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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 Iamanaka 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 (Al-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 × 10^6 conidia was measured photometrically at A_{530}. It is known that 1 to 5 × 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 ß-mercaptoethanol) 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 ($A_{260\ nm}$). 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 µl 0.1 × TE (TrisEDTA) was used. Fungal DNAs were diluted 1:10 to 0.5 ng/µl and 5 µl of each DNA sample was added to 25 µl of master mix containing 2 µl of (25 pmole) ITS-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer mix, 75 mM of dNTPs, 1.5 mM $MgCl_2$ and 1.5U Taq DNA polymerase.

The thermocycler ([3]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 µl of each PCR product was analyzed by electrophoresis on a 1% agarose gel containing 0.5 µg/ml 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 cycle-sequencing 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 fungi, forming dark filaments (hyphae) 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 greyish 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; in Sukkary dates, *Aspergillus niger* was 39.7%, *Aspergillus tubingensis* was 24%, *Talaromyces 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

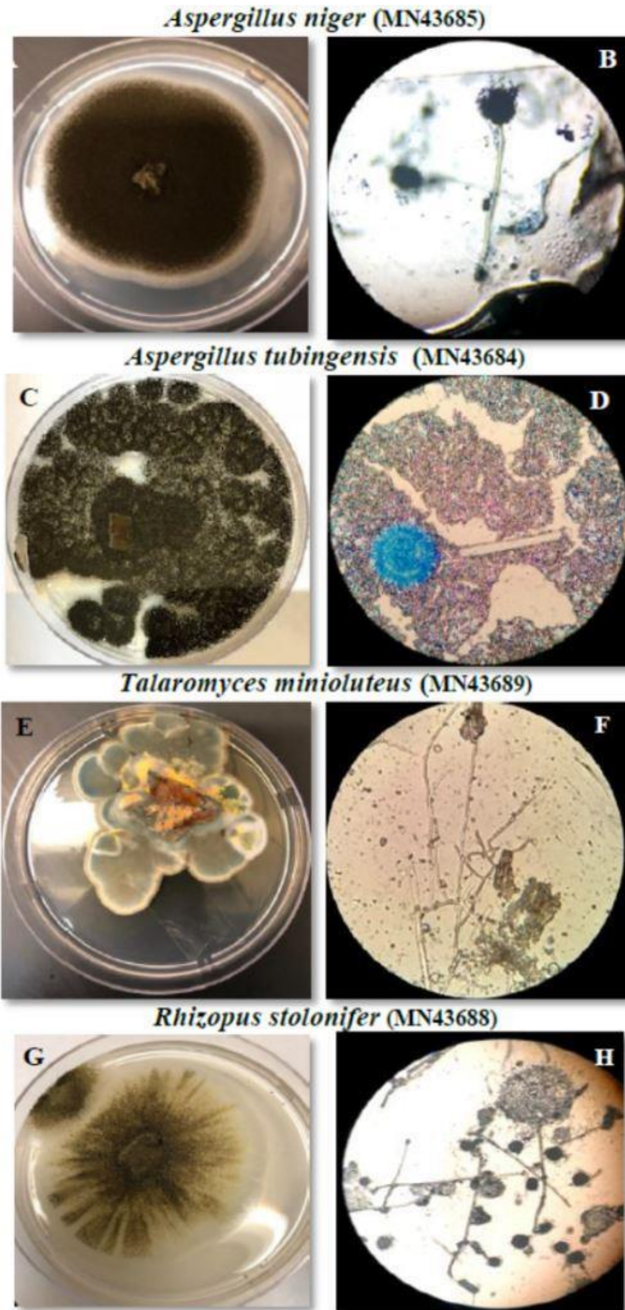

Figure 1. Morphological and microscopic examination of fungi isolated from contaminated soft dates. (A & B) *Aspergillus niger,* (C & D) *Aspergillus tubingensis*, (E & F) *Talaromyces minioluteus*, (G & H) *Rhizopus stolonifer.*

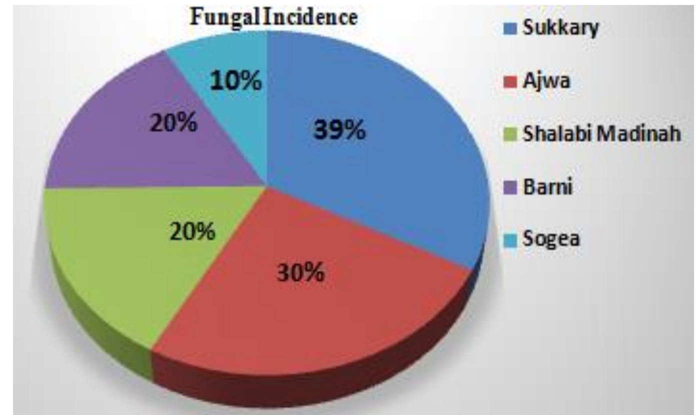

Figure 2. Percentages (%) of spoiled soft dates types contaminated with post-harvest fungi collected from different markets at Al Madinah Almunawarah.

