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MOLECULAR CHARACTERISATION OF PATHOGENIC FUNGI OF MAIZE GRAINS

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ABSTRACT

Fungi differ from each other phylogenetically, ecologically, metabolically, biochemically, physiologically and morphologically. Despite industrial, medicinal and agricultural importance of fungi, their identification remains daunting for young mycologists. A study was conducted to identify the common fungal pathogens causing post-harvest contamination of stored maize grains purchased from the Rumuokoro daily market in Port Harcourt, Rivers State. Fungal cultures were isolated and morphologically identified using both standard blotter method and Potato Dextrose Agar (PDA) media. The DNA of the most common fungal isolate, coded M-11, was molecularly characterised using Internal Transcribed Spacer 1 (ITS-1) molecular marker. The M-11 isolate DNA sequence was aligned using the Basic Local Alignment Search Tool for nucleotide (BLASTN) 2.8.0 version of the National Centre for Biotechnology Information (NCBI) database. The amplified DNA sequence of the millet isolates yielded 569 base pairs. The results indicated that the M-11 isolate sequence was 89.34% identical to *Aspergillus flavus* var. flavus strain CSS 130026 and 88.11% identical to *Aspergillus flavus* isolate AA 133. The above outcome shows that one of the causal organisms associated with stored maize grains is *Aspergillus flavus.* It is anticipated that these results will improve on the current information available, provide information for developing effective disease control strategy for alleviating the post-harvest losses caused by *Aspergillus flavus* and also provide a foundation for further study of potential mycotoxic effect of consuming diseased maize seeds.

Keywords: Post-harvest Diseases, *Zea mays*, molecular characterisation, sequencing, *Aspergillus flavus*

INTRODUCTION

Maize, *Zea mays (L.)*, is a principal cereal in the world and ranks third in terms of production after wheat and rice (Mahmoodi and Rahimi, 2009). Maize is an important staple food that provides income for farmers and food. It also provides food for one-third of all malnourished children and 900 million poor people worldwide (CGIAR, 2016). According to Gtorni (2009), during storage, several kinds of fungi are associated with maize seeds which either cause their deterioration or simply remain viable to infect germinating seedlings. Fungi play important roles in nature and despite their importance, their identification remains overwhelming. It is necessary for fungi to be properly differentiated using the molecular method of characterisation because according to Pryce (2003), the traditional identification method of fungi is a cumbersome method that takes time and may lack specificity. The molecular method of identification helps researchers to identify other closely related species and it is rapid and accurate. It is important in the treatment of plant diseases as it promises better and faster laboratory diagnosis

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compared with the traditional method. Therefore, this study was aimed at identifying the fungal pathogens associated with the post-harvest diseases of stored maize grains using macro- and micro-morphological identification and authenticating the identification using molecular characterisation technique.

MATERIALS AND METHODS

Study Area

This study was carried out at the Mycology/Pathology Laboratory of the Department of Plant Science and Biotechnology, University of Port Harcourt, Choba, Nigeria. It is located on latitude 40 35″ 14″ N through 40 54″42″ N and longitude 60 54″ 00″E through 60 55″ 50″E. The DNA of the organism was extracted at Regional Centre for Biotechnology and Biofuel Research Laboratory, located at the University of Port Harcourt. Purification and sequencing of the PCR products were carried out at the International Institute of Tropical Agriculture (IITA), Ibadan. Maize grains were obtained in May 2019 from Rumuokoro Daily Market in Obio/Akpor Local Government Area in Rivers State.

Isolation of Fungi

Standard Blotter Method recommended by International Seed Health Testing Association (ISTA, 2016) was used to isolate fungal pathogens associated with stored maize. Petri dishes were lined with 3 layers of Sterile Whatman's 9 cm filter papers and moistened with sterile distilled water. The stored maize grains used were sorted to remove diseased ones, then soaked in 70% ethanol for 2-3 minutes and rinsed twice in sterilized distilled water, after which 10 seeds were placed in the Petri dishes equidistantly and incubated at 25$\pm$ 2^{o}C for 3-7 days at the Pathology/Mycology laboratory of the Department of Plant Science, University of Port Harcourt, Choba, Rivers State, Nigeria. The most common maize isolate was coded M-11.

