

Occurrence, Molecular Characterization and Phylogenetic Relationship of *Aspergillus* Species Isolated from Garri Sold in Benue State, North Central, Nigeria

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Abstract

Characterization and identification of molds based on cultural and morphological characteristics are often not reliable and frail with limitations. The occurrence of *Aspergillus* species in garri on sale in markets in Benue State, Nigeria, was studied by molecular techniques. *Aspergillus* species were isolated and purified on Potato Dextrose Agar. DNA from the purified isolates was extracted using the ZR fungal DNA miniprep and amplified by PCR mix made up of 12.5 µL of Taq 2X Master Mix. Primer sequences for the fungi characterization were internal transcribed spacers ITS 4 and ITS 5. The phylogenetic tree was plotted between the isolated organisms and reference sequences and evolutionary analysis was conducted in MEGA X. Result revealed that one thousand, six hundred and forty-six *Aspergilli* were isolated comprising of 980 and 666 isolates from the white and yellow garri respectively. *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. aculeatinus*, and *A. aculeatus* were identified. Twenty percent (20%) of the strains had aflatoxin D structural gene, 50% amplified AFLP and 70% of the strains expressed AFLQ genes needed for the biosynthesis of aflatoxin B1. Majority of the strains that showed the expression of these structural genes were consistent with *Aspergillus flavus*. Phylogenetic tree showed a close relationship among the isolates and their most identical sequence in the NCBI database.

Keywords

Aflatoxin, *Aspergillus* species, Aspergillosis, Garri, Polymerase Chain Reaction (PCR), Benue State

1. Introduction

The genus *Aspergillus* has significant presence in a variety of ecosystems and different substrates such as the soil, textiles, food and feed [1] [2] [3] [4] [5]. *Aspergillus* species cause the disease aspergillosis. The disease caused by these filamentous fungi has been a major threat [6]. The human aspergillosis is caused by fungi of the *Aspergillus* taxonomy. The disease involved a broad range of clinical presentations including the fungus ball of the lung (aspergilloma) [7]. Saprophytic fungi of the *Aspergillus* genus are a common contaminant of food and soil, and their spores are ubiquitous. It is estimated that approximately 10 million patients are considered at risk of developing invasive aspergillosis each year [8]. The most frequent species involved in human disease is *Aspergillus fumigatus*, followed by *Aspergillus flavus* [8].

In addition to the general human aspergillosis, a disease condition produced by molds of the *Aspergillus* genera, another major issue arising from mold contamination of food and feed is aflatoxicosis. Aflatoxicosis is a pathological condition resulting from the consumption of aflatoxin. Aflatoxins are metabolites of some *Aspergillus* species that display acute immune and hepatotoxic effects in humans, and very high carcinogenic potential following chronic exposure [9] [10]. Aflatoxins contaminate food/feed and could occur before and after harvest and its occurrence is favored by conditions such as storage, transportation and food processing, even when the best practices are followed [11]. It causes liver cancer: responsible for 5% - 28% of liver cancer cases, stunted growth in children, immunosuppression, and decreases resistance to infections [11] [12]. In animals, aflatoxins can lead to liver damage, impaired productivity and reproductive efficiency, and increased susceptibility to diseases. It has been estimated that 7761 out of 10,130 liver cancer cases in Nigeria in 2010 were attributable to aflatoxins [11] [12]. The US Food and Drug Administration (FDA) has set limits of 20 µg/kg for total aflatoxins for human and animals, and 0.5 µg/kg for milk and its products [13]. The Nigeria's National Agency for Food and Drugs Administration and Control (NAFDAC) enforces a standard of 4 µg/kg for ready-to-eat foods and 10 µg/kg for raw food items, for packaged goods and export-bound products [14].

Chemically, aflatoxins are metabolites which are derivative of polyketide and require the formation of hexanoate units in their biosynthesis [15]. Aflatoxin production is mediated by a cascade of structural and some regulatory genes. The biosynthetic pathway of aflatoxin involves different clustered genes, including structural, regular, and unassigned genes [16]. DNA-based detection system is a powerful, quick, reliable and more precise method for detecting aflatoxin and includes polymerase chain reaction (PCR) and the expression of aflatoxin specific genes including *aflD* (*nor-1*), *aflP* (*omtA*), *aflO* (*omtB*), *aflQ* (*ordA*), *aflR* [16] [17] [18].