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 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.

Fungal isolates	Date types				
	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%	-	-	-

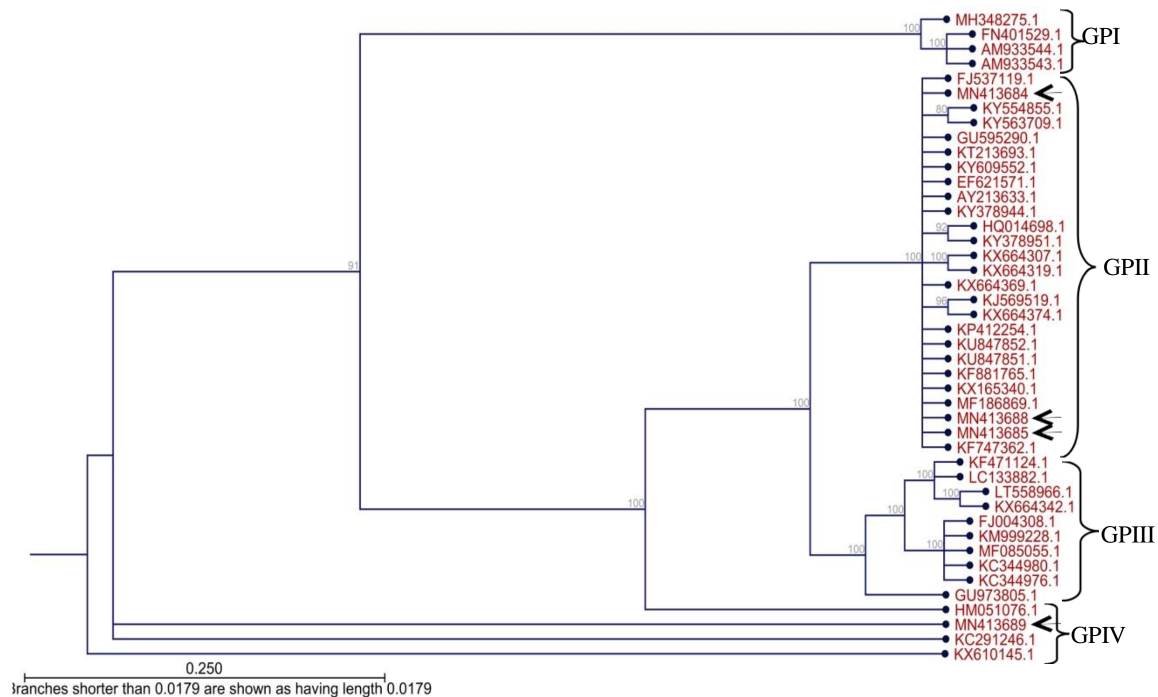

Figure 3. Phylogenetic relationships based on the ITS rDNA regions of the spoilage fungi isolated from soft date fruits. This tree was obtained by neighbor-joining (NJ). The numbers at the branch nodes represent the bootstrap values.

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

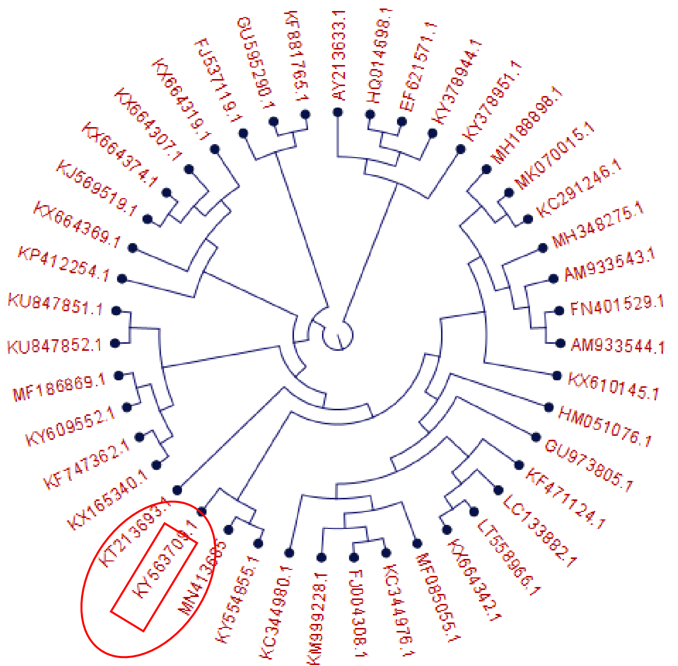

Figure 4. Phylogenetic tree of accession number MN413685 (*Aspergillus niger*) isolate MJU-2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene with other fungal sp.18S ribosomal RNA gene.

