

# Molecular identification of some fungi associated with soft dates (*Phoenix dactylifera* L.) in Saudi Arabia

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# Abstract

The Kingdom of Saudi Arabia is considered one of the major dates producing zones in the world, contributing 13% of the total world production. Most of the dates are delivered to local markets without any treatment thus, may be leading to microbial contamination which may increase the incidence of microbial growth. Some pathogenic fungi associated with soft dates (Phoenix dactylifera Linn.) collected from Almadinah Almunawarah, local markets, was isolated and molecularly identified during summer 2019. Contaminated date samples (Sukkary, Ajwa, Shalabi madinah, Barni and Sogea) were placed on PDA plates and incubated at 26°C for 5 days. Morphological and molecular identification of the resulting fungi were investigated. Molecular identification of fungal isolates was conducted using the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA). Fungal DNAs were amplified by PCR using ITS1-F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The purified PCR products were sequenced and the obtained nucleotide sequences were submitted to GenBank on the NCBI. The results showed that the most dominant isolated fungi were Aspergillus niger (100%) associated with "Sogea", followed by Aspergillus tubingensis (66%) isolated from "Shalabi Almadinah" (40%), Rhizopus stolonifer (36.3%) associated with "Ajwa", Talaromyces minioluteus (14.2%) isolated from "Sukkary", respectively. Soft dates are most likely contaminated due to the artificial rise in moisture content and the related inappropriate storage conditions. In conclusion, the study has declared that Aspergillus spp. considered the most fungal contamination of date fruits. Fungal contamination may have an effect on the public health by their mycotoxins which may lead to severe poisoning, diarrhea, emesis, and death. Most date fruits sold and marketed are neither covered nor protected in any way from environmental contamination. If these dates are consumed without thoroughly washing that may induce imminent mycotic infection. The study recommended that prevention of dates fruit mold spoilage should be avoided by application of hazard analysis, critical control point programs and adherence to hygienic good manufacturing practices.

Keywords: Ajwa, Aspergillus niger, Aspergillus tubingensis, PCR, ITS sequencing, molecular identification.

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# INTRODUCTION

Date fruit is considered one of the most common fruits in the Middle East, especially in the Arabian Peninsula. Dates are consumed fresh (Rutab) or after partial drying and storage (Tamar) during off-season (Al-Bulushi et al., 2017). The Kingdom of Saudi Arabia is considered one of the major dates producing zones in the world, contributing 13% of the total world production. Therefore, the Kingdom of Saudi Arabia exerts great effort to maximize dates production and maintain this market share through product quality and safety assurance (Gherbawy et al., 2012).

Date is marketed throughout the world as a high-value fruit crop and low-cost food. Production of dates increased from 4.60 million tons in 1994 to 6.9 million tons in 2004 worldwide (FAO, 2008). Mycotoxins, especially Aflatoxins (AFs) and OTA, may be found in date fruit (Abdel-Sater and Saber, 1999; Alghalibi and Shater, 2004; Iamanaka et al., 2005). OTA contamination of 20 data samples sold in Brazil of global origin was investigated by lamanaka et al. (2005) only two samples found to have 0.1 to 5 µg/kg contaminations with OTA. The presence of Aflatoxin-B1 (AFB1), OTA and zearelenone has been reported in Egyptian dates analyzed by thin-layer chromatography (TLC) (Abdel-Sater and Saber, 1999). Two specimens were infected with AFB1 and OTA at levels of 300 to 390 µg/kg, respectively, and 360 to 450 µg/kg, with three date samples being contaminated with Zearelenone at 500 to 1000 µg/kg. The criteria of General Standardization Organization (GSO) Policy (2010) for the Exposure Assessment of Contaminants and Toxins in Food and Food Groups should be consulted when the meaning of certain foods in total exposure to the contaminant is identified.

Date is marketed throughout the world as a high-value fruit crop and low-cost food (Mirza et al., 2019). They pass through five main stages of maturity as per Arabic tradition and internationally accepted terminology; Hababouk, Kimri, Khalal, Rutab, and Tamr (Al-Shahib and Marshall, 2003). The moisture content varies in the maturity stage of dates. The moisture content is relatively low in dried dates due to the drying process. Carbohydrates are the major source of energy in dates and average dates contain 54.9 g/100 g of dry weight and dried dates contain 80.6 g/100 g. Protein and fats are present in small amount in dates (Elhindi et al., 2017).

Moreover, most of the dates are delivered to local markets and consumed by Saudi people without any treatment thus, may lead to microbial contamination which may increase the incidence of microbial growth due to high levels of sugars and nutrients element and their low pH values make them particularly desirable to fungal decayed (Singh and Sharma, 2007).

Recent studies found that fungal contamination, yeast and mold counts were higher than bacterial counts in both fresh and treated date (Shenasi et al., 2002). Therefore, fungi are considered the major causative agent of the spoilage of date fruits (Hasnaoui et al., 2010). Fungi are also responsible for off-flavor formation and production of allergenic compounds which lead to qualitative losses (Kader and Hussein, 2009).