Morphological and Microscopic Characterisation and Identification

The fungal mycelium of the isolate M-11 was cultured on Potato Dextrose Agar medium at room temperature for 7 days to obtain pure culture using the modified method described by Ikechi–Nwogu and Elenwo (2012). The fungi found growing on the maize grains were physically identified using CBS Laboratory Manual Series Food and Indoor Fungi by Samson *et al*. (2010) and Descriptions of Medical Fungi by Kidd *et al.* (2016). The morphological identification of isolate M-11 was conducted by visually observing the mycelium and comparing it with the pictorial guide by Snowdon (1990). Colonies were compared for their diameters, overall colour of conidia, reverse colour, texture, zonation and sporulation. The isolate was also subjected to microscopic analysis for its characterisation and identification using an electron binocular microscope at X40.

Molecular Characterisation Using the Internal Transcribed Spacer (ITS) Marker and Identification

The Genomic DNA of the isolate M-11 was extracted following the protocol of Quick-DNA^{TM}Fungal/Bacterial MiniPrepKit (Zymo Research Group, California, USA) as described by the manufacturer, with modifications at the Regional Centre for Biotechnology and Bioresources (RCBB), University of Port Harcourt, Rivers State, Nigeria. The M-11 isolate DNA quantity and concentration were measured using the Nanodrop 2000c

spectrophotometer (Thermo fisher Scientific Inc. Wilmington, Delaware, USA). The DNA purity was measured as the ratio of absorbance at 280 nm to that of 260 nm. The quality of the DNA of the isolate M-11 was further quantified using the Agarose gel electrophoresis, performed according to the modified method of Saghai-Maroof *et al.* (1984). The DNA sample of the M-11 isolate was shipped to the International Institute of Tropical Agriculture (IITA) Bioscience Centre, Ibadan, Nigeria for amplification and sequencing. The primers used to amplify fragments of the nuclear ribosomal DNA (rDNA) of the M-11 isolate were the Internal Transcribed Spacer 4 (ITS4) with the sequence TCCTCCGCTTATTGATATGS and ITS5 with the sequence GGAAGTAAAAGTCGTAACAAGG. The amplicons were sequenced using the ABI 3500 capillary electrophoresis sequencer. The DNA sequence file was saved in the Bioedit file with extension .ab1. The sequence was analysed using the Molecular Evolutionary Genetic
Analysis (MEGA) version 7.0.26 software, and aligned using the Basic Local Alignment Search Tool for nucleotide (BLASTN) 2.8.0 version of the National Centre for Biotechnology Information (NCBI) database.

RESULTS

Isolation, Morphological and Microscopic Description of Fungi Associated with Maize

One unidentified fungus coded M-11 found to be associated with maize was isolated from the stored maize grain as shown in Plate 1. Characteristically, the isolate was distinguished by rapid growth and a greyish green to olive yellow conidial colour, which turned green with age. The conidia were variable in shape and size, had somewhat thin walls and ranged from smooth to moderately rough, the majority being finely rough.

Plate 1a: Pure culture of fungus isolated from maize grain on Potato Dextrose Agar

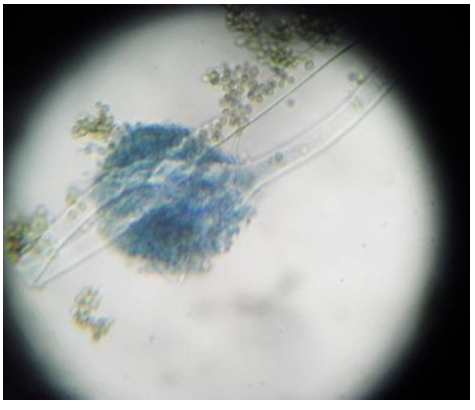


Plate 1b: The microscopic appearance of isolate

Molecular Characterisation Using the Internal Transcribed Spacer (ITS) Marker and Identification

The genomic DNA of the isolate M-11 of maize was successfully extracted. The NanoDrop result showed that the concentration of the DNA of the isolates was 15.3 ng/µl, while the absorption peak of the 260 nm/280 nm readings was 1.97 and the 260 nm/230 nm reading was 1.74. DNA bands were observed on gel when viewed under UV light as presented in Figure 1. The DNA of the fungal isolates was successfully amplified to yield amplicons of size over 569 base pairs.

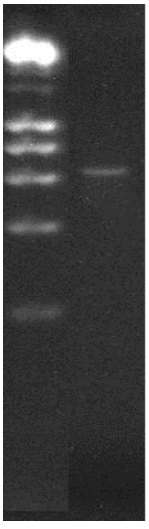


Figure 1**:** PCR products generated from fungal DNA isolates

The alignment results (BLAST) are presented in Figure 2. Figure 2 shows the alignment scores presented as red lines. The scores of the alignments of all aligned sequences were below 200.

