P71: MOLECULAR IDENTIFICATION AND AFLATOXIN SCREENING OF *ASPERGILLUS* ISOLATED FROM PHILIPPINE DRIED FISH PRODUCTS

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Abstract – Mycotoxin contamination due to toxigenic fungi occurs frequently in various food commodities. These can have serious human and animal health risks. In the Philippines, dried fish is widely distributed and are considered as an important commodity due to its availability in the market, ease of processing and cheapness. Testing of these market food products ensures food and consumers safety. This study therefore evaluated the occurrence of aflatoxin-producing *Aspergillus* from Philippine dried fish products found on its local markets. Thirty-one samples of eleven types of dried fish products from nine local markets were collected for the isolation of toxigenic fungi. A total of 115 *Aspergillus* isolates were recorded from all dried fish products. Morphological characterization grouped the isolates into nine morphospecies. Identification of these fungi using morphocultural characterization and sequence analysis of the ITS genes confirmed its identities as *Aspergillus alliaceus, Aspergillus clavatus, Aspergillus flavus, Aspergillus niger, Aspergillus ochraceous, Aspergillus oryzae, Aspergillus steynii, Aspergillus tamarii var. 1, and Aspergillus tamarii var. 2.* The nine morphosphecies were cultured and mass produced on Malt Extract Broth for 14 days for the detection of aflatoxin using TLC and HPLC. Aflatoxin was initially observed only in *A. oryzae* crude culture extracts with TLC, but absence was not confirmed by the HPLC analysis.

Keywords: *aflatoxin, Aspergillus, dried fish products, mycotoxin*

1. INTRODUCTION

The Philippines ranked as 8th among the top fish-producing countries in Asia in 2014 with a recorded total production of 4.7 million metric tons of fish including crustaceans, mollusks, and aquatic plants [1]. The Philippines is also one of the highest per capita in fish consumption in the world at 39.8 kg per year of fish and fish products. For the dried fishes, an average of 9.9 g per day is consumed by an ordinary Filipino [2]. Thus, dried fish products are among the most common food commodities that most of the market goers would often purchase in the country [3].

However, in spite of their dry nature, dried fish products could also be contaminated with microorganisms. The usage of poor quality raw materials, poor handling and storage, and inability to meet required parameters for product analysis could be grounds in considering the product unsafe. Knowing that these products are readily available in the market and could be sold even with minimal processing, this may entail consumer health risks [4].

Molds are widely distributed in different types of environment and have the capability to adapt and grow in many diverse substrates, especially in food [5]. Another problem associated with the growth of fungi in food is the contamination of mycotoxins. These mycotoxins are not easily degraded by conventional method of food processing. Thus, mycotoxins present food safety challenges to the farm-to-table food range [6].

2. EXPERIMENTAL

Collection of Dried Fish Products. Approximately 250 grams of different dried fish products were purchase from the nine local markets in Luzon, Philippine. A total of 31 dried fish samples were collected in this study.

Isolation of *Aspergillus* from Dried Fish Products. Ten grams of dried fish products were selected from the bulk samples. To avoid bias, the samples were initially grouped into three sub-samples. Then, representative samples from each sub-sample were chosen randomly to comprise the composite samples. The dried fish products were cut into 20 mm size and placed in a sterile container. Half-strength Malt Extract Agar (MEA) was prepared and ssupplemented with antibiotic.

All culture plates were incubated at room temperature for 3–7 days. *Aspergillus* growing out of the food samples were re-isolated.

Molecular Identification of *Aspergillus*. *Aspergillus* isolates were sent to MACROGEN, Korea for DNA extraction, PCR amplification, purification and sequencing. The primer pair used was for the ITS gene: ITS-5 (5'-GGAAGTAAAAGTCGTAACAAGG -3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3'). After the DNA Sequences were gathered, the forward and reverse sequences were checked and edited using the software BioEdit Sequence Alignment Editor. The combined sequences were uploaded to the BLAST search engine (https://blast.ncbi.nlm.nih.gov/) where sequences of the related taxonomic genera were downloaded. The published sequences, along with the samples' sequences, were aligned and edited using BioEdit via the accessory application ClustalW multiple alignment. Lastly, the phylogenetic trees were constructed using MEGA Software based on a maximum likelihood analysis. The identities of the isolates were revealed based on the bootstrap values of the isolates to the related sequences downloaded from the Genbank.