However, dates are prone to contamination in the field during harvesting, transporting, storage, marketing and/or by the consumer. Fungi play a substantial role in spoilage of dates because of their pathogenicity to the harvested products. Fungi, that is, *Aspergillus* spp. and *Rhizopus* spp. may grow on high-moisture dates, especially when harvested following rain or high humidity periods (AI-Farsi and Lee, 2008).

Aspergillus sp. is found to be the most common fungi contaminating date fruits. However, microbial research on date fruits is limited in most of the date producing countries. The presence of *Aspergillus niger* damages date fruits and produces not only a loss of value but food safety concerns because of the potential of mycotoxins produced inside the fruit (Colman et al., 2012). There is a big shortage in research studies which examined the dates in Saudi Arabia and studying the different types of fungal spoilage affecting them. Therefore, the aim of current research is to detect the incidence (%) of fungal infection in each type of tested date samples in Saudi Arabia under unfavorable storage conditions and to characterize those fungi using morphological and molecular techniques.

### MATERIALS AND METHODS

### Sample collection

Samples were collected from Al-Madinah Al-Munawarah markets in Saudi Arabia, based on their ripening stage which is related to the moisture and sugar content of the dates. About 50 kg of apparently infected date samples from five different date types (Ajwa, Barni, Sukkari, Shalabi Madina and Sogea) were collected from different markets in Al Madinah Al-Munawarah and were subjected for lab examination. About 150 g of dates packaged in polyvinyl chloride plastic films were transferred within several minutes after collection to an ice box container; aseptically handled and moved promptly to microbiology laboratory, College of science, University of Jeddah.

### Isolation and identification fungal isolates

Infected dates were identified by physical examination according to the method of Jha (1995). Nevertheless, five samples from each variety were cut into pieces and a small piece (1cm) of infected date fruit was surface sterilized by 2% sodium hypochlorite solution for 2 min, washed twice with sterile water and cultured on PDA plates then incubated at 26 to 28°C for 5 days. When colonies of different shape and colors was observed on the plates, single hyphen tips were cultured on PDA medium and incubated at 26 to 28°C for 5 days, sub-culturing each of the different colonies onto the SDA plates were done. Purified cultures were examined microscopically by staining with lactophenol dye. All fungal isolates were maintained in 4°C until used. Molecular identifications of isolated fungi were confirmed using ITS rDNA sequence analysis (Raja et al., 2017).

### Molecular identification

### DNA isolation

Fungal genomic DNAs were isolated using QIAamp DNA Mini Kit according to QIAGEN kit handbook. Fungal isolates were grown on PDA plates at 28°C for 72 h. The plate surface was flood with 10 ml fungal saline (0.9% w/v NaCl), to harvest the conidia. 1 ml saline suspension containing 1 to  $5 \times 10^6$  conidia was measured photometrically at A<sub>530</sub>. It is known that 1 to  $5 \times 10^6$  cells yield about 20 to 30 µg fungal genomic DNA. Fungal cells were centrifuged and

500 µl lysis solutions (50 mM Tris, pH 7.5, 10 mM EDTA, 28 mM ßmercaptoethanol) was added to the collected cell pellet and incubated at 37°C for 30 min. The tubes were centrifuged at full speed for 10 min and the supernatants were discarded. The pellets were resuspended in 180 µl Buffer AL and 20 µl Proteinase K and the tubes were incubated at 56°C for 10 min. 200 µl ethanol (96 to 100%) was added to each sample, and mix well by vortexing. QIAamp Spin Column was placed in a 2 ml collection tube and the mixture solution was carefully applied to the QIAamp Spin Column. The spin columns were closed and centrifuged at 6000 xg for 1 min. The filtrates in the QIAamp collection tubes were discarded. The QIAamp Spin Column was carefully opened and 500 µl of Buffer AW1 was added and centrifuged at 6000 xg for 1 min. The QIAamp Spin Column was placed in a clean 2 ml collection tube, and the filtrate was discarded. 500 µl of AW2 Buffer was added to QIAamp Spin Column and centrifuged at full speed for 3 min. The QIAamp Spin Columns were placed in a clean 1.5 ml microcentrifuge tube, and 150 µl of AE Buffer was added to silica filter to elute the DNA. The Spin Columns were Incubated at room temperature for 1 min then centrifuged at 6000 xg for 1 min. Eluted in DNAs were kept at -20°C until use.

## Quantification of nucleic acids

The DNA was quantified spectrophotometrically by measuring the absorbance at 260nm (A  $_{260 \text{ nm}}$ ). The A $_{260}$ : A $_{280}$  ratio of a nucleic acid extract was measured. Nucleic acid extracts were diluted 100 to 500 µl with water before assay and the extinction coefficient for the DNA was calculated according to the following equation: A $_{260} \times 50 \times$  dil. factor (µg/ml). The integrity of the DNA was checked by agarose gel electrophoresis.

