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Original article

Molecular heterogeneity in the 18s DNA gene of *Alternaria* sp. and *Fusarium* sp. producing mycotoxins in rice and maize grains

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ABSTRACT

Background: Food contaminated with fungi and their toxins is a problem that threatens many developing countries. Kingdom of Saudi Arabia depends on the exported grain and legume seeds.
Materials and methods: The study involved examination of 160 samples of rice and maize seeds collected from different locations in the Kingdom of Saudi Arabia. Heterogeneity in the 18s rRNA gene of toxigenic *Alternaria* sp. and *Fusarium* sp. was unraveled. The seeds were disinfected and cultured on Potato Dextrose Agar (PDA), Yeast Extract Sucrose (YES) media and incubated at 25 °C/7 days. The isolated fungi were subjected to 18s rRNA gene sequencing. Five toxins were extracted from maize and rice grains infected with isolated fungi.
Results: The isolated fungi were identified based on morphological and spores characters as *Fusarium* sp. and *Alternaria* sp. Molecular identification based on18s rDNA barcode' was performed due to its high degree of inter specific variability, conserved primer sites and multi-copy nature in the genome. *Fusarium* sp. produced the highest detected (2070 μg/kg) fumonisin especially in cereal production season 2011. The collected grain from Dammam recorded the highest percentage (5485.2 g/kg) of toxins.
Conclusion: This work highlights that 50% of samples were found contaminated with toxins in various concentrations which impose a threat for public health and necessitate rapid identification methods for toxigenic fungi such as 18s rDNA sequencing.

1. Introduction

Several fungi attack the corn grains during harvest and storage. While more than 25 different fungi species known to invade stored grains and legumes (Duan et al., 2007), some species such as *Aspergillus*, *Fusarium*, *Penicillium* are responsible for most spoilage and germ damage during storage (Pitt, 2000a). They cause reduction in nutritive values, produce undesirable odors, and changes appearance of stored food grade seeds and decrease germination ability and total decay (Castillo et al., 2004). Besides their mycotoxins, they are considered as health hazard for man and animals,

render products unacceptable for edible purposes or lower their market grade (Pitt, 2000b; Richard et al., 2009). Mycotoxins are secondary metabolites that have no biochemical significance in fungal growth and development. Mycotoxins are produced mainly by the mycelial structure of filamentous fungi, or more specifically, the molds. Toxigenic molds are known to produce one or more of these toxic secondary metabolites. Examples of mycotoxins of greatest public health and agro-economic significance include aflatoxins (AF), ochratoxins (OT), trichothecenes, zearalenone (ZEN), fumonisins (F), tremorgenic toxins, and ergot alkaloids (Hamada et al., 2012). The storage, environmental, and ecological conditions are factors contributing to the presence or production of mycotoxins in foods or feeds. The carcinogenic potential of AF, OT, trichothecenes, ZEN, and F was evaluated. Naturally occurring AF were classified as carcinogenic to humans (Group 1) while OT and F were classified as possible carcinogens (Group 2B). Trichothecenes and ZEN, however, were not classified as human carcinogens (Group 3). Metabolism and defense mechanisms are important factors in understanding mycotoxins toxicity in specific species or individual animals (Hamada et al., 2012). Understanding the metabolic pathways of mycotoxins in ruminants and

non-ruminants could enable researchers and public health officials to gain insight on how to assess the associated risks of mycotoxin exposure in various species (Abbas et al., 2010). PCR technique was used for detection of myco-toxigenic fungi and sequences of mycotoxin-related genes (Hamada et al., 2012; Sanzani et al., 2012b). Several studies have reported a correlation between the biomass of producing fungi and specific mycotoxin contaminations (Boutigny et al., 2012; Sanzani et al., 2013). However, as the toxigenic ability of each strain needs to be considered, various mycotoxin genotyping assays have been developed to directly quantify genes responsible for mycotoxin synthesis, from both fungal culture and plant material (Kulik et al., 2011). This study aims to examination of fungi and its toxins contaminating rice and maize seeds collected from locations in the Kingdom of Saudi Arabia and unraveling heterogeneity in the 18s rDNA gene of *Alternaria* sp. and *Fusarium* sp. producing mycotoxins.