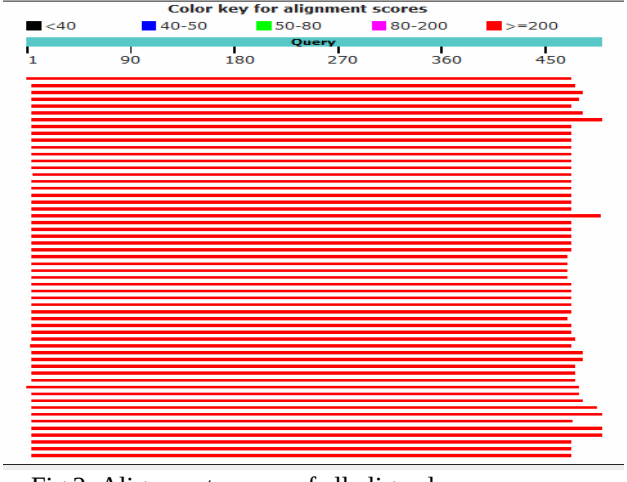
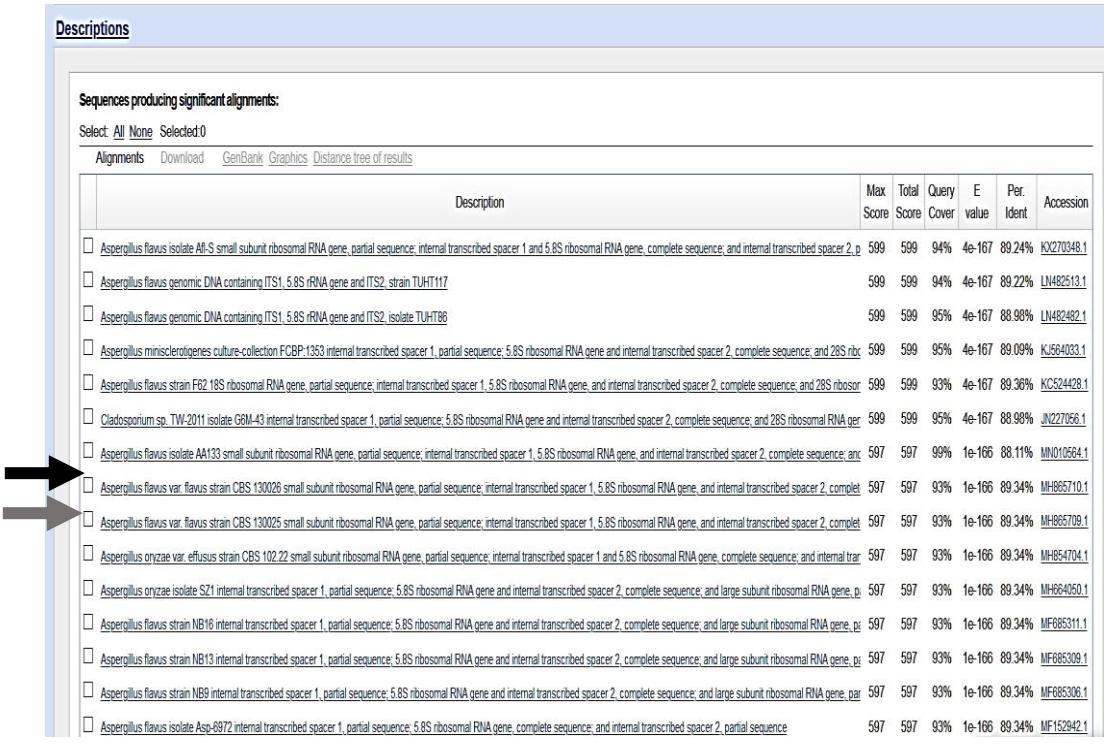


Fig 2: Alignment scores of all aligned sequences

The sequencing result after blasting revealed the species identity of the fungal isolates to be *Aspergillus flavus.* The results indicated that the M-11 isolate sequence was 89.34%, identical to *Aspergillus flavus* var. flavus strain CSS 130026 (grey arrow) because of the query cover of 99% and 88.11% identical to *Aspergillus flavus* isolate AA 133 (black arrow) as shown in Fig. 3 below.



Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Aspergillus flavus isolate Afl-S small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, p	599	599	94%	4e-167	89.24%	KX270348.1
Aspergillus flavus genomic DNA containing ITS1, 5.8S rRNA gene and ITS2, strain TUHT117	599	599	94%	4e-167	89.22%	LN482513.1
Aspergillus flavus genomic DNA containing ITS1, 5.8S rRNA gene and ITS2, isolate TUHT86	599	599	95%	4e-167	88.98%	LN482482.1
Aspergillus minisclerotigenes culture-collection FCBP:1353 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribo	599	599	95%	4e-167	89.09%	KJ564033.1
Aspergillus flavus strain F62 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosor	599	599	93%	4e-167	89.36%	KC524428.1
Cladosporium sp. TW-2011 isolate G6M-43 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA ger	599	599	95%	4e-167	88.98%	JN227056.1
Aspergillus flavus isolate AA133 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; anc	597	597	99%	1e-166	88.11%	MN010564.1
Aspergillus flavus var. flavus strain CBS 130026 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complet	597	597	93%	1e-166	89.34%	MH865710.1
Aspergillus flavus var. flavus strain CBS 130025 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complet	597	597	93%	1e-166	89.34%	MH865709.1
Aspergillus oryzae var. effusus strain CBS 102.22 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal trar	597	597	93%	1e-166	89.34%	MH854704.1
Aspergillus oryzae isolate SZ1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, p	597	597	93%	1e-166	89.34%	MH664050.1
Aspergillus flavus strain NB16 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, pa	597	597	93%	1e-166	89.34%	MF686311.1
Aspergillus flavus strain NB13 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, pa	597	597	93%	1e-166	89.34%	MF686309.1
Aspergillus flavus strain NB9 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, par	597	597	93%	1e-166	89.34%	MF686306.1
Aspergillus flavus isolate Asp-6972 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	597	597	93%	1e-166	89.34%	MF152942.1

Fig 3: The Sequence Alignments of M-11 isolate Sequence with NCBI Database Sequences

DISCUSSION

Traditionally, mycologists use morphology as the only means of fungal species identification to understand the evolution of morphological characters (Huzefa *et al.,* 2017). Although morphological approaches are routinely used in fungal taxonomic studies for classification of fungi, it may not always perform well for lower-level (species) classifications (Huzefa *et al.,* 2017). Again, identifying fungi based on morphology alone can be challenging particularly when non-experts are dealing with cultures of fungi, since there are inadequate number of morphological characters that can be used for identification. As a result, DNA sequence-based methods have emerged for classifying species within the mega diverse fungi (Huzefa *et al.,* 2017).

In this study, the DNA sequence alignment result indicated that the M-11 isolate sequence was 89.34% identical to *Aspergillus flavus* var. flavus strain CSS 130026 because of the query cover of 99% and 88.11% identical to *Aspergillus flavus* isolate AA 133.

It is evident that *Aspergillus flavus* was identified as the fungal pathogen associated with stored maize grains. According to Jackson and Dobson (2011), this pathogen is the most reported food-borne fungus and a dominant species found on stored products, particularly grains. Diener *et al.* (1987) reported that *Aspergillus flavus* is a facultative, parasitic pathogen of plants that has the ability to inhabit a number of crops such as corn, cotton, peanut and others. Dominant fungal species such as *Alternaria alternate*, *Aspergillus flavus*, *Curvularia lunata* and *Fusarium moniliforme* have caused diverse diseases which include rotting, leaf spotting, wilting and general spoilage in maize and other grains such as sorghum, rice and wheat (Jonar *et al.*, 2011).

Economic losses due to the infection of maize grain by *A. flavus* is primarily due to the contamination of the grain with the fungal aflatoxin. *Aspergillus flavus* is the main producer of carcinogenic aflatoxins in maize. Aflatoxins have a negative economic impact due to loss of crop value and is also associated with numerous diseases and disorders in humans and livestocks (Okoth *et al.*, 2018). *A. flavus* causes deterioration of stored wheat, corn, rice, barley, flour and soybeans.

In general, it does not invade the seeds of living wheat or intact peanuts but growth occurs primarily when products are stored under conditions of relatively low moisture. It has been reported that aflatoxins cause an annual loss of $163 million to maize producers in the United States (Wu, 2015). Ayeni (2015) reported that ingesting aflatoxin-contaminated foods is a chief cause of the fifth most common cancer (liver cancer) globally. There is, therefore, need to limit the consumption of *A. flavus*-contaminated grains.

CONCLUSION

The use of molecular techniques is very essential, as it aids to identify disease-causing organisms and provides information for developing effective disease-control strategies, which can alleviate the post-harvest losses caused by *Aspergillus flavus.* This can improve the productivity and availability of safe maize grains all over the world.

RECOMMENDATION

It is recommended that maize grains be ware-housed under strict hygienic conditions to avoid microbial contamination which may cause diseases and allergic reactions.

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