### PCR amplification

Qiagen® *Taq* PCR Master Mix Kit was used to amplify fungal DNA samples via PCR and generate ITS rDNA gene product. A negative control of 2  $\mu$ I 0.1 × TE (TrisEDTA) was used. Fungal DNAs were diluted 1:10 to 0.5 ng/ $\mu$ I and 5  $\mu$ I of each DNA sample was added to 25  $\mu$ I of master mix containing 2  $\mu$ I of (25 pmole) ITS-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer mix, 75 mM of dNTPs, 1.5 mM MgCl<sub>2</sub> and 1.5U Taq DNA polymerase.

The thermocycler (<sup>3</sup>Prime Thermal Cycler, 3PRIMEX/02, Bibby Scientific Ltd) was used for amplification of the samples for a total of 35cycles as follows: one cycle at 95°C for 3 min, followed by 35 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min, followed by final extension cycle at 72°C for 10 min, and a 10°C soak. An aliquot of 10  $\mu$ I of each PCR product was analyzed by electrophoresis on a 1% agarose gel containing 0.5  $\mu$ g/mI ethidium bromide and visualized on UV transilluminator.

### Automated DNA sequencing

The PCR products were purified by QIAquick PCR Purification Kit (QIAGEN) based on manufacturer's instructions. The purified products were sequenced using an automated DNA sequence (ABI PRISM 3700) using the Big Dye Deoxy Terminator cyclesequencing kit (Applied Biosystems) following the manufacturer's instructions. Sequences were submitted to GenBank on the NCBI website (http://www.ncbi.nlm.nih.gov). Sequences obtained in this study were compared with the GenBank database using the BLAST software on the NCBI website (http://www.ncbi.nlm.nih.gov /BLAST/). ITS Sequence and Phylogenetic Analysis DNA sequences were aligned using BLAST program. Phylogenetic trees were constructed after multiple sequence alignments using Clustal W embedded in MEGA6 program and neighbor-joining method with 500 bootstrap replicates (Tamura et al., 2013).

### ITS sequence and phylogenetic analysis

DNA sequences were aligned first with Clustal Omega, Multiple Sequence Alignment (EMBL, EBI). CLC Main Workbench V8.1.3 (Qiagen, Bioinformatics) was used to construct a neighbor-joining tree using Jukes-Cantor model (Jukes and Cantor, 1969).

# RESULTS

Four spoilage fungi were isolated and identified from four types of soft dates morphologically and microscopically is shown in Figure 1. The isolated fungi were Aspergillus niger, Aspergillus tubingensis, Talaromyces minioluteus and Rhizopus stolonifer. Aspergillus niger are filamentous forming filaments (hyphae) fungi, dark and microscopically appeared as; a smooth and colorless conidiophores and spores with dark brown globose heads. While, Aspergillus tubingensis yellow-green dense colony clear radial lines, the back of colony is brown, with white mycelium and spherical spore. Talaromyces minioluteus is cylindrical with clear, vivid orange red to orange red stipe, and a grevish green capitulum with globose of smooth to slightly roughened conidia. Rhizopus stolonifer appeared as white, green and black colonized fungi.

The percentages of fungal contamination are illustrated in Figure 2 as following; Sukkary, Ajwa, Shalabi Madinah, Barni and Sogea soft dates were 39, 30, 20, 20 and 10%, respectively.

The incidence of fungal isolates detected in each type of tested date samples were shown in Table 1 as follows; Sukkary dates, Aspergillus niger was 39.7%, in Asperaillus tubingensis was 24%, Talaromvces minioluteus was 14.2% and Rhizopus stolonifer was 22.1%. However, the incidence of Aspergillus niger, Aspergillus tubingensis Talaromyces minioluteus and Rhizopus stolonifer isolates from Ajwa were; 0%, 54.5%, 9.2%, 36.3% respectively. However, the incidence of Aspergillus niger, Aspergillus tubingensis detected from Shalabi Madinah were 34 and 66% respectively and free from Talaromyces minioluteus and Rhizopus stolonifer. In case of Barni, the contamination by A. niger and A. tubingensis was 37.5 and 62.5%, respectively and free from other fungal spp. Sogea dates were contaminated only with A. niger (100%).

The PCR product sizes of the ITS rDNA regions were of variable lengths, from 536 to 596 bp. *A. niger* isolate MJU-2 had the longest ITS rDNA region (596 and 600 bp), whereas *Talaromyces minioluteus* isolate MJU-6 had the shortest one (536 bp).