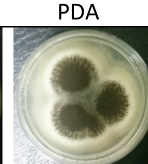
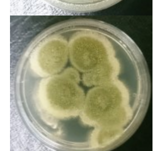
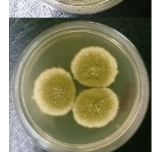
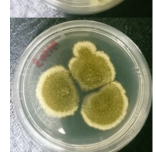
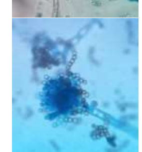
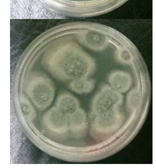
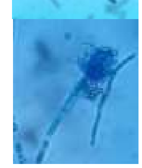
Assessment of Aflatoxin by *Aspergillus* on Culture Medium. The extraction and estimation of aflatoxins from the fungal cultures were based on the procedure of Shantha *et al.* (1990) and Josyn & Lahai (1992). Fungal isolates were initially mass produced by transferring an agar block cut from the margin of 5- to 7-day old fungal colony onto glass bottles containing 200 mL of Malt Extract Broth (MEB). The culture flasks were maintained under stationary condition at room temperature for 14 days. Following culture, the culture medium was filtered with cotton wefts and the mycelium was crushed with the stirring rod and immersed with EtOAc. Filtrate of the culture medium and the mycelium was collected in a sterile bottle and was extracted three times with EtOAc at ratio of 1:1 (v/v). The organic phase was transferred together and the supernatant, approximately 200 mL, was evaporated at 40°C, 121 rpm by a rotary evaporator. The extracts were then transferred in a clean vial and stored at 27°C before TLC and HPLC analysis.

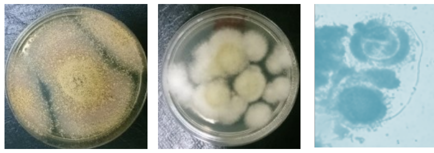
3. RESULTS AND DISCUSSION

Molds are widely distributed in different kinds of environment and has the ability to adapt and grow in many different substrates including foods. When molds grow on food, they produce enzymes for spoilage and mycotoxins that if inhaled or ingested are toxic to both humans and animals [7]. Among the existing fungal toxins, the common and considered to be the most important are the aflatoxins. These compounds are products of the species belonging to the genus *Aspergillus* [8].

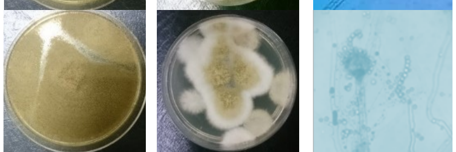
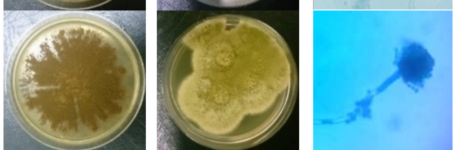
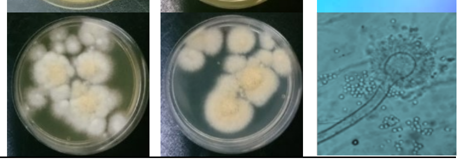
In this study, a total of 115 *Aspergillus* strains were recorded from 31 dried fish samples collected from nine different local markets in Luzon, Philippines. The colony descriptions of the *Aspergillus* isolates were observed based on its growth on MEA and PDA and the fungi were grouped into nine morphospecies (Table 1). The nine *Apergillus* morphospecies, previously identified based on colony descriptions and spore morphologies were subjected to a molecular characterization by sequencing their ITS genes. Results of the molecular analysis were in agreement with the data obtained from the morphological identification, thereby validating the identity of the *Aspergillus* isolates. However, one morphospecies, MS_01, identified as *A. niger,* has not been able to be amplified due to the multiple variation in its DNA sequences, and hence, its identity was based solely on their morphocultural characters.

Table 1. Morphocultural characters of nine morphospecies of *Aspergillus*.

Morpho-species	Growth on MEA	Growth on PDA	Slide Culture
MS_01 (*A.niger*)			
MS_02 (*A.oryzae*)			
MS_03 (*A.tamarii*)			
MS_04 (*A.clavatus*)			

MS_05 (*A.steynii*)	

Continuation Table 1.

