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Mycotoxin-Producing Fungal Species Recovered from Melon Seed Marketed in Nigeria

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ABSTRACT

Agricultural products may be contaminated by mycotoxin-producing pathogenic fungi while on the field or during harvesting and storage. A field survey was conducted in order to isolate and identify the mycotoxin-producing fungal strains in melon seeds collected from major markets in eight States in Nigeria. Samples per site were pooled, processed and screened for the presence of mycotoxin-producing fungi by culture technique and Internal transcribed spacer sequencing (ITS). Mycotoxin production was determined using Thin-Layer Chromatography (TLC) with a scanning densitometer. Eighteen producing fungi species: *Macrophomina phaseolina*, *Aspergillus foetidus*, *Aspergillus flavipes*, *Rhizopus oryzae*, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium aurantiocandidum*, *Aspergillus awamori*, *Neurospora crassa*, *Aspergillus fischeri*, *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus oryzae*, *Lichtheimia hyalospora*, *Aspergillus terreus*, *Rhizopus oryzae*, *Aspergillus welwitschiae*, *Aspergillus corrugatu*s were identified. These sequences with accession numbers between MN423295-MN423331 have been deposited at the National Center for Biotechnology Information (NCBI) database. The genera *Macrophomina* and were the most dominant. The identified fungi have been associated with production of mycotoxins that could exert toxic effects on animals and humans. The results suggest that strict measure and safe hygienic practices should be adopted to reduce the risks associated with consumption of contaminated melon seed and its final products.

INTRODUCTION

Melon seeds (*Colocynthis citrullus* L. 'Egusi', *Citrullus vulgaris* 'Ahu-elu' and *Citrullus lanatus* (Thumb) Matsun 'Egusi kirikiri') are frequently used as condiment in making soups in Nigeria (Chiejina, 2006). Due to its popularity in large scale catering in the country, efforts have been made to store them for sale in times of scarcity (Bankole, 1993; Chiejina, 2006). It is an important food crop in many sub-Saharan African countries. The seeds are rich in oils, which can be extracted for cooking purposes, and the seeds can also be ground into a powder and used as a soup thickener or flavouring agent (Van der Vossen *et al*., 2004; Ayodele and Salami, 2006; Brisibe *et al*., 2011).

The residue from oil extraction is made into balls that are fried to produce a local snack called 'robo' in Nigeria. With a high moisture content of these seeds, mould invasion becomes life-threatening and the germinability of the seeds is an issue. Melons are usually harvested and kept for about 2weeks to undergo post-harvest ripening during which deterioration by pathogens occur (Snowdow, 1990). Findings have shown that seeds are important disease reservoirs (Neergaard, 1977; Chiejina, 2006) and many fungi are pathogenic to the seeds. In this connection, there is a reduction in the yield in terms of quality and quantity. Seed-borne pathogens have the ability to infect so-called crops raised from such seeds (Surganarayana, 1978). Because of the importance of melon seeds in the diet of Nigerians, this study is, therefore, an avenue to screen for fungal isolates from melon seeds for mycotoxin production in a bid to decrease the high risk associated with consumption of melon seed products in the developing economy of Nigeria.

MATERIALS AND METHODS

Sample Collection for Isolation of Fungi:

For the fungal isolation, one hundred and twenty (120) samples of melon seed were randomly collected from selected major markets in each of the sampling sites from two geopolitical zones (North Central and South West) in Nigeria. The following States were sampled: Abuja, Nasarawa, Kogi, Kwara, Lagos, Ogun, Oyo and Ekiti. Figure 1 shows the map of Nigeria showing the location of the study area and sampling points.

