoculation by dilutions prepared from the tested cereal grains and their products. The species of *Aspergillus* and *Rhizpous* were more abundant while, few belonged to *Trichoderma* and *Fusarium*. **Table 1** represents the isolated fungi from samples collected from the locations of El-Gharbia governorate. *Rhizopus*, *Aspergillus* and *F. chlamydosporum* were detected in wheat flour and corn flour samples collected from Kafr Al-Zayat. From another location; Mahalla Abo-Ali, two samples were collected (rice and barely) from which *Aspergillus*, *Rhizopus*, *F. anthophylum* and *Trichoderma* were observed**.** Also, two samples (wheat and rice) were collected from El Mahalla El Kubra where *F. oxysporum* and unknown *Fusarium* species were isolated and identified. *Aspergillus* and *Rhizopus* were detected from the two samples whereas *Trichoderma* was isolated from rice sample only **(Table 1)**. *F. brachygibbosum* were isolated from rice samples collected from Mahalla Abo-Ali and El-Azhar. While, *F. chlamydosporum* was isolated from two samples (corn flour and barely) from Kafr Al-Zayat and El-Ghoria. In the meantime, *F. equiseti* was recognized from barely and starch samples collected from El-Ataba. *F. moniliforme* was isolated only from wheat samples in El-Azhar location.

From Cairo governorate, eight species of *Fusarium* namely; *F. solani*, *F. chlamydosporum*, *F. oxysporum*, *F. subglutinans*, *F. brachygibbosum*, *F. moniliforme*, *F. verticillioides and F equiseti* were isolated and identified from 19 samples. The samples were collected from four locations (Al-Hussein, El-Ghoria, El-Azhar and El-Ataba) 3, 4, 5 and 7 samples, respectively **(Table 1)**. Three *Fusarium* species; *F. oxysporum*, *F. solani* and *F. equiseti* were more abundant. The remaining isolated *Fusarium* species (5 identified and some unidentified species) were listed only in one sample. Five starch samples in addition to one sample of barley were free of *Fusarium* species, which were collected from three locations in Cairo. *Aspergillus and Rhizopus* were highly occurred. They were isolated from 14 and 15 samples representing 73.7% and 78.9% of the

samples, respectively. The two genera were isolated from different locations in Cairo governorate. The genus *Trichoderma* was rarely occurred where it was isolated from one starch sample only (5.3% of the samples) at Al-Hussein location.

3.2 Morphological characterization between *Fusarium* species

The *Fusarium* cultures were described morphologically after 7 to 10 days after heavy growth according to color, texture and reverse **(Table 2 and Fig.1)**. After the macroscopic description of cultures, the microscopic features of each isolate were investigated and identified according to [11] and [12] **(Fig.2)**. Eighteen strains of 10 *Fusarium* species were recognized and identified morphologically as: *F. oxysporum, F. solani, F. equiseti, F. chlamydosporum* and *F. brachygibbosum, F. verticillioides, F. subglutinans, F. anthophylum***,** *F. moniliforme* and unknown *Fusarium* species. Morphological identification failed to distinguish some isolated species which later were identified clearly by Molecular techniques. In addition, two species which identified morphologically as *F. moniliforme* and *F. chlamydosorum* were identified by molecular techniques as *F. fujikuroi* and *F. verticillioides*. Our results considering isolation and identification of fungi from cereals agree with the results of [14] who isolated and identified different *Fusarium* species occurring on fruits, vegetables and food grains.

3.3 Molecular Identification of *Fusarium* isolates

Specific PCR reactions were carried out using the forward (ITS1) and reverse (ITS4) primers to amplify the ITS 5.8S rDNA region from genomic DNA extracted from the tested fungal isolates. The electrophoretic profile indicated amplification of DNA fragments of ranged from 500 to 600 bp as shown in **Fig.3**. PCR fragments of conserved ribosomal ITS regions were sequenced, aligned and deposited in NCBI GenBank with the following accession numbers. *F. fujikuroi* (MG211161), *F. fujikuroi* (MG211162), *F. verticillioides* (XM 018895258.1), *F. sporotrichioides* (MG211163), *F. solani* (MG211159), *F. solani* (MG211160) and *F. equiseti* (KM246255.1) as represented in **Table 3**.