The DNA sequence and phylogenetic analysis presented in Figure 3 declared the sequence analysis of the ITS regions of the nuclear encoded rDNA showed significant alignments of 98 to 100% of accession number



**Figure 1.** Morphological and microscopic examination of fungi isolated from contaminated soft dates. (A & B) *Aspergillus niger,* (C & D) *Aspergillus tubingensis,* (E & F) *Talaromyces minioluteus,* (G & H) *Rhizopus stolonifer.* 

MN413684 isolate MJU-1 for *A. tubingensis* with 579/580 (99%) identities. Alignment for accession number MN413685 for *A. niger* isolate MJU-2 with 589/596, (99%) identities. Alignment for accession numbers MN413688 for *Rhizopus stolonifer* isolate MJU-5 with 584/590 (99%) identities. Alignment for accession number MN413689 for *Talaromyces minioluteus* isolate MJU-6 with 536/537 (99%) identities. Similarities within



**Figure 2.** Percentages (%) of spoiled soft dates types contaminated with post-harvest fungi collected from different markets at AI Madinah Almunawarah.

studied fungal accessions (MN413684 isolate MJU-1, MN413685 isolate MJU-2, MN413688 isolate MJU-5, MN413689 isolate MJU-6 were relatively high, within the range of 96 to 100%. Figures 4, 5, 6 and 7, and Table 2 showed the dendogram generated based on the similarity percentages of the four fungal strains as well as reference strains obtained from Gen-Bank. Isolates within each species were clustered together and distinctly separated from the others. The generated dendogram shows that the studied isolates were clustered in three groups. The first group (I) includes MN413689 isolate MJU-6, the second group include the accession number MN413684 isolate MJU-1. The third group includes two accession numbers MN413685 isolate MJU-2 and MN413688 isolate MJU-5.

# DISCUSSION

This study was carried out to identify fungi isolated from soft dates collected from Almadinah Almunawarah using morphological and molecular examination methods. Four fungal species were isolated and identified at the species level using rDNA ITS sequences comparison and analysis. The Isolated species belong to two classes as the following: Eurotiomycetes (*A. niger, A. tubingensis* and *Talaromyces minioluteus*) and Mucoromycotina (*R. stolonifer*). The percentages of fungal contamination found in collected samples of soft dates; Sukkary, Ajwa, Shalabi Madinah, Barni and Sogea soft dates were 39, 30, 20, 20 and 10%, respectively.

Our results are in agreement with the results of another study conducted by Anjili et al. (2015) who reported about 1000 fruits of date palm obtained from four (4) markets from Yola Town Market which showed that the highest incidence fungal isolates from the samples were *A. niger* (40.42%) and *R. stolonifer* (22.45%). These have also been reported by Atia et al. (2009), Hashem (2009),

Table 1. Incidence (%) of fungal contamination among collected soft dates.

Fundal isolatos	Date types					
Fungarisolates	Sukkary	Ajwa	Shalabi madinah	Barni	Sogea	
Aspergillus niger (MN43685)	39.7%	-	34%	37.5%	100%	
Aspergillus tubingensis (MN43684)	24.0%	54.5%	66%	62.5%	-	
Talaromyces minioluteus (MN43689)	14.2%	9.2%	-	-	-	
Rhizopus stolonifer (MN43688)	22.1%	36.3%	-	-	-	



Figure 3. Phylogenetic relationships based on the ITS rDNA regions of the spoilage fungi isolated from soft date fruits. This tree was obtained by neighbor-joining (NJ). The numbers at the branch nodes represent the bootstrap values.

Abass (2013) and Al Hazzani et al. (2014), while Al-Jasser (2010) found these fungi on date palm fruits.

In this study, the fungal incidence in certain types of dates are indicated as follows: 39.7% A. niger, 24% T. minioluteus, 14.2% A. tubingensis and 22.1% R. stolonifer found in Sukkary dates. While, the incidence of A. niger, A. tubingensis, T. minioluteus and R. stolonifer were 0, 54.5, 9.2 and 36.3% in Ajwa, respectively. Surprisingly, the incidence of *A. niger* and *A. tubingensis* in Shalabi Madinah and in Barni were 34, 66, 37.5 and 62.5%, respectively, and were free from T. minioluteus and R. stolonifer. Only Sogea dates were contaminated mostly with Aspergillus niger (100%). These results concur with those reported by Colman et al. (2012) which collected about 360 date fruits from Maiduguri metropolis markets for the detection of the presence of 327 (90.83%) fungal species and noted that the highest percentage was A. niger (39.17%), followed by other

Aspergillus spp.

Our results have shown that soft date fruits are much more prone to contamination by Aspergillus spp. than other fungal species. The results were similar to those reported in Egypt by Abdel-Sater and Saber (1999) which found that Aspergillus was the predominant genus isolated from date samples, Aspergillus niger were the most isolated Aspergillus spp. Rhizopus stolonifer was 25% of the samples which considered the second most common fungus isolated from dried fruits in Yemen (Saeed et al., 2004). Other results recorded in Iraq by Hameed and Abass (2006), Abass et al. (2007) and Abass (2013) showed about 27% A. niger, which was the most predominant species as contaminants of the different dates. Moreover, Al Hazzani et al. (2014) found that the variety Sukhari was found to be the most contaminated fruit with fungi and A. niger was the most predominant fungi present in date fruits collected from the



**Figure 4.** Phylogenetic tree of accession number MN413685 (*Aspergillus niger*) isolate MJU-2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene with other fungal sp.18S ribosomal RNA gene.