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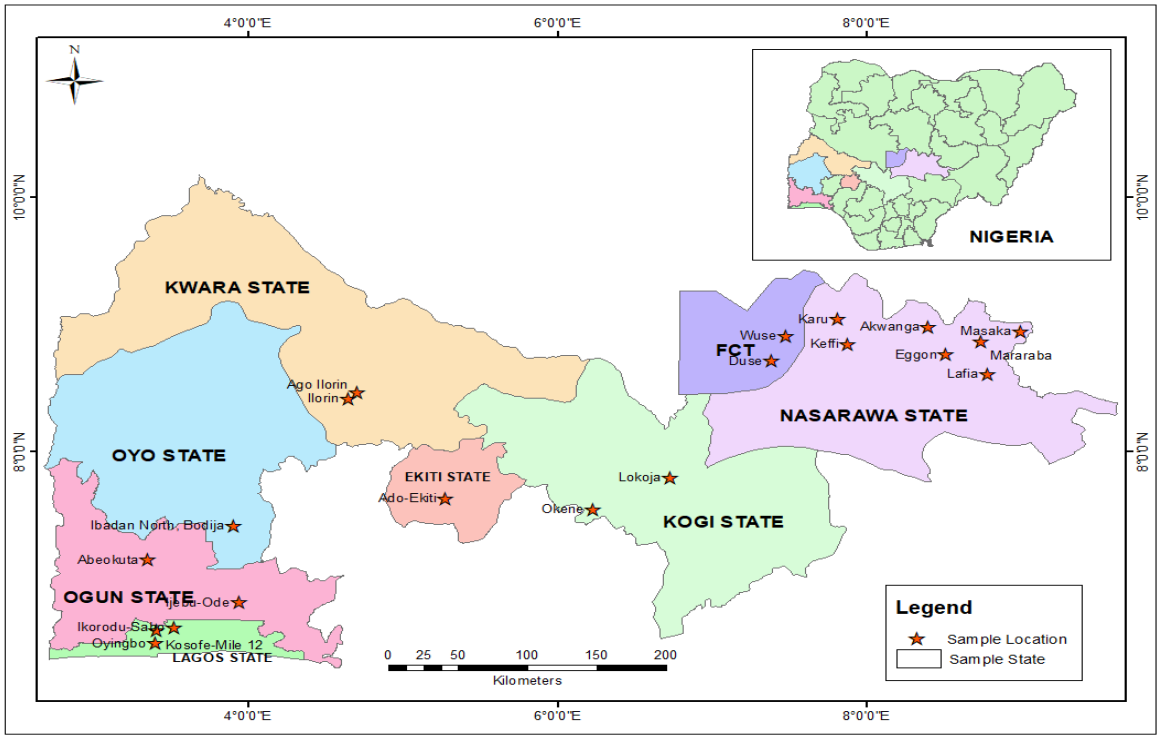

Fig. 1: Map of Nigeria showing the location of the study area and sampling points

Aflatoxin Extraction and Detection of Aflatoxin in Melon Seed Samples:

The melon seed samples were collected from major markets in each of the sampling sites. The melon seed samples were carefully grounded with a commercial waring blender (Warring Commercial, Springfield, MO) and thoroughly mixed. Aflatoxin extraction was done using the modifications of Bankole *et al*. (2004), Countryman *et al*. (2009) and Odoemelam and Osu (2009). Twenty grams (20g) of the grounded sample was weighed out (2 replicates) for extraction purposes. Each

weighed sample was blended with 100 ml of 80 % methanol for three minutes using a waring blender. The blended mixture was poured into a 250 ml Pyrex conical flask and seal flask with Parafilm. Then the sample was shaken using orbit shaker at 4 x 100 rpm for 30 minutes. The filtrate was obtained using No 1 quantitative Whatman filter paper, 185 mm. The mixture was shaken for 30 minutes and filtered using Whatman paper No. 1. The solution was extracted twice; first with 25 ml n-hexane and then 25 ml chloroform. After separation, the chloroform layer which contained the toxin was filtered through anhydrous sodium sulphate into polypropylene cups and allowed to evaporate to dryness. The extracts were dissolved using 1-2 ml of chloroform and spotted with aflatoxin standard (4 µL) on TLC plates (silica gel 60,250 µm) using the capillary tube. The spotted extracts were separated on Thin-Layer Chromatography (TLC) plates (silica gel 60,250 µm) and developed using chloroform, acetone, and isopropanol (90:10:1). The plates were scanned using the densitometer CAMAG TLC Scanner 3 with win CATS 1.4.2 software (Camag AG, Muttenz, Switzerland) to quantify the aflatoxin extracted from the melon kernels (Aquino *et al*., 2005; Suhagia *et al*., 2006; Atehnkeng *et al*., 2008b; Leslie *et al*., 2008).