Aflatoxin production normally starts with the transformation of the starter unit into the first stable metabolite norsolorinic acid (NOR). The *nor-1* is the old

name for aflD and it codes a norsolorinic acid ketoreductase needed to convert 1'-keto group of norsolorinic acid to 1'-hydroxyl group of averantin [19]. Aflatoxin P gene omtA is an old name for aflP and codes for the enzyme O-methyltransferase A. It functions in the conversion of sterigmatocystin (ST) to O-methylsterigmatocystin (OMST). Another key gene in the aflatoxin biosynthetic pathway is the aflO formerly called omtB. This codes for the enzyme O-methyltransferase B responsible for the conversion of dihydrodemethylsterigmatocystin (DHDMST) to dihydrosterigmatocystin (DHST). Similarly, aflQ was formerly called ordA. It codes for Oxydoreductase which converts O-methylsterigmatocystin (OMST) to AFB1 and AFG1, dihydro-Omethylsterigmatocystin (DHOMST) to AFB2 and AFG2. Finally, aflR is a transcription activator and regulates aflatoxin biosynthesis [20] [21] [22].

Conventional mold identification in the clinical laboratory relies on microscopic and macroscopic morphological features [23]. Due to the unusual poor sporulation and anti-fungal resistance phenotypes, clinical isolates formerly identified as *A. fumigatus* have been re-identified using multi-locus sequence analysis. DNA-sequencing enabled the identification of “cryptic” species [24]. The success of molecular identification largely depends on the choice of the right primer sequence for the amplification of the DNA. Schoch *et al.* [25] had evaluated six DNA regions as potential DNA barcodes for fungi by a multi-national, multi-laboratory consortium. They discovered among the regions of the ribosomal cistron, that the internal transcribed spacer (ITS) region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intra-specific variation. They further proposed that ITS be adopted as the primary fungal barcode marker [25]. In the present study, ITS was used as the primer sequence for the amplification of the genes from *Aspergillus* species.

Garri is a staple food prepared from the roots of cassava (*Manihot esculenta* Crantz). It is made from peeled, washed, grated, fermented and toasted fresh cassava tuber [26] [27]. Garri is stored and marketed in a ready to eat form [28] [29]. The major biological agents that contaminate and spoil garri are molds, insects and mites [30] [31]. Garri is rich in carbohydrate and therefore suitable for fungal growth. Molds such as *Aspergillus*, *Penicillium*, *Fusarium*, *Rhizopus*, *Cladosporium* and *Mucor* have been associated with garri during storage and distribution [32] [33].

There is information on the occurrence, contamination and distribution of the *Aspergillus* in garri in many localities of Nigeria and the health implications of consumption of such garri. Several researchers have isolated *Aspergillus* species from both white and yellow garri [29] [32]-[38]. However, several of these authors did not characterize this major spoilage agent *Aspergillus* by molecular method.

The present study therefore aimed to determine the occurrence, molecular characterization and phylogenetic relationship of *Aspergillus* species isolated from

garri sold in Benue State, North Central, Nigeria. *Aspergillus* species were isolated and molecularly characterized via DNA amplification and sequencing. Amplification of specific genes required for aflatoxin B1 production were also carried out.

2. Materials and Methods

2.1. Preparation of Potato Dextrose Broth for Molecular Analysis

Briefly, the Potato Dextrose broth (Himedia Laboratories^(R)) was prepared by measuring and dissolving 39 g in 1000 mL of distilled water, and filtering through the Whatman number 1 filter paper. This was sterilized by autoclaving at a temperature of 121°C for 15 minutes using saturated steam of 15 psi of pressure. This was allowed to cool to 45°C - 50°C and supplemented with chloramphenicol (0.05 g/L) before dispensing into the already sterilized bottles. Thereafter, the pure isolates were inoculated into the broth and incubated for 48 - 72 hours and the growth was used for molecular analysis.

2.2. Extraction of Fungal DNA

The DNA from the purified isolate was extracted using the ZR fungal DNA miniprep (Zymo Research Cat Number: D6005). Two milliliters of the fungal suspension were added to Zymo Research BashingTM lysis tube. This was followed by the addition of 750 µL lysis solution. This was secured in a bead fitted with 2 mL tube holder assembly and processed at maximum speed (10,000 x g) for five minutes. Centrifugation of the Zymo Research Bashing beadTM lysis tube in a microcentrifuge at >10,000 x g for 1 minute was done. Thereafter, 400 µL supernatant was transferred to a Zymo-Spin IVTM Spin Filter (orange top) in a collection tube and centrifuged at 7000 x g for 1 minute. In addition, 1200 µL of fungal DNA Binding Buffer was added to the filtrate in the collection tube. Then, 800 µL of the mixture was transferred to a Zymo-SpinTM IIC column in a collection tube and centrifuged at 10,000 x g for 1 minute. The flow through from the collection tube was discarded and the above step repeated. Also, 200 µL DNA pre-washed buffer was added to the Zymo-SpinTM IIC column in new collection tube and centrifuged at 10,000 x g for 1 minute. Then, 500 µL fungal DNA wash buffer was added to the Zymo-SpinTM IIC column and centrifuged at 10,000 x g for 1 minute. Finally, the Zymo-SpinTM IIC column was transferred to a clean 1.5 mL microcentrifuge tube and 100 µL DNA elution buffer added directly to the column matrix. This was centrifuged at 10,000 x g for 30 seconds to elute the DNA.