**Figure 5.** Phylogenetic tree of accession number MN413684 (*Aspergillus tubingensis*) isolate MJU-1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene with other fungal sp.18S ribosomal RNA gene.



**Figure 6.** Phylogenetic tree of accession number MN413689 (*Talaromyces minioluteus*) isolate MJU-6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene with other fungal sp.18S ribosomal RNA gene.



**Figure 7.** Phylogenetic tree of accession number MN413688 (*Rhizopus stolonifer*) isolate MJU-5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene with other Fungal sp.18S ribosomal RNA gene.

open markets of Riyadh, Medina and Kharj, Saudi Arabia.

On other hand, the results of the current study disagreed

Table 2. Information and identification for ITS rDNA sequences of the species of spoilage fungi isolated from sweet date fruits.

		<b>.</b>	ITS rDNA				
No. Isolate ID Species and strai number		Species and strain accession number	Identification	Identity (%)	Length (bp)	Reference	
1.	MJU-1	MN413684	Aspergillus tubingensis	99%	579	This study	
2.	MJU-2	MN413685	Aspergillus niger	99%	589	This study	
3.	MJU-5	MN413688	Rhizopus stolonifer	99%	584	This study	
4.	MJU-6	MN413689	Talaromyces minioluteus	99%	536	This study	
5.		Aspergillus tubingensis strain HKAS 93727	Aspergillus tubingensis	99%	592	GenBank: KX165340	
6.		<i>Aspergillus niger</i> strain SCAU-F- 99	Aspergillus niger	99%	597	GenBank: KF881765	
7.	129B	<i>Aspergillus niger</i> isolate 129B	Aspergillus niger	99%	590	GenBank: KU847851	
8.		<i>Aspergillus tubingensi</i> s strain DGY03	Aspergillus tubingensis	99%	591	GenBank: MF186869	
9.	FIS17	<i>Aspergillus niger</i> isolate isolate FIS17	Aspergillus niger	99%	594	GenBank: KY378951	
10.	F03-05	<i>Aspergillus tubingensi</i> s isolate F03-05	Aspergillus tubingensis	99%	597	GenBank: KX664319	
11.	F01-01	<i>Aspergillus tubingensis</i> isolate F01-01	Aspergillus tubingensis	99%	599	GenBank: KX664307	
12.	UACH-198	<i>Rhizopus stolonifer</i> isolate UACH-198	Rhizopus stolonifer	99%	587	GenBank: KY563709	
13.	UACH-146	Aspergillus niger isolate UACH-146	Aspergillus niger	99%	587	GenBank: KY554855	
14.	ZJ13	<i>Aspergillus tubingensi</i> s isolate ZJ13	Aspergillus tubingensis	99%	595	GenBank: KP412254	
15.	FIS2	<i>Aspergillus tubingensis</i> isolate FIS2	Aspergillus tubingensis	99%	594	GenBank: KY378944	
16.	132	<i>Aspergillus tubingensis</i> isolate 132	Aspergillus tubingensis	99%	590	GenBank: KU847852	
17.	TFR29	<i>Aspergillus tubingensis</i> isolate TFR29	Aspergillus tubingensis	99%	598	GenBank: KX664374	
18.	F33-01	Aspergillus tubingensis isolate F33-01	Aspergillus tubingensis	99%	594	GenBank: KT213693	
19.		<i>Aspergillus tubingensis</i> strain LYF12	Aspergillus tubingensis	99%	594	GenBank: KJ569519	
20.		Aspergillus niger strain CICR3	Aspergillus niger	99%	597	GenBank: KF747362	
21.		Aspergillus tubingensis strain 3.4342	Aspergillus tubingensis	99%	582	GenBank: EF621571	
22.		Aspergillus niger strain WM10.76	Aspergillus niger	99%	590	GenBank: HQ014698	
23.		Aspergillus niger strain EN2	Aspergillus niger	99%	583	GenBank: KY609552	
24.		Aspergillus tubingensis strain GX1-5E	Aspergillus tubingensis	99%	584	GenBank: GU595290	
25.		Aspergillus niger strain UWFP 515	Aspergillus niger	99%	584	GenBank: AY213633	
26.	South- west0056	Aspergillus tubingensis isolate South-west 0056	Aspergillus tubingensis	99%	530	GenBank: FJ537119	
27.	F28-03	<i>Aspergillus tubingensis</i> isolate F28-03	Aspergillus tubingensis	99%	602	GenBank: KX664369	
28.	PILE 14-5	<i>Talaromyces minioluteus</i> isolate PILE 14-5	Talaromyces minioluteus	99%	545	GenBank: KF471124	