Isolation of Mycotoxin-Producing Fungi:

To 2g of sample, 10ml of distilled water was added and was well mixed. Out of the sample solution, 1ml was plated out on the surface of the solidified Potato Dextrose Agar and Sabouraud Dextrose Agar (Fluka, Sigma-Aldrich, USA) and incubated at 37 °C for 72-120 h. The microorganisms isolated were sub-cultured by repeated streaking until pure cultures were obtained.

Identification of Mycotoxin Producing Fungi:

The isolates were then identified using appropriate microscopic and macroscopic characteristics and ITS gene sequences and stored as agar slants at 4°C until needed.

Cultural and Morphological Characteristics:

The fresh culture samples were used for the microscopy. The samples were subcultured on a freshly prepared Potato Dextrose Agar and Sabouraud Dextrose Agar plates and further processed by Lactophenol staining. They were examined for spore formation and other characteristics by using a compound microscope (Hitachi S–3500N model, ThermoNaran, Hitachi technologies, Schaumburg, Illinois, USA). The pure cultures of the fungal isolates were identified according to the identification criteria of mycology (Barnett and Hunter, 1972; de Hoog *et al*., 2000).

Purification of Cultures through Single Spore Isolation:

The test organisms were purified through a single spore isolation technique (Samapundo *et al*., 2007). The single conidial isolates were maintained on low nutrient medium for further studies.

Molecular Identification:

Genomic Extraction and ITS Gene Detection:

The genomic DNA of the strains was extracted and purified following a standard protocol for fungal genomic DNA preparations using Quick DNA™ fungi/bacterial miniprep kit D6005 (USA). The ITS gene was amplified by Polymerase Chain Reaction (PCR) (94 °C for 5 min, 30 cycles consisting of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 45 s followed by a terminal incubation at 72 °C for 7 min) using universal ITS-4 (5'TCCTCCGCTTATTGATATGS3') and ITS-5 (5'GGAAGTAAAAGTCGTAACAAGG3') primers. The PCR product purification of isolates was carried by adding 2.5µL of PCR cocktail mix to 10.0µL of the PCR product, mixed well and incubated at room temperature for 30 min. The reaction was stopped by heating the mixture at 94 °C for 5 min. The PCR amplified product was purified and the nucleotide sequence was determined with an automated sequencing apparatus (3130XL, Applied Biosystems). The ITS sequences of the strains were

searched for homology with the sequences in public databases using the BLAST search program (http://www.ncbi.nlm.nih.gov/) to find closely related fungal ITS gene sequences. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura *et al*., 2007). The neighbor-Joining evolutionary model was used to construct the phylogenetic tree.

Agarose Gel Electrophoresis of DNA Fragments:

The PCR amplified DNA segments were separated by electrophoresis on a 1.5 % agarose gel stained with ethidium bromide, using 100 bp DNA marker (Promega, USA) as DNA standard, Millipore water (blank) was used as a negative control. The gel was run for 80 min at 100 V, and the amplified products were observed and imaged by Kodak fluorescent imaging equipment, model IS 4000R (Kodak image station, care stream molecular imaging health Inc. Rochester, NY, USA.).

RESULTS AND DISCUSSION

Aflatoxin Extraction and Detection in Melon Seed Samples:

The results of aflatoxin concentrations in market melon seed samples were presented in Figure 2. The aflatoxin B1 and B2 concentrations were in the order: Oyo (652 and 116 ppb) > Ekiti (574 and 202 ppb) > Nasarawa (319 and 70 ppb) > Abuja (182 and 80 ppb) > Kogi (144 and 13 ppb) > Ogun and Lagos (0 and 0 ppb). While all samples from respective States recorded 0 ppb for aflatoxin G1 and G2. However, most results of this study were above the international permissible level, and pose a source of concern on the safety level of melon seeds sold in our local markets. Limits vary according to the commodity around the world. For instance, the European Union (EU) sets 2-4µg/kg as standard limit (FAO, 2007), while USA, the U.S. Department of Agriculture and the U.S. Food and Drug Administration (FDA) has established an "actionable" level of 15-20 ppb or 20µg/kg for foods except for milk and a limit of 0.5µg/kg for AFM1 in milk (FAO, 2007). Similarly, Bankole *et al*. (2004) investigated the storage practices and aflatoxin B_1 contamination of 'egusi' melon seeds in Nigeria and they found out that aflatoxin B_1 contamination was detected at levels above 5µg/kg in 35.6% of the forest and 27.4% of savanna samples with mean levels of 13.7 and 12.1µg/kg respectively.