Table 1: Fungal species isolated from different locations in Egypt.

Host	**Location**	**Isolated Fungi**			
		Aspergillus	***Rhizopus***	***Trichoderma***	***Fusarium***
Wheat Flour	Kafr Al-Zayat	+	+	-	-
Corn flour	Kafr Al-Zayat	-	+	-	*F. chlamydosporum*
Rice	Mahalla Abo-Ali	+	+	-	*F. anthophylum*
Barely	Mahalla Abo-Ali	+	+	+	-
Wheat	Betyna	+	+	-	*F. oxysporum*
Rice	Betyna	+	+	++	*F. sp*
Starch	Al-Hussein	+	-	-	-
Starch	Al-Hussein	+	+	-	-
Starch	Al-Hussein	+	+	+	-
Rice	El-Ghoria	+	-	-	*F. solani*
Barely	El-Ghoria	-	-	-	*F. chlamydosporum*
Wheat	El-Ghoria	-	+	-	*F. oxysporum*
Barely	El-Ghoria	-	+	-	-
Corn flour	El-Azhar	+	+	-	*F. subglutinans*
Corn flour	El-Azhar	+	+	-	*F.* sp
Rice	El-Azhar	+	+	-	*F. brachygibbosum*
Wheat	El-Azhar	-	+	-	*F. moniliforme*
Flour	El-Azhar	+	+	-	*F. verticillioides*
Starch	El-Ataba	+	+	-	*F. oxysporum*
Starch	El-Ataba	-	-	-	-
Barely	El-Ataba	+	+	-	*F.oxysporum*
Barely	El-Ataba	+	+	-	*F. equiseti*
Flour	El-Ataba	+	+	-	*F. solani*
Starch	El-Ataba	+	+	-	*F. equiseti*
Starch	El-Ataba	+	+	-	-

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Table 2: Morphological features of tested *Fusarium* species.

	Identified *Fusarium* species	**Culture Appearance**	
		Above	**Reverse**
1	*F. chlamydosporum*	White hyphae	Yellow red pigmented
2	*F. verticillioides*	White violet	White pigmented violet
3	*F. equiseti*	White, yellow with red	Red yellow
4	*F. brachygibbosum*	Dark red with white hyhea	Yellow dark red
5	*F. oxysporum*	White	Yellow red
6	*F. equiseti*	White, yellow with red	Red yellow
7	*F. oxysporum*	White	White
8	*F. chlamydosporum*	White pigmented violet mycelia	Yellow bright violet
9	*F. subglutinans*	White	White pigmented dark violet
10	*F. anthophylum*	Yellowish	Yellowish few pigment red (orange)
11	*F. oxysporum*	White	Dark red violet
12	*F. solani*	white yellow	Creamy
13	*Fusarium* sp.	White	White pigmented blue brown
14	*F. moniliforme*	white hyphae	Deep dark violet white
15	*F. oxysporum*	White pink	White red pigment
16	*F. solani*	Creamy Yellow	Creamy to brown
17	*Fusarium* sp.	White yellow pigmented brown	Yellow pigmented dark brown
18	*F. brachygibbosum*	White pigmented red	Yellow pigmented red