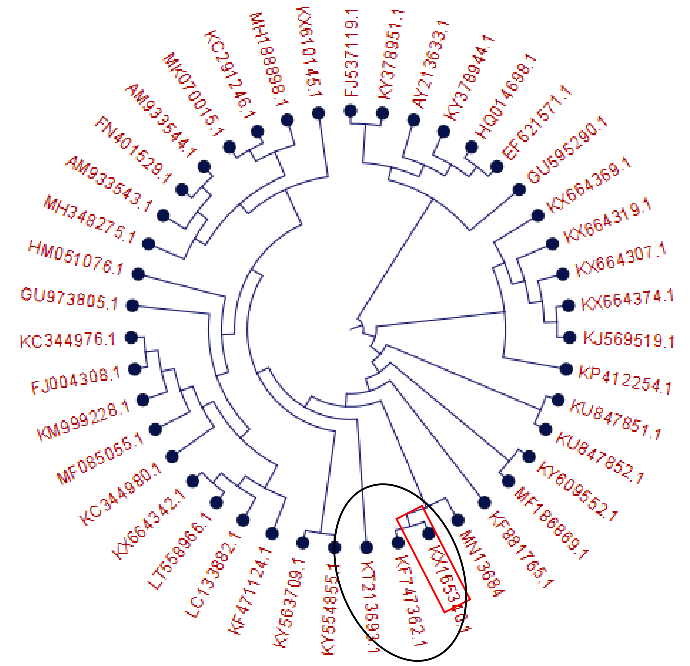

Figure 5. Phylogenetic tree of accession number MN413684 (*Aspergillus tubingensis*) isolate MJU-1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene with other fungal sp.18S ribosomal RNA gene.

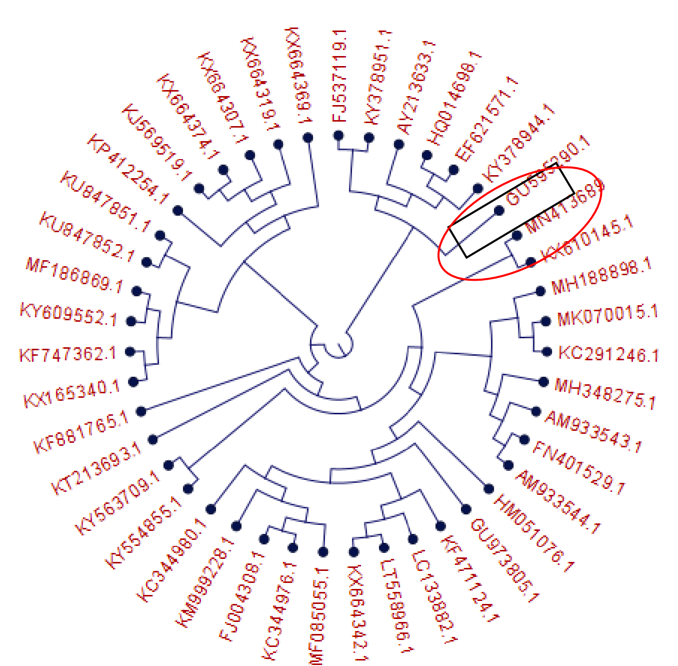

Figure 6. Phylogenetic tree of accession number MN413689 (*Talaromyces minioluteus*) isolate MJU-6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene with other fungal sp.18S ribosomal RNA gene.