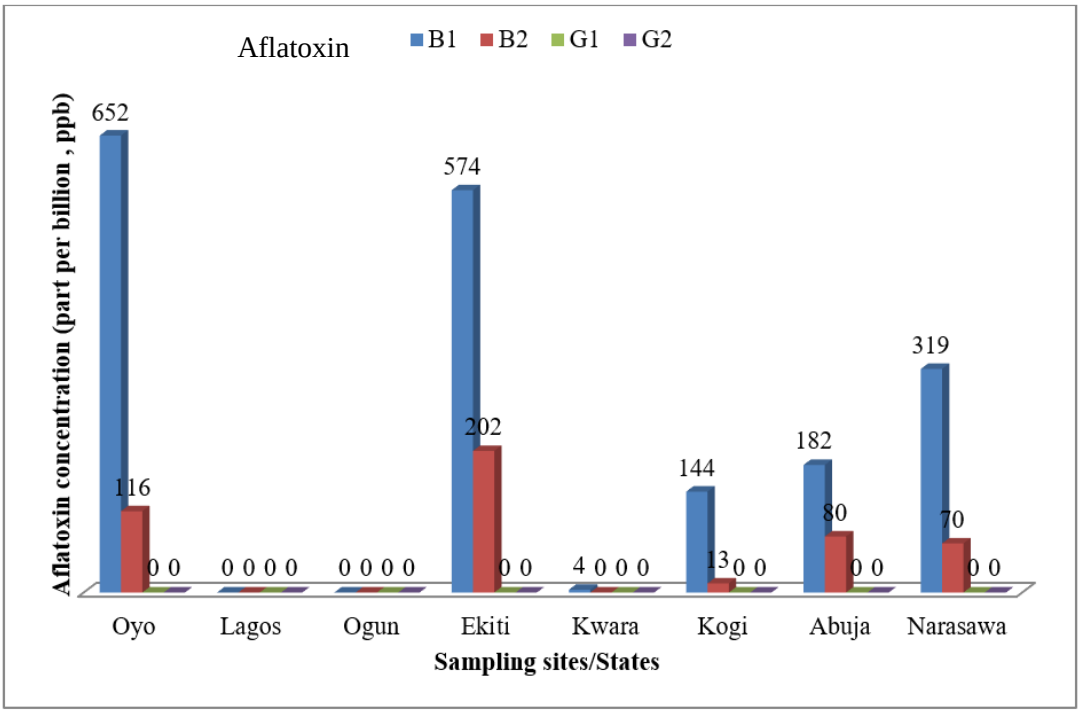

Fig. 2: Quantification of Aflatoxin in the melon seed samples

Isolation and Identification of Mycotoxin-Producing Fungi:

In the present study, eighteen mycotoxin-producing fungal species were isolated from melon samples, which were identified as *Macrophomina phaseolina* strain SKO5 (MN423295), *Aspergillus foetidus* strain SKO9 (MN423298), *Aspergillus flavipes* strain SKO10 (MN423299), *Rhizopus oryzae* strain SKO20 (MN423306), *Aspergillus flavus* strain SKO21 (MN423307), *Aspergillus niger* strain SKO22 (MN423308), *Penicillium aurantiocandidum* strain SKO25 (MN423311), *Aspergillus awamori* strain SKO27 (MN423313), *Neurospora crassa* strain SKO29 (MN423314), *Aspergillus fischeri* strain SKO30 (MN423315), *Aspergillus flavus* strain SKO31 (MN423316), *Aspergillus tamarii* strain SKO32 (MN423317), *Aspergillus oryzae* strain SKO33 (MN423318), *Lichtheimia hyalospora* strain SKO34 (MN423319), *Aspergillus terreus* strain SKO40 (MN423323), *Rhizopus oryzae* strain SKO46 (MN423328), *Aspergillus welwitschiae* strain SKO47 (MN423329), *Aspergillus corrugatu*s strain SKO49 (MN423331) (accession numbers of MN423295-MN423331). In this study, the most predominant strains out of the whole fungi strains were *Rhizopus oryzae* and *Aspergillus flavus* (Table 1 and Fig. 3).