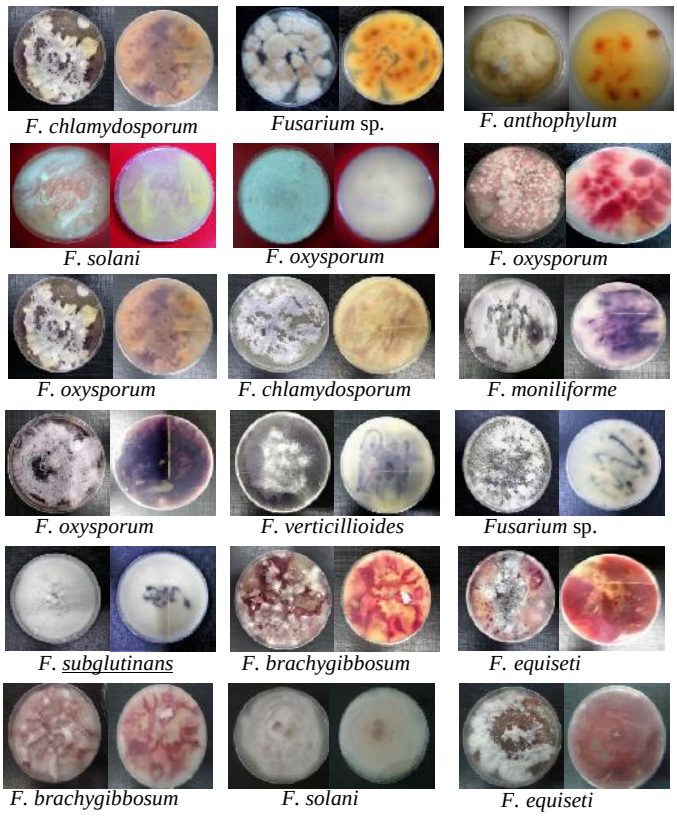

Fig.1: Culture appearance of tested *Fusarium* species; reverse at right of each photo.

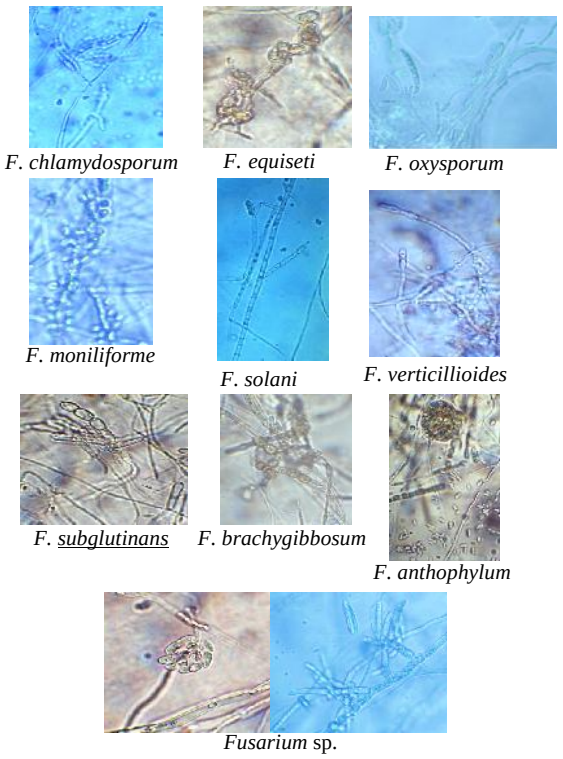

Fig.2: Microscopic features of tested *Fusarium* species.

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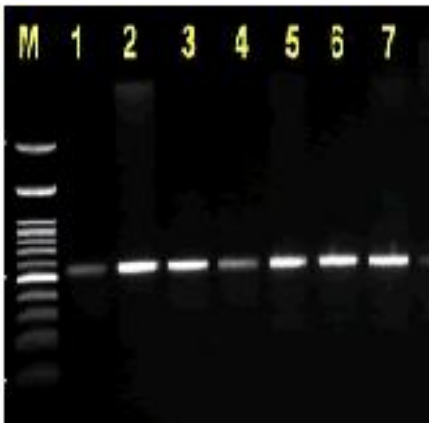

Fig.3: Amplification of ITS conserved ribosomal regions of *Fusarium* species using the primers ITS1 and ITS4. M: 100 bp DNA ladder. 1: *Fusarium* sp, 2: *F. solani*, 3: *F. chlamydosporum*, 4: *F. moniliforme*, 5: *F. verticillioides*, 6: *Fusarium* sp. and 7: *F. equiesti*.