2. Material and methods

2.1. Sampling collection

Samples of rice grains (120) and maize grains (40) were collected from locations in the Kingdom of Saudi Arabia in plastic bags and brought to the laboratory. These samples were sterilized by submerging in Clorox solution concentration of 5% for 1–2 min and washed with sterile distilled water. The sterilized grains were placed on the surface of the Potato Dextrose Agar (PDA) (Emmons, 1980). The plates were incubated at 25 °C for 7 days. The grown isolated fungi were transferred onto the surface of Yeast Extract Sucrose (YES) agar medium (Davis et al., 1967) by streaking method and incubated at 25 °C for 7 days for isolation and identification.

2.2. Isolation and identification of fungi

The isolated fungi were purified on Czapeks medium and identified following the classification keys as mentioned by Pitt and Hocking (2009) and Muhadiet al. (2007). The isolated fungi were identified as described by Koneman (1978) through the investigation of the morphological characteristics and microscopy of the colony form, color, strength and type spinning innate as well as shape of spores, color and dimensions.

2.3. Molecular characterization of isolated fungi

The mycelium mass of fungal isolates grown on PDA broth medium was harvested by centrifugation at 6000 pm for 10 min. The pellets were washed twice by PBS buffer and stored at 20 °C. Total DNA of the three isolates was isolated using lysozyme – dodecyl sulfate lysis method as described by Leach et al. (1990). The supernatant was saved for PCR. PCR primers capable of amplifying 18s rDNA gene and nucleotide sequences of the 18s rDNA gene of all isolated fungi were used. 18srDNA gene amplicons were obtained by using eukaryotic primers 1A (5′-AACCTGGTTGATCCTGCCAGT-3′) and 564R (3′GGCACCAGACTTGCCCTC-5′) (with modifications in reference to (Medlin et al., 1988). PCR conditions were modified for 18s amplification, an initial denaturation at 94 °C for 5 min, followed by 25 cycles at 94 °C for 50 s, 53 °C for 50 s and 72 °C for 50 s; for 18S amplification, an initial denaturation at 94 °C for 5 min, followed by 25 cycles at 94 °C for 50 s, 59 °C for 50 s and 72 °C for 50 s. 10 μl of PCR product was electrophoresed on 1% agarose gel to determine the size of the product. The negative control consisted of all PCR components except for the template DNA.

3. Results and discussion

The isolated fungi from rice and maize grains collected randomly from different locations in the Kingdom of Saudi Arabia were identified based on Morphology characters, growth on Czapeks plates and results of wet mount microscopic observation were tabulated in Table 1. Fungi are remarkable organisms that readily produce a wide range of natural products (secondary metabolites) some are deleterious (e.g. mycotoxins). Fungi that exhibit filamentous growth and have a relatively complex morphology produce many secondary metabolites. The production of these secondary metabolites usually commences late in the growth of the fungus, often upon entering the stationary phase (Sekar et al., 2008). For identification by morphology, LCB wet mount was prepared and the following morphologies were observed. The results of LCB wet mount preparation are shown in Table 1. Mold spores present in biscuit survive for several years, and therefore, care should be taken in the storage of biscuit (Christensen and Cohen, 1950). Table 1 showed the mean values of total fungal counts obtained with the direct plating technique. These results are in agreement with the results reported by Gashgari et al. (2010). Dilution plating is the technique recommended for fungal enumeration in flours and direct plating is considered to be the more effective technique for mycological examination of particulate foods such as grains or nuts and wheat samples (Cabañas et al., 2008). Cabañas et al. (2008) reported that the total mold counts obtained from wheat flour samples in Spain are similar to those reported by other authors. In Malaysia, total fungal count in wheat flour samples ranged from 102 cfu/g sample to slightly more than 104 cfu/g sample (Abdullah et al., 1998). In Spain, the maximum mold count limit for wheat flour for human consumption is 104 cfu/g.