A reliable finding from ITS gene sequence analyses as summarized (Table 1) showed that the ITS genes of the strains had 90-100% similarity to other referenced strains from the GenBank. The phylogenetic tree showed two distinct clusters; strains are closely related by being in the same cluster and having genetic distance (Fig. 4). However, the mycotoxin producers recovered in this study were further stratified into three different subgroups within their common cluster. The sequence analysis of ITS gene and unrooted phylogenetic tree showed that fungal species are likely to have evolved from the same ancestor (Figure 4). The cultural characteristics, as well as the ITS gene sequences, confirmed the association of strains to the genera. The characteristic features of the strains were similar to those reported previously by Alexopoulus *et al*. (1996) and Obani *et al*. (2019). To the best of our knowledge, this is the first report on the identification of tropical toxigenic fungi isolates obtained without any precedent in Nigeria from melon seeds through morphological characters and ITS gene sequence analysis. Oyedele *et al* (2018) obtained one hundred and eight fungal species from contaminated melon seeds marketed in major major markets in Southwest, Nigeria. The same authors identified the fungal isolates as *C lunata*, *A flavus*, *A oryzae*, *C geniculate*, *F equiseti*, *N sitophila*, *L pseudotheobromae* and *P simplicissimum* from the same melon seeds. However, Oyedele *et al*. (2018) and this present study isolated and identified *A. oryzae*, *A. flavus* and *Penicillium* sp. as common fungal species from contaminated melon seeds.

Table 1: Results of sequence comparisons of isolates with those available in the NCBI database

Isolates	Strain	Size of contig sequences (bp)	Accession numbers	Corresponding species in the NCBI database	Identity	Query
Macrophomina phaseolina	SKO5	553	MN423295	NR_160622	98	96
Aspergillus foetidus	SKO9	588	MN423298	NR_077143	96	94
Aspergillus flavipes	SKO10	578	MN423299	NR_135457	92	87
Rhizopus oryzae	SKO20	610	MN423306	NR_103595	98	90
	SKO46	625	MN423328	NR_111041	98	83
Aspergillus flavus	SKO21	595	MN423307	NR_160622	95	93
	SKO31	583	MN423316	NR_111041	96	93
Aspergillus niger	SKO22	586	MN423308	NR_077143	94	94
Penicillium aurantiocandidum	SKO25	568	MN423311	NR_163548	94	93
Aspergillus awamori	SKO27	584	MN423313	NR_077143	90	87
Neurospora crassa	SKO29	580	MN423314	NR_155109	94	95
Aspergillus fischeri	SKO30	573	MN423315	NR_137486	91	84
Aspergillus tamarii	SKO32	588	MN423317	NR_160622	98	96
Aspergillus oryzae	SKO33	555	MN423318	NR_137519	92	79
Lichtheimia hyalospora	SKO34	754	MN423319	NR_111440	97	90
Aspergillus terreus	SKO40	589	MN423323	NR_111440	97	90
Aspergillus welwitschiae	SKO47	593	MN423329	NR_163668	92	94
Aspergillus corrugatus	SKO49	558	MN423331	NR_151791	94	86