2.3. Electrophoresis for DNA and Polymerase Chain Reaction

One gram (1 g) of agarose for DNA and 2 g of agarose for PCR were measured. Agarose powder was mixed with 100 mL 1xTAE in a microwavable flask. These were microwaved for 2 minutes until the agarose was completely dissolved but the solution was not allowed to overboil, as some of the buffer would evaporate

and thus alter the final percentage of agarose in the gel. The agarose solution was allowed to cool to 50°C for 5 minutes. Then, 10 µL EZ vision DNA stain was added. The agarose gel was poured into a gel tray with the well comb in place. The newly poured gel was placed at 4°C for 15 minutes.

Loading Samples and Running the Agarose Gel

The loading buffer was added to each of the DNA samples. The agarose gel was placed into the gel box once it solidified. The gel box was filled with 1xTAE until gel was covered. Then, a molecular weight ladder was carefully loaded into the first lane of the gel. Also, the samples were carefully loaded into additional wells of the gel. The gel was run at 150 Volts for 1 hour. The power was then turned off, the electrodes disconnected and gel carefully removed from the gel box. The DNA fragments was visualized under the ultra violet (UV) transilluminator.

2.4. Polymerase Chain Reaction Mix Components

The PCR mix was made up of 12.5 µL of Taq 2X Master Mix (M0270, New England Biolabs); 1 µL each of 10 µM forward and reverse primer; 2 µL of DNA template and then made up with 8.5 µL Nuclease free water. Primer sequences for fungi characterization:

Forward ... ITS4: TCCTCCGCTTATTGATATGC

Reverse ... ITS5: GGAAGTAAAAGTCGTAACAAGG

2.4.1. Cycling Conditions for Gene Specific Primers (AFLD and AFLP)

The initial denaturation at 94°C for five minutes was done, followed by 36 cycles of denaturation at 94°C for thirty seconds. This was thereafter followed by annealing at 55°C for thirty seconds and elongation at 72°C for forty-five seconds. In addition, a final elongation was achieved at 72°C for seven minutes and was held at a temperature of 10°C.

2.4.2. Cycling Conditions for Gene Specific Primer (AFLQ)

The initial denaturation for five minutes at 94°C was done. This was followed by denaturation by thirty-six cycles for thirty seconds at 94°C, and annealing for thirty seconds at 50°C; then elongation for forty-five seconds at 72°C. These were followed by the final elongation for seven minutes at 72°C and a holding temperature at 10°C. For the cycling of gene specific primers, negative and positive control molds were *Penicillium* species (non-aflatoxigenic) and *A. flavus* previously known to produce aflatoxin respectively.

2.5. Phylogenetic Tree Analysis

The evolutionary history was inferred by using the Maximum Likelihood method and [39] model. Initial tree for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was

drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X [40].

3. Results

Molecular characterization of *Aspergillus* species with potential of causing aflatoxin contamination of garri in selected Local Government Areas (LGA) of Benue State, North Central Nigeria is presented in **Table 1**. The ITS gene amplified all the *Aspergillus* isolates at between 600 to 700 base pair (bp). *A. flavus* was most notable fungi in aflatoxin causation identified in samples from four (4) LGA including Oju, Buruku, Vandeikya and Ushongo, Prevalence and distribution of *Aspergillus* species in Garri in 12 LGAs of Benue State is shown in **Table 2**. Guma LGA had the highest *A. fumigatus* isolate (49 in white garri and 36 in yellow garri). Buruku had no *A. fumigatus* isolate. Vandeikya LGA had the highest *A. niger* isolates of 54 in white garri and 35 in yellow garri. Okpokwu had the least isolate of *A. niger* of 12 in white garri. Ogbadigbo LGA had the least *A. niger* isolates of 6 in yellow garri. *A. aculeatinus* was isolated only in white garri from Okpokwu and Gboko LGA. Oju LGA had the highest *A. flavus* isolates of 51 from white garri and 33 isolates from yellow garri. Kwande LGA had the least isolates of *A. flavus* of 3 in white garri and 4 in yellow garri. Konshisha LGA had the least isolates of *Aspergillus*. *Aspergillus aculeatus* was also not predominant across the LGAs. **Table 3** shows the percentage distribution of *Aspergillus* in the studied population with *Aspergillus niger* having the highest distribution across the LGAs and *Aspergillus aculeatus* having the least distribution, especially in yellow garri. *Aspergillus aculeatinus* was not found in yellow garri across the LGAs.