29.	F21-01	<i>Talaromyces diversus</i> isolate F21-01	Talaromyces diversus	97%	596	GenBank: KX664342
30.		<i>Talaromyces minioluteus</i> strain E93	Talaromyces minioluteus	99%	556	GenBank: KX610145
31.		<i>Talaromyces sp</i> DI16-144	Talaromyces sp.	97%	592	GenBank: LT558966
32.		<i>Talaromyces sp</i> JCM 28523	Talaromyces sp.	97%	575	GenBank: LC133882
33.		<i>Talaromyces purpureogenus</i> strain CFRM02	Talaromyces purpureogenus	94%	523	GenBank: MF085055
34.	LN2R560	<i>Talaromyces purpureogenus</i> isolate LN2R560	Talaromyces purpureogenus	94%	513	KM999228
35.		<i>Talaromyces purpureogenus</i> strain ATHUM 5090	Talaromyces purpureogenus	94%	522	FJ004308
36.		Talaromycespurpurogenusstrain A3S3-40	Talaromyces purpurogenus	94%	556	KC344980
37.		<i>Talaromyces purpurogenus</i> strain A4S1	Talaromyces purpurogenus	94%	513	KC344976
38.		Aspergillus niger strain TFR-4	Aspergillus niger	94%	513	GU973805
39.		Rhizopus stolonifer	Rhizopus stolonifer	99%	590	KC291246
40.	wxm171	<i>Rhizopus stolonifer</i> isolate wxm171	Rhizopus stolonifer	92%	243	HM051076
41.	RWP-47	<i>Rhizopus stolonifer</i> isolate RWP- 47	Rhizopus stolonifer	87%	145	MH348275
42.		<i>Rhizopus stolonifer</i> strain TUR5	Rhizopus stolonifer	87%	145	AM933544
43.	TUR8	Rhizopus stolonifer isolate TUR8	Rhizopus stolonifer	87%	145	FN401529
44.		Rhizopus stolonifer strain TUR1	Rhizopus stolonifer	87%	145	AM933543

Table 2. Information and identification for ITS rDNA sequences of the species of spoilage fungi isolated from sweet date fruits.

with the results of Ibrahim and Rahma (2009), who found that *Rhizopus* spp. was the dominant isolate; however, Deeb et al. (2006) failed to isolate any of the mentioned fungus spp. These differences could be attributed to the variation in geographical location.

A. niger is a filamentous ascomycete fungus that is ubiguitous in different environments and the most common member of the microbial communities found in soil, air and many other environments (Samson et al., 2002; Al-Mayahi et al., 2010). Hence, its saprophytic activity with a wide range of oxidative and hydrolytic enzymes enables this fungus to grow wherever there is a suitable source of food and moisture (Schuster et al., 2002). These black Aspergilli can produce Ochratoxin A (OTA) in various food commodities (Schuster et al., 2002). Ochratoxin is identified as a renal carcinogen to particular animal species (Kuiper-Goodman and Scott, 1989) and can cause nephrotoxic, teratogenic and immunosuppressive effects in multiple animal species (Kuiper-Goodman and Scott, 1989; O'Brien and Dietrich, 2005).

Date fruits sold in shops or market places were neither

covered nor protected in any way from dust or atmospheric contamination, and when consumed without any form of washing or clearing could enhance mycotic infection.

In this study, the fungal isolates were first identified at the level of the genus by morphological examination, depending on the colors of the colony formed on both sides, on the top and on the back of the fungal cultures. The morphological examination and identification of fungi are useful for the identification of isolates up to the family or genus level (Wang et al., 2016). However, this identification is not adequate to identify the isolated fungi up to the species level (Lutzoni et al., 2004).

The application of molecular identification is quick, adequate, reproducible and highly specific, in contrast to morphological and biochemical tests for the diagnosis of fungi in the laboratory (Liu et al., 2000). The molecular identification of isolated fungi was carried out by DNA amplification using the ITS region sequencing. The ITS rDNA sequences were compared to those in the databases using NCBI-BLAST. The sequence of ITS rDNA region is known to be one of the key tools to identify fungal species from environmental sources (Anderson and Parkin, 2007) and was therefore used extensively to enhance the classical identifications. Our results of PCR amplification was conducted using internal transcribed spacer (ITS) PCR products amplified from the predominant fungi using ITS1 and ITS4 primers. The results showed that the PCR product sizes of the ITS rDNA regions were of variable lengths, from 536 to 596 bp. The ITS rDNA PCR product obtained from A. niger isolate was (596 bp), whereas it was 536 bp from T. minioluteus isolate. The obtained results were in agreement with that reported by Abass (2013) who molecularly characterized the most abundant fungal contaminants of date palm in which, the Aspergillus species were found to be the most frequently isolated species, followed by the species of Alternaria and Penicillium.