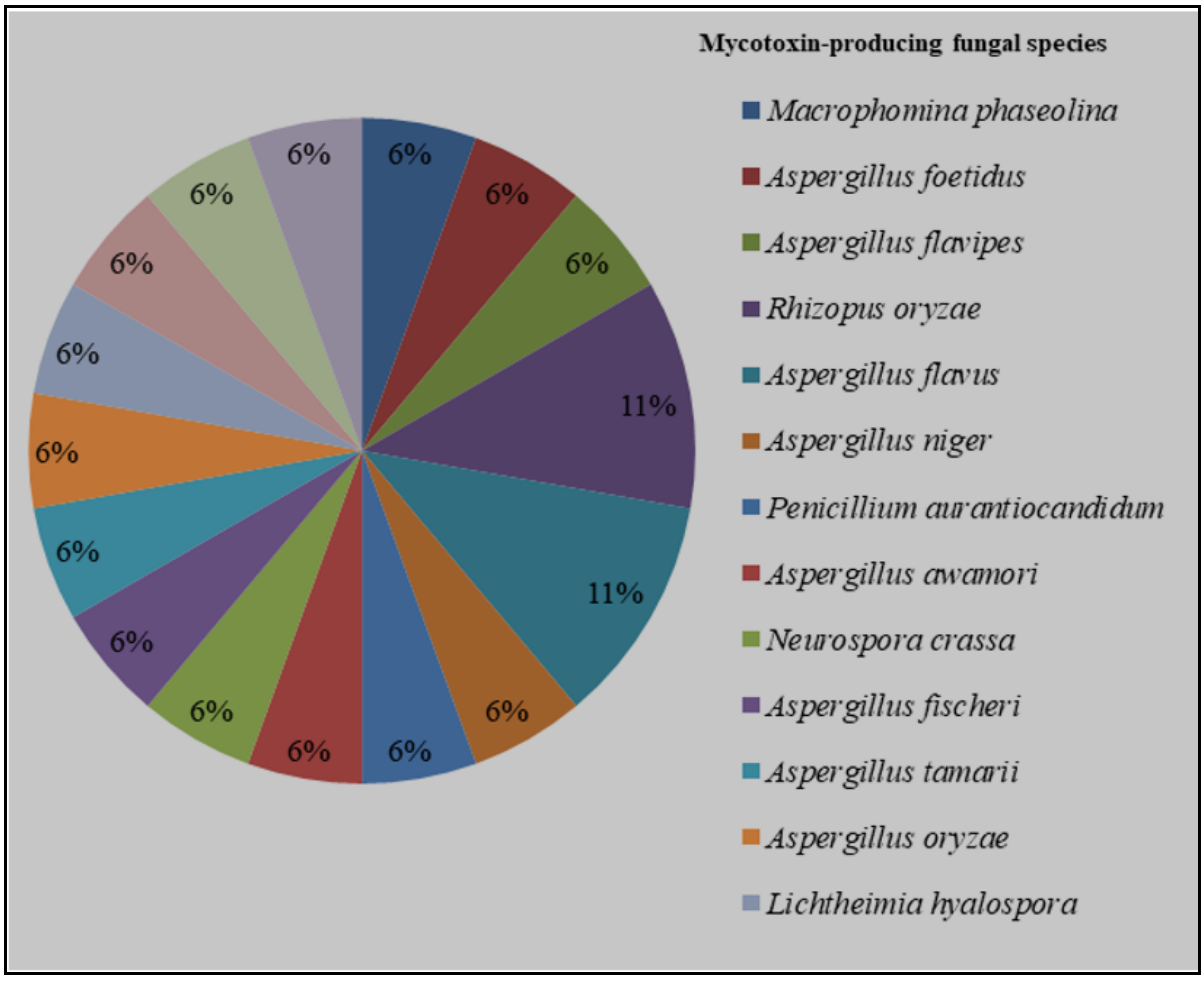

Fig. 3: Prevalence of mycotoxin-producing fungi in melon seed samples

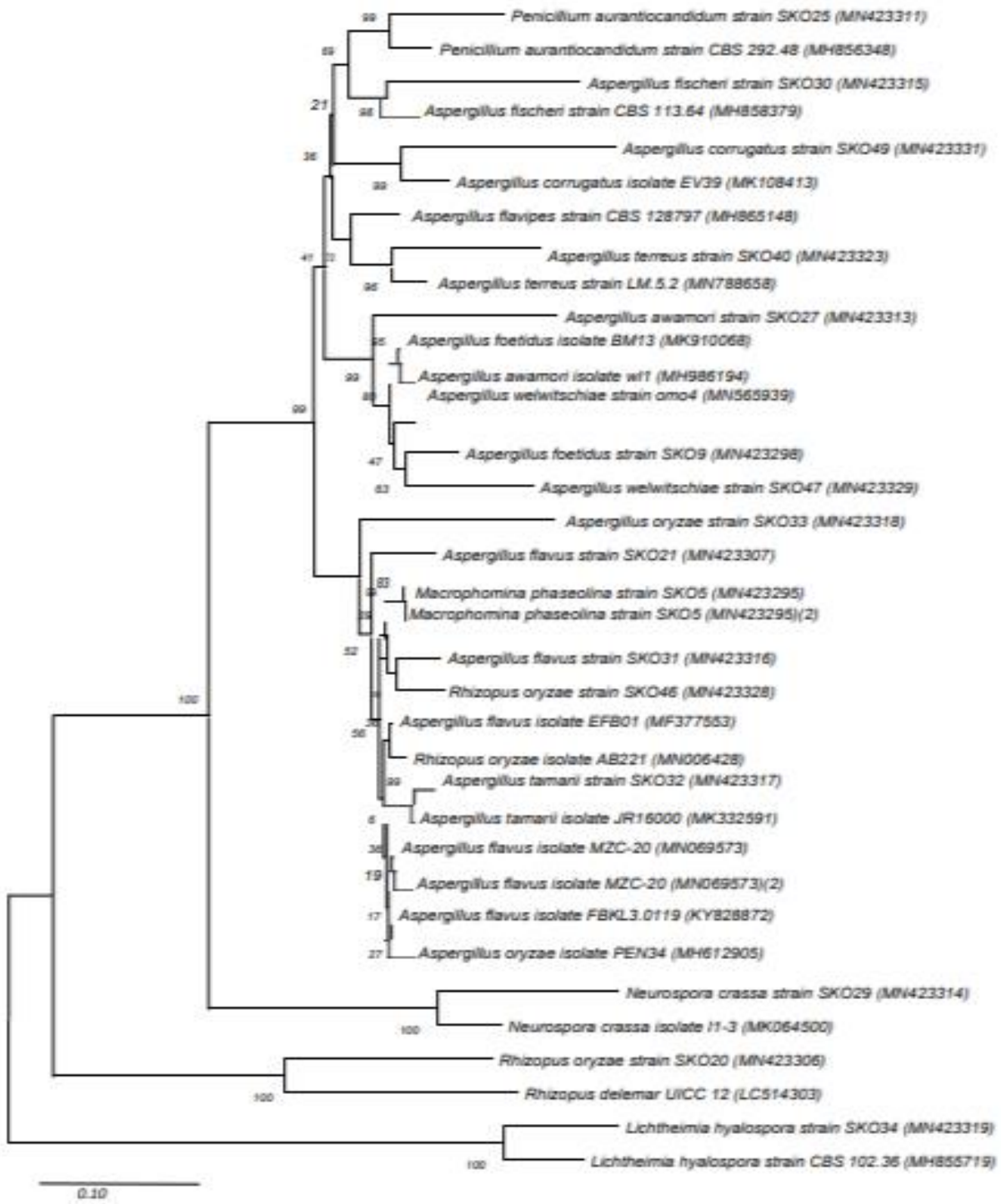

Fig.4: Phylogenetic tree (dendrogram) of mycotoxin-producing fungal species ITS sequences using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap test = 1000 replicates. The evolutionary distances were computed using Tamura-Nei (Saitou and Nei, 1987) parameter method. Analysis involving 40 nucleotide sequences was computed using Mega 5 software.

CONCLUSIONS

The results showed the isolation of mycotoxin-producing fungal species. The combination of morphological identification and molecular approach based on sequencing of rDNA-ITS region of fungi has strengthened our knowledge about fungi microflora associated with melon seed diseases in Nigeria. This study reveals the occurrence of eighteen fungi associated with melon seed diseases. Occurrence of the fungi represents a statement of unhygienic matrix in which the crop is normally stored.

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