Table 3: *Fusarium* species accession number identified by BLAST software.

Name	Description	Accession number in GeneBank
F. chlamydosporum	*F. fujikuroi*	MG211161
F. verticillioides	*F. fujikuroi*	MG211162
F. moniliformie	*F. verticillioides*	XM_018895258.1
Fusarium sp,13	*F. sporotrichioides*	MG211163
Fusarium sp, 7	*F. solani*	MG211159
F. solani	*F. solani*	MG211160
F.equesti	*F. equiseti*	KM246255.1

3.4 Determination of genetic diversity within *Fusarium* species:

The ITS partial sequence of the *Fusarium* species is presented in **Fig.4**. As a result of phylogenetic tree *F. solani* (MG211159) and *F. verticillioides* (XM_018895258.1) were grouped in one cluster. While, each of the other *Fusarium* species sequence *F. fujikuroi* (MG211162), *F. sporotrichioides* (MG211163), *F. solani* (MG211160) and *F. equiseti* (KM246255.1) were clustered in separate groups.

In the present study, 18 strains of *Fusarium* species were isolated and identified. Morphological identification of these strains failed to distinguish between two *F. solani* strains. These two strains can be identified clearly by Molecular techniques. One of them was introduced to the GeneBank and registered by the accession number (MG211159).

Our results indicated that the ITS region is powerful technique to identify fungal strains. This is agree with other researchers [15] who indicated that ITS region is the most frequently sequenced genetic marker of fungi and it is routinely used to address research questions relating to systematics, phylogeny and identification of strains and specimens at and below the species level. Moreover, others isolated and identified different isolates of *Fusarium sp* from wheat grains samples and identified at the molecular level by ITS-rDNA regions amplification [16].

In the present study, morphological and microscopic investigations were not able to identify *F. sporotrichioides* (MG211163), while molecular technique using ITS rDNA region was able to identify.

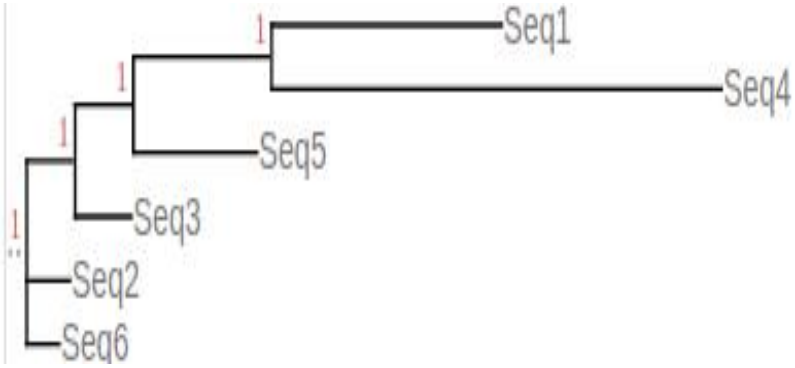

Fig.4: Dendrogram illustrating the genetic distances between different isolated *Fusarium* species. Seq.1: *F. solani* (MG211159), Seq 2: *F. solani* (MG211160), Seq 3: *F. equiseti* (KM246255.1), Seq 4: *F. verticillioides* (XM_018895258.1), Seq 5: *F. fujikuroi* (MG211162) and Seq 6: *F. sporotrichioides* (MG211163).

4. Conclusions

In the present work, some *Fusarium* species were isolated from different cereal grains and their products collected from different places in Cairo and El-Gharbia governorates, Egypt. The isolated species were identified morphologically using the macroscopic and microscopic features of each species. Then the tested species were identified genetically by sequencing of rRNA gene using ITS1 and ITS4 primers.

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