Four fungal species were identified using DNA sequencing with an identity range between 87 and 99%. The DNA sequence and phylogenetic analysis indicated that the sequence analysis of the ITS regions showed significant alignments for accession number MN413684, MN413685, MN413688 and MN413689 respectively, with other 44 reference sequences of related species obtained from Gen-Bank. Isolates within each species were clustered together and distinctly separated from the others. The generated dendogram shows that the studied isolates were clustered in three groups. The first group (I) includes MN413689 isolate MJU-6, the second group include the accession number MN413684 isolate MJU-1. The third group includes two accession numbers MN413685 isolate MJU-2 and MN413688 isolate MJU-5. The same clustering method used in the current study were also reported by Awa and Oguntade (2015) who conducted a quick molecular test based on the amplification of fragments of the internal transcribed spacer (ITS1) region to distinguish Colletotrichum isolates from other pathogenic fungi. The analysis of the nucleotide sequences of the ribosomal DNA (rDNA) fragment showed sufficient variability to clearly classify the 7 fungal species isolated from symptomatic mango fruits into Colletotrichum gloeosporioides, Fusarium verticillioides Lasiodiplodia and theobromae. Chromatogram produced from their rDNA data confirmed the identities of the fungal isolates. Chul-Kwon et al. (2018) identified 18 spoilage fungi isolated from mulberry fruits and molecularly characterized based on the comparisons of the ITS rDNA sequences.

# CONCLUSION

The isolation and identification of filamentous fungi from soft dates in Saudi Arabi showed existence of some economically-important fungi. The study specifically employed the molecular technique in fungal identification which added great benefits to the process of distinguishing between similar species of fungi in Al-Mutarrafi et al. 105

comparison with the traditional techniques.

The research also discusses date fruits sold in open areas which are not shielded from contamination and may be consumed without hygiene, might increase the risk of mycotic infection.

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### REFERENCES

- Abass MH, Al-Abadi UAM, AlKaby AMS, 2007. The efficiency of Henna leaves extracts and some fungicides to reduce the fungal contamination of date palm *Phoenix dactylifera* L tissue culture Iraqi J Biotech, 62: 1-40.
- Abass MH, 2013. Microbial contaminants of date palm *Phoenix dactylifera* L in Iraqi tissue culture laboratories. Emir J Food Agric, 25(11):875-882.
- Abdel-Sater MA, Saber SM, 1999. Mycoflora and Mycotoxins of some Egyptian dried fruits. Bull Fac Sci Assiut Univ, 28:91-107.
- Al Hazzani A, Al-Rizwana H, Moubayed NMS, Alshatwi AA, Munshi A, Elgaaly G, **2014.** Postharvest fruit spoilage bacteria and fungi associated with date palm *Phoenix dactylifera L* from Saudi Arabia. Afr J Microbiol Res, 8(11): 1228-1236.
- AI-Bulushi IM, Bani-Uraba MS, Guizani NS, AI-Khusaibi MK, and Al-Sadi AM, 2017. Illumina MiSeq sequencing analysis of fungal diversity in stored dates. BMC Microbiol, 17, 72.
- Al-Farsi MA, Lee CY, 2008. Optimization of phenolics and dietary fibre extraction from date seeds Food Chemistry, 108(3): 977-985.
- Al-Jasser MS, 2010. Effect of storage temperatures on microbial load of some date palm fruits sold in Saudi Arabia market. African Journal of Food Science 4(6):359–363.
- Al-Mayahi AM, Ahmed AN, AlKhalifa AA, 2010. Isolation and identification of associated fungi with the micropropagation of five different date palm cultivars and the effect of Benlate fungicides in their control. Basra J Date Palm Res, 92: 79-97.
- Al-Shahib W, Marshall R, 2003. The fruit of the date palm: Its possible use as the best food for the future. Int J Food Sci Nutrit, 544: 247-259.
- Anderson IC, Parkin PI, 2007. Detection of active soil fungi by RT-PCR amplification of precursor rRNA molecules. J Microbiol Methods, 68: 248–253.
- Atia MM, El-Mahmodi AM, El-Shili M, 2009. Fungi Associated with Some Stored Date Palm Fruits in Libya. The 3rd National Conference on Basic Science Under the Title: Basic Sciences are the Main Source of Creativity Gharian Libya from. 25 - 27.
- Awa OC, Oguntade O, 2015. Molecular Identification of fungal species associated with mango anthracnose in Southwest Nigeria. J Agric Environ Sci, 42: 99-103.
- **Chul-Kwon** O, Ju WT, Bok Kim H, Sung GB, Kim YS, **2018**. Isolation and identification of postharvest spoilage fungi from mulberry fruit in Korea. Korean J Environ Agric, 373: 221-228.
- **Colman** S, Spencer THI, Ghamba PE, Colman E, **2012**. Isolation and identification of fungal species from dried date palm *Phoenix dactylifera* fruits sold in Maiduguri metropolis. Afr J Biotechnol, 11(57): 12063-12066.
- Deeb HM, Lashin SM, Arab YA, 2006. Distribution and pathogenesis of date palm fungi in Egypt. ISHS Acta Horticultural, 736: 22-46.
- Elhindi KM, Sharaf El-Din A, Elgorban AM, 2017. The impact of arbuscular mycorrhizal fungi in mitigating salt-induced adverse

effects in sweet basil Ocimum basilicum L. Saud J Biol Sci, 24(1): 170-179.

FAO, 2008. "Date Palm" http://faostatefaoorg/ site/340/default.