The result in Table 2 showed that five types of toxins and their quantity (Zeara* Fum* DON and T-2 from *Fusarium* sp. and *Alternaria* from *Alternaria* sp.) were extracted by *Fusarium* sp. and *Alternaria* sp. in maize and rice grains. *Fusarium* sp. recorded the highest presence reached 2070 μg/kg of Fumonisin especially in cereal production season 2011. The collected grains from Dammam recorded the highest percentage Micrograms per 5485.2/kg. The results indicated that 50%contamination of samples with toxins in concentrations ranging from showing 13–722 μg per/kg was observed, where the presence of toxins in extracts from samples collected from Dammam was found at highest concentrations, this may be due to contamination of the source or poor storage and exportation. These ratios of DON toxin is very high compared to previous studies and pose a threat to humans, as several studies have indicated that the contamination concentration of 0.06–0.04 mg/kg caused harmful effects to the liver and the low level of albumin in the blood (Bergsjø et al., 1993; Zöllner and Mayer-Helm, 2006). The selected poison samples are less contaminated as compared to most contaminated samples.

3.1. Molecular characterization of Fusarium isolates

The Genetic Profiling of *Fusarium* sp. producing mycotoxins in rice and maize grains based on heterogeneity in the 18s DNA gene was performed. The molecular phylogenetic analysis of different *Fusarium* isolates was performed by the use of 18s rRNA gene sequencing and subsequent sequence analysis. The small subunit of rRNA (18S) was amplified using conventional PCR and sequenced to obtain the partial nucleotide sequences characteristic of the three *Fusarium* isolates. Sequence homology-based analysis of the three isolates with published reference sequences on GenBank Database using BLASTn tool confirmed that the three fungal isolates belong to the genus, *Fusarium* with greatest homology to *F. oxysporum*, *Fusarium* sp. and *F. proliferatum* published reference

Table 1
Morphological characters of isolated fungi on Czapeks (Dox) plates and results of wet mount microscopic examination.

Probable organism	Colony morphology	Morphological growth		
		Mycelium	Spores	Conidiophores/Sterigmata
Fusarium	White then changing to pink color	White/clear less	Spherical	Conidiophores may be single or branched with conidia
Alternaria	Darker color black	Darker black color		Conidiophores Pale brown to olive brown Straight or flexuous

Table 2
Determination of different mycotoxins contaminated the grain and seeds.

NO	Sample code	Fusarium Toxin				Alternaria Toxin
		Zeara*	Fum*	DON	T-2	Alternariol
1	Indian rice – Kuwaiti Company for Supply (2013)	478.9	670.4	168.9	670.5	478.9
2	Indian rice – Kuwaiti Company for Supply (2014)	498.2	1473.8	489.3	683.9	172.5
3	Rice Shalane – (2011)	702.9	2076.5	689.4	963.6	244.3
4	Rice Shalane – (2012)	434.0	1284.0	426.3	595.9	149.6
5	Corn Yemeni White	382.0	1130.1	375.2	524.4	133.8
6	Indian corn	693.2	2044.9	678.9	949.0	238.6
7	Habash Indian 2014	588.3	1740.4	577.8	810.6	204.4
8	Rice Almayor – Pakistan	391.2	1157.2	384.2	537.0	136.9

sequences. Therefore, the three studied isolates were designated as *F. oxysporum* IS1, *Fusarium* sp. IS2 and *F. proliferatum* IS3 based on their highest similarity to the reference sequences inferred by multiple sequence alignment and phylogenetic analysis. The multiple sequence alignment was constructed using Clustal W software between the three studied isolates and reference published strains. The multiple sequence alignment revealed regions with same conserved sequences as well as variation sites among all aligned 18s rRNA sequences belonging to *Fusarium*. Phylogenetic analysis was performed by construction of phylogenetic tree using a neighbor joining method to unravel the relationships among all target fungal isolates. The phylogenetic tree (Fig. 1(consisted of two large clusters with five sub-clusters in which two targeted *Fusarium* isolates (*F. oxysporum* ISI and *Fusarium* sp. IS2) were categorized in the same cluster along with the reference *Fusarium* sp. KU382625.1

and *F. oxysporum* KY073256.1. In addition, *F. proliferatum*IS3 was separated along with the highly close reference isolate, *F. proliferatum* KU204757.1 as well as other *Fusarium* species such as *Fusarium* sp. KT996077.1 in a single cluster. Lastly, the close relatedness phylogenetically of the 18s rRNA gene in the three *Fusarium* isolates along with reference published strains helped assigning them into the proper taxa and obtain some inferences about their phylogenetic characteristics.