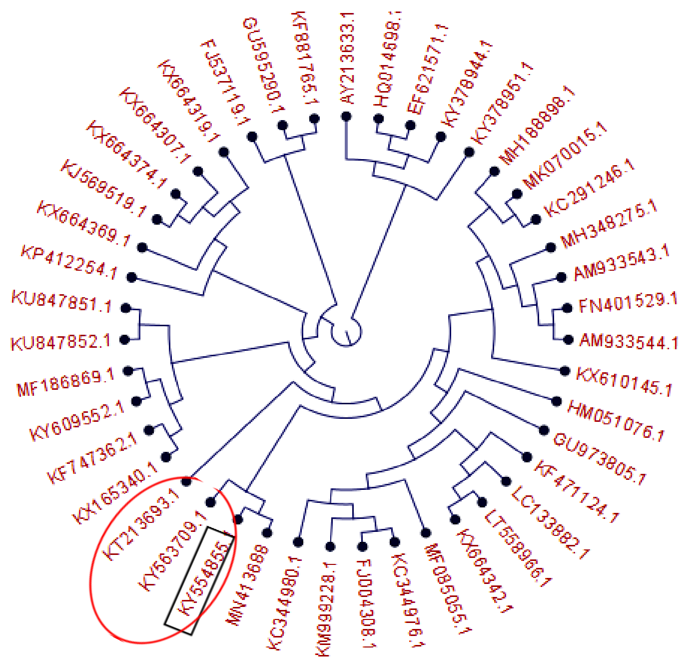

Figure 7. Phylogenetic tree of accession number MN413688 (*Rhizopus stolonifer*) isolate MJU-5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene with other Fungal sp.18S ribosomal RNA gene.

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.

No.	Isolate ID	Species and strain accession number	ITS rDNA			Reference
			Identification	Identity (%)	Length (bp)	
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.		*Aspergillus niger* strain SCAU-F-99	*Aspergillus niger*	99%	597	GenBank: KF881765
7.	129B	*Aspergillus niger* isolate 129B	*Aspergillus niger*	99%	590	GenBank: KU847851
8.		*Aspergillus tubingensis* strain DGY03	*Aspergillus tubingensis*	99%	591	GenBank: MF186869
9.	FIS17	*Aspergillus niger* isolate isolate FIS17	*Aspergillus niger*	99%	594	GenBank: KY378951
10.	F03-05	*Aspergillus tubingensis* isolate F03-05	*Aspergillus tubingensis*	99%	597	GenBank: KX664319
11.	F01-01	*Aspergillus tubingensis* isolate F01-01	*Aspergillus tubingensis*	99%	599	GenBank: KX664307
12.	UACH-198	*Rhizopus stolonifer* 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	*Aspergillus tubingensis* isolate ZJ13	*Aspergillus tubingensis*	99%	595	GenBank: KP412254
15.	FIS2	*Aspergillus tubingensis* isolate FIS2	*Aspergillus tubingensis*	99%	594	GenBank: KY378944
16.	132	*Aspergillus tubingensis* isolate 132	*Aspergillus tubingensis*	99%	590	GenBank: KU847852
17.	TFR29	*Aspergillus tubingensis* isolate TFR29	*Aspergillus tubingensis*	99%	598	GenBank: KX664374
18.	F33-01	*Aspergillus tubingensis* isolate F33-01	*Aspergillus tubingensis*	99%	594	GenBank: KT213693
19.		*Aspergillus tubingensis* 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	*Aspergillus tubingensis* isolate F28-03	*Aspergillus tubingensis*	99%	602	GenBank: KX664369
28.	PILE 14-5	*Talaromyces minioluteus* isolate PILE 14-5	*Talaromyces minioluteus*	99%	545	GenBank: KF471124

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

29.	F21-01	*Talaromyces diversus* isolate F21-01	*Talaromyces diversus*	97%	596	GenBank: KX664342
30.		*Talaromyces minioluteus* strain E93	*Talaromyces minioluteus*	99%	556	GenBank: KX610145
31.		*Talaromyces sp* DI16-144	*Talaromyces sp.*	97%	592	GenBank: LT558966
32.		*Talaromyces sp* JCM 28523	*Talaromyces sp.*	97%	575	GenBank: LC133882
33.		*Talaromyces purpureogenus* strain CFRM02	*Talaromyces purpureogenus*	94%	523	GenBank: MF085055
34.	LN2R560	*Talaromyces purpureogenus* isolate LN2R560	*Talaromyces purpureogenus*	94%	513	KM999228
35.		*Talaromyces purpureogenus* strain ATHUM 5090	*Talaromyces purpureogenus*	94%	522	FJ004308
36.		*Talaromyces purpurogenus* strain A3S3-40	*Talaromyces purpurogenus*	94%	556	KC344980
37.		*Talaromyces purpurogenus* 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	*Rhizopus stolonifer* isolate wxm171	*Rhizopus stolonifer*	92%	243	HM051076
41.	RWP-47	*Rhizopus stolonifer* isolate RWP-47	*Rhizopus stolonifer*	87%	145	MH348275
42.		*Rhizopus stolonifer* 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

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 ubiquitous 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* and *Lasiodiplodia 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 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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