Gherbawy YA, Elhariry HM, Bahobial AAS, 2012. Mycobiota and

mycotoxins (aflatoxins and ochratoxin) associated with some Saudi date palm fruits. Foodborne Pathog Dis, 9(6): 561-567

- Hameed MA, Abass MH, 2006. Study of cytological changes associated with contaminated date palm *Phoenix dactylifera* L tissue cultures with fungi. Basra Res J, 32: 127.
- Hashem A, 2009. Date palm fruit spoilage and seed-born fruit of Saudi Arabia. Res J Microbiol, 4:208-213.
- Hasnaoui A, Elhoumaizi MA, Asehraou A, Hakkou A, **2010**. Chemical composition and microbial quality of main varieties of dates grown in Figuig oasis of Morocco. Int J Agric Biol, 12: 311-314.
- **Ibrahim** S, **Rahma** MA, **2009**. Isolation and identification of fungi associated with date palm fruits *Phoenix dactylifera* sold at Beyaro University, Kano, Nigeria. Bayero. J Pure Appl Sci, 2: 127-130.
- Iamanaka BT, Taniwaki MH, Menezes HC, Vicente E, Fungaro MHP, 2005. Incidence of toxigenic fungi and ochratoxin A in dried fruits sold in Brazil. Food Additives and Contaminants, 22: 1258–1263.
- Jha D, 1995. Laboratory manual on seed pathology. Villas Publishing House PVT Ltd. pp. 13 30.
- Jukes TH, Cantor CR, 1969. Evolution of protein molecules Munro HN Ed Mammalian protein metabolism III New York: Academic Press, 121-132.
- Kader AA, Hussein AM, 2009. Harvesting and postharvest handling of dates, ICARDA, Aleppo, Syria , 15 pp.
- Liu D, Coloe S, Baird R and Pedersen J. 2000. Application of PCR to the identification of dermatophyte fungi. J Med Microbiol, 49: 493-497.
- Lutzoni F, Kauff F, Cox CJ, Mc Laughlin D, Celio G, Dentinger B, Padamsee M, Hibbett D, James TY, Baloch E, Grube M, Reeb V, Hofstetter V, Schoch C, Arnold AE, Miadlikowska J, Spatafora J, Johnson D, Hambleton S, Crockett M, Shoemaker R, Sung GH, Lücking R, Lumbsch T, O'Donnell K, Binder M, Diederich P, Ertz D, Gueidan C, Hansen K, Harris RC, Hosaka K, Lim YW, Matheny B, Nishida H, Pfister D, Rogers J, Rossman A, Schmitt I, Sipman H, Stone J, Sugiyama J, Yahr R, Vilgalys R, 2004. Assembling the fungal tree of life: progress, classification and evolution of the subcellular traits. Am J Bot, 91: 1446–1480.
- Kuiper-Goodman T, Scott PM, 1989. Risk assessment of the mycotoxin ochratoxin A. Biomed Environ Sci, 2: 179–248.
- Mirza MB, Sayed FQ, Khan F, Elkady AI, Al-Attar AM, Hakeem KR, 2019. Ajwa Dates: A Highly Nutritive Fruit with the Impending Therapeutic Application Chapter in Plant and Human Health Volume 3: Pharmacology and Therapeutic Uses edited by Münir Ahmet Öztürk Khalid Rehman Hakeem. 211-229.
- **O'Brien** E, **Dietrich** DR, **2005**. Ochratoxin A: The continuing enigma. Crit Rev Toxicol, 35: 33–60.

- Raja HA, Miller AN, Pearce CJ, Oberlies NH, 2017. Fungal identification using molecular tools: A primer for the natural products research community. J Nat Prod, 80(3): 756-770.
- Saeed M, Alghalibi S, Abdul-Rahman MS, 2004. Mycoflora and mycotoxin contamination of some dried fruits in Yemen Republic. Ass Univ Bull Environ Res, 72: 19-27.
- Samson RA, Houbraken J, Summerbell RC, Flannigan B, Miller JD, 2002. Common and important species of fungi and actinomycetes in indoor environments In: B Flannigan, RA Samson and JD Miller Eds, 287-292.
- Schuster E, Dunn-Coleman N, Frisvad JC, Van Dijck PW, 2002. On the safety of Aspergillus niger - A review. Appl Microbiol Biotech, 59(4-5): 426-435.
- Singh D, Sharma RR, 2007. Postharvest diseases of fruit and vegetables and their management. In: Prasad, D Ed., Sustainable Pest Management Daya Publishing House: New Delhi, India.
- Shenasi M, Kofi A, Candlish A, 2002. Microflora of date fruits and production of aflatoxins at various stage of maturation. Int J Food Microbiol, 79: 113-119.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S, **2013**. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol, 30(12): 2725-2729.
- Wang Z, Nilsson RH, James TY, Dai Yand Townsend JP, **2016**. Future Perspectives and Challenges of Fungal Systematics in the Age of Big Data. Biology of Microfungi. Springer, pp. 25–46.

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