4. Molecular characterization of *Alternaria* isolates

The molecular identity and phylogenetic criteria of *Alternaria* isolates were determined using 18s rRNA gene sequencing and sequence analysis. The small subunit of rRNA (18S) was amplified and sequenced to yield a partial nucleotide sequences for the two
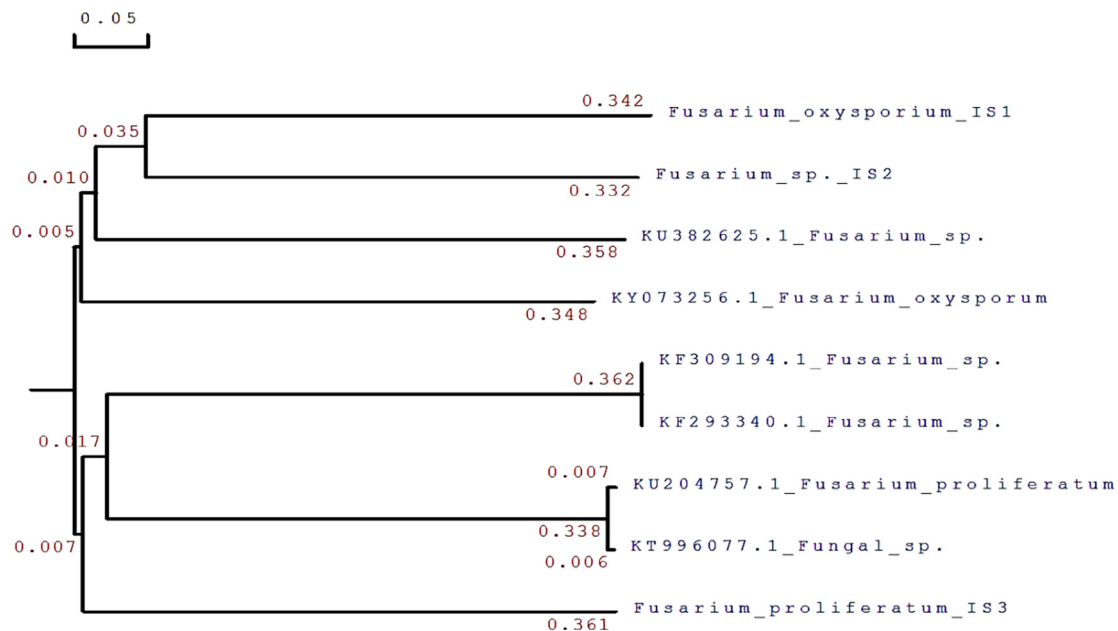