MS_06 (*A.flavus*)	
MS_07 (*A.alliaceus*)	
MS_08 (*A.tamarii*)	
MS_09 (*A.ochra-ceus*)	

Comparison of the sequences by BLAST search showed high percent similarities between the sequences of the isolates and those deposited in the GenBank. Homology was supported by the phylogenetic tree constructed based on maximum likelihood with high bootstrap values (Figure 1).

Among the isolated *Aspergillus*, *A. niger* was the most predominant with 54 isolates, followed by *A. oryzae* (18), *A. alliaceus* (11), *A. tamarii* var. 1 (11), and then, by *A. clavatus* (6), and *A. flavus* (6). Interestingly, *A. flavus, A. niger, A. tamarii* and *A. ochraceus* were previously reported from smoked and dried fishes [9, 10, 11, 12, 13]. Salted and sun-dried fish from India were frequently contaminated with *A. flavus, A. niger,* and *A. oryzae* species [14]. *Aspergillus niger* was also the most common species isolated from Malaysian salted dried fish [15]. *Aspergillus flavus* was also isolated in dried salted fish as reported by Andrew & Pitt [16], and Pitt & Hocking [17].

In this study, representative isolates of the nine *Aspergillus* morphospecies were subjected to aflatoxin screening. Following mass production of *Aspergillus* on MEB for 14 days, TLC and HPLC analyses were done on the crude culture extracts. TLC analysis showed the possible presence of aflatoxin only in *A. Oryzae.* The positive result TLC gave was confirming the presence of other member of the coumarin group of compounds where aflatoxin belongs. Other metabolites could have been produced by the *Aspergillus* species in the MEB culture medium. To confirm further the presence of aflatoxin, HPLC was used.

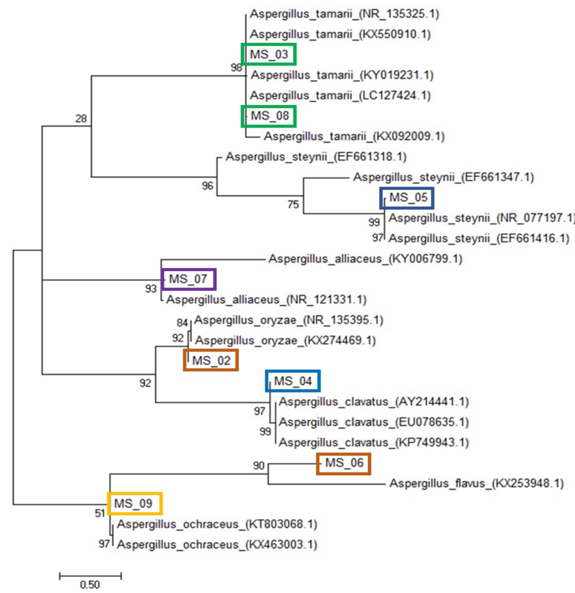


Figure 1. Phylogenetic tree constructed for the isolated *Aspergillus* species. The related taxonomic sequences were downloaded from the Genbank and can be retrieved through the following accession numbers: *A. alliaceus* (NR121331), *A. alliaceus* (KY006799), *A. flavus* (KX253948), *A. tamarii* (NR135325), *A. tamarii* (LC127424), *A. tamarii* (KX550910), *A. tamarii* (KY019231), *A. tamarii* (KX092009), *A. oryzae* (NR135395), *A. oryzae* (KX274469), *A. ochraceus* (KX463003), *A. ochraceus* (KT803068), *A. steynii* (EF661416), *A. steynii* (NR077197), *A. steynii* (EF661347), *A. steynii* (EF661318), *A. clavatus* (KP749943), *A. clavatus* (EU078635), and *A. clavatus* (AY214441).

However,results of the HPLC contradicted the results of TLC. The HPLC chromatograms of the nine crude culture extracts showed absence of any aflatoxins. When the chromatograms of the samples and standard were compared, the presence of aflatoxin in the extracts was not confirmed as none of the extracts matched with the retention time of the standard due to high matrix effect. Aflatoxin could still be existent in the crude extracts but at ppb levels.

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