Table 1. Molecular characterization of *Aspergillus* species contamination of garri in selected LGA in Benue State.

Sample	Location/LGA	<i>Aspergillus</i> identified	Strain	Pairwise Identity	NCBI Accession number
1	Gboko	<i>Aspergillus aculeatinus</i>	B2819	89.30	MK204521
2	Ogbadigbo	<i>A. niger</i>	LS1614	94.30	KT335957
3	Oju	<i>A. flavus</i>	BRM051244	99.50	MK461562
4	Buruku	<i>A. flavus</i>	JN-YG-3-5	99.00	MG554231
5	Guma	<i>A. flavus</i>	SMLF30	99.00	MGG840796
6	Vandeikya	<i>A. flavus</i>	BRM051244	99.30	MK461562
7	Makurdi	<i>A. flavus</i>	SMLF30	99.80	MK281555
8	Otukpo	<i>A. aculeatus</i>	AN5	98.10	KY859793
9	Ushongo	<i>A. flavus</i>	CBS	98.20	MH864264
10	Okpokwu	<i>A. aculeatus</i>	JO6	95.90	EU645733

Table 2. Prevalence and distribution of *Aspergillus* species in garri in 12 LGA in benue state.

LGA	<i>A. fumigatus</i>		<i>A. niger</i>		<i>A. aculeatinus</i>		<i>A. flavus</i>		<i>A. aculeatus</i>		Total	%
	WG	YG	WG	YG	WG	YG	WG	YG	WG	YG		
Makurdi	32	21	34	10	-	-	6	6	6	-	115	6.99
Ogbadigbo	20	24	34	6	-	-	10	8	-	-	102	6.20
Kwande	25	18	32	14	-	-	3	4	-	-	96	5.83
Ushongo	24	30	16	22	-	-	24	8	-	-	124	7.53
Vandeikya	32	24	54	35	-	-	34	18	-	-	197	11.97
Oju	12	31	28	14	-	-	51	33	-	-	169	10.27
Buruku	-	-	24	30	-	-	30	24	-	-	108	6.56
Guma	49	36	18	20	-	-	32	12	-	-	167	10.15
Okpokwu	29	32	12	16	8	-	22	10	21	14	164	9.96
Gboko	16	20	42	18	4	-	32	18	-	-	150	9.11
Otukpo	14	10	46	18	0	-	20	15	28	15	166	10.09
Konshisha	20	14	36	18	0	-	-	-	-	-	88	5.34
Total	273	260	376	221	12	-	264	156	55	29	1646	100

KEY: A: *Aspergillus*; WG: White garri; YG: Yellow garri.

Table 3. Total distribution of *Aspergillus* species isolated from the garri.

Isolate	Type of Garri	Frequency	Percentage
<i>Aspergillus fumigatus</i>	White	273	16.58
<i>Aspergillus fumigatus</i>	Yellow	260	15.80
<i>Aspergillus niger</i>	White	376	22.84
<i>Aspergillus niger</i>	Yellow	221	13.43
<i>Aspergillus aculeatinus</i>	White	012	0.73
<i>Aspergillus aculeatinus</i>	Yellow	000	0.00
<i>Aspergillus flavus</i>	White	264	16.04
<i>Aspergillus flavus</i>	Yellow	156	9.48
<i>Aspergillus aculeatus</i>	White	055	3.34
<i>Aspergillus aculeatus</i>	Yellow	029	1.76
Total		1646	100

The agarose gel picture of the amplified DNA extracted from 10 fungal *Aspergilli* strains is shown in **Figure 1**. The lane M was 200 bp whereas the highest point was 1350 base pair (bp). Most of the isolates contained genes that fell within 600 to 800 bp. The strains involved were 1 from Gboko (*Aspergillus aculeatinus*); strain 2 from Ogbadigbo (*Aspergillus niger*); strain 3 from Oju (*Aspergillus flavus*); strain 4 from Buruku (*Aspergillus flavus*); strain 5 from Guma (*Aspergillus flavus*); strain 6 from Vandeikya (*Aspergillus flavus*); strain 7 from Makurdi (*Aspergillus flavus*); strain 8 from Otukpo (*Aspergillus aculeatinus*); strain 9 from Ushongo (*Aspergillus flavus*) and strain 10 from Okpokwu (*Aspergillus aculeatus*).