Fig. 1. Phylogeny of the three studied *Fusarium* isolates (*Fusarium oxysporum* IS1, *Fusarium* sp. IS2 *and Fusarium proliferatum* IS3) as compared to reference published strains on GenBank. Minimum bootstrap frequency used during analysis was 1000.
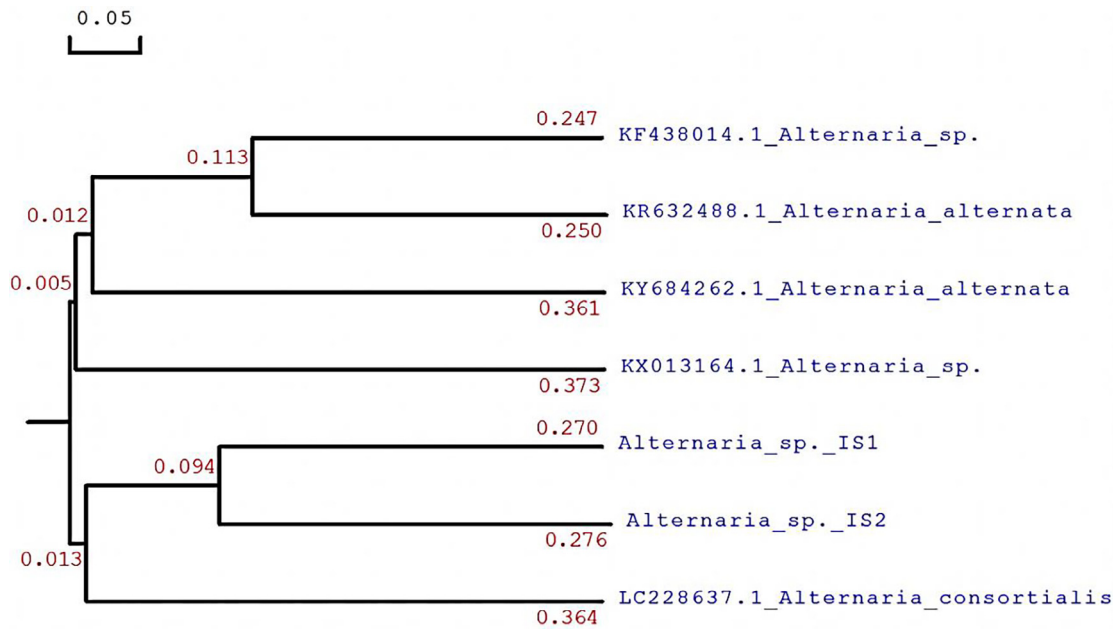
Fig. 2. Phylogeny of the two studied *Alternaria* isolates (*Alternaria* sp. IS1 and *Alternaria* sp. IS2) as compared to reference published strains on GenBank. Minimum bootstrap frequency used during analysis was 1000.

Alternaria isolates. Sequence homology analysis of the two isolates with published reference sequences on GenBank Database using BLASTn tool indicated that the two fungal isolates belong to the genus, *Alternaria* with greatest homology to *Alternaria alternate*, *A. consortialis* and *Alternaria* sp. published sequences. Therefore, the two studied isolates were designated as *Alternaria* sp. IS1 and *Alternaria* sp. IS2. A multiple sequence alignment was constructed using Clustal W software between the two studied isolates and reference strains. The multiple sequence alignment showed the conserved regions in all sequences as well as distinguished the variable positions among the aligned sequences. Phylogenetic analysis was performed by construction of phylogenetic tree using a neighbor joining method to resolve the relationships among all target fungal isolates (Fig. 2). The phylogenetic tree displayed three clusters in which *Alternaria* sp. IS1 and *Alternaria* sp. IS2 were separated in the same cluster which emphasize their close relatedness phylogenetically along with *A. consortialis* (accession number; LC228637.1). In addition, the target *Alternaria* isolates showed high analogous similarity to different *Alternaria* species such as *A. alternata* (accession numbers; KR632488 and KY013164) and other *Alternaria* sp. (accession number; KF438014.1 and KR632488.1), respectively. Thus, the molecular characterization based on sequence homology of the 18s rRNA gene affirmed the phylogeny and identity of the studied two *Alternaria* isolates.

Because of its higher degree of variation than other genic regions of rDNA (for small- and large-subunit rRNA), variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions. In addition to the standard ITS1 + ITS4 primers (White et al., 1990) used by most studies, several taxon specific primers have been described that allow selective amplification of fungal sequences (Gardes and Bruns, 1993). There are some indications suggesting that ITS sequences are conserved irrespective of the life history evolution in plants. First confirmation was reported on marine green algae, *Monostromala tissimum* (Bast et al., 2009) in which ITS sequence data were identical between sexual (heteromorphic alternation) and asexual (no alternation) strains.

This work led to isolation and molecular identification of mycotoxins producing *Fusarium* and *Alternaria* species from grains collected from different stores in Saudia Arabia which triggers an interest in developing rapid detection and intervention methods for these toxigenic fungi.

Competing interests

Funding

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