Figure 2 presents the agarose gel picture of the amplification of AFLP struc-

tural gene at 650 bp. The marker lane is 50 bp molecular weight. Out of the 10 fungal strains loaded in the agarose comb, five (5) (50%) of the strains showed clear amplification at 650 bp and showed possession of AFLP structural genes. The strains were *Aspergillus flavus* (from Buruku), *Aspergillus flavus* (from Vandekya), *Aspergillus flavus* (from Ushongo), *Aspergillus flavus* (from Guma) and *Aspergillus flavus* (from Makurdi). *Aspergillus aculeatinus* (from Gboko), *Aspergillus niger* (from Ogbadigbo), *Aspergillus flavus* (from Oju), *Aspergillus aculeatus* (from Otukpo) and *Aspergillus aculeatus* (from Okpokwu) respectively have no AFLP genes in them.

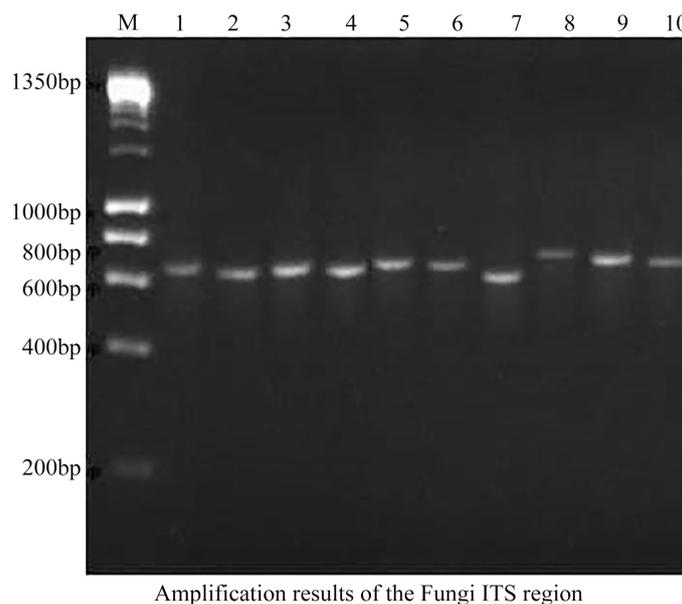


Figure 1. Gel picture for the amplification of Fungi at ITS region.

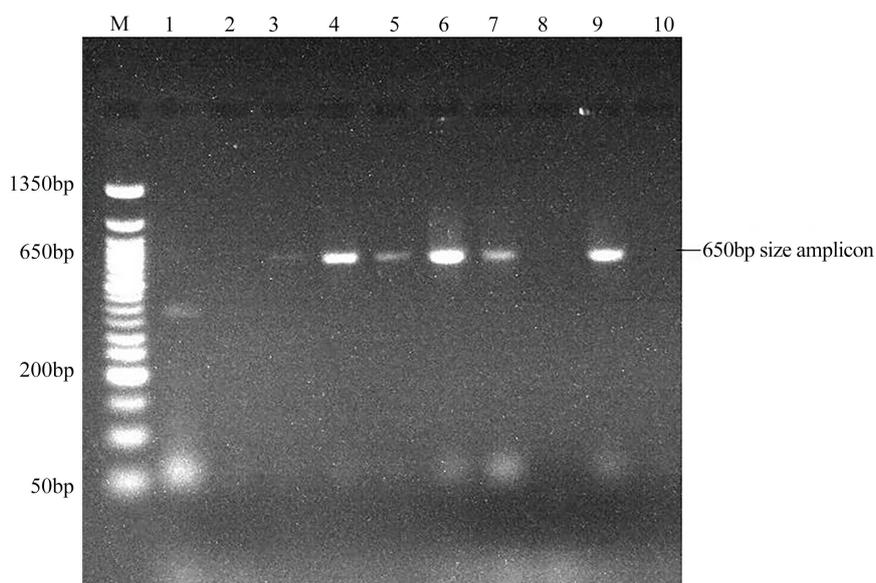


Figure 2. Gel image showing amplification of AFLP gene at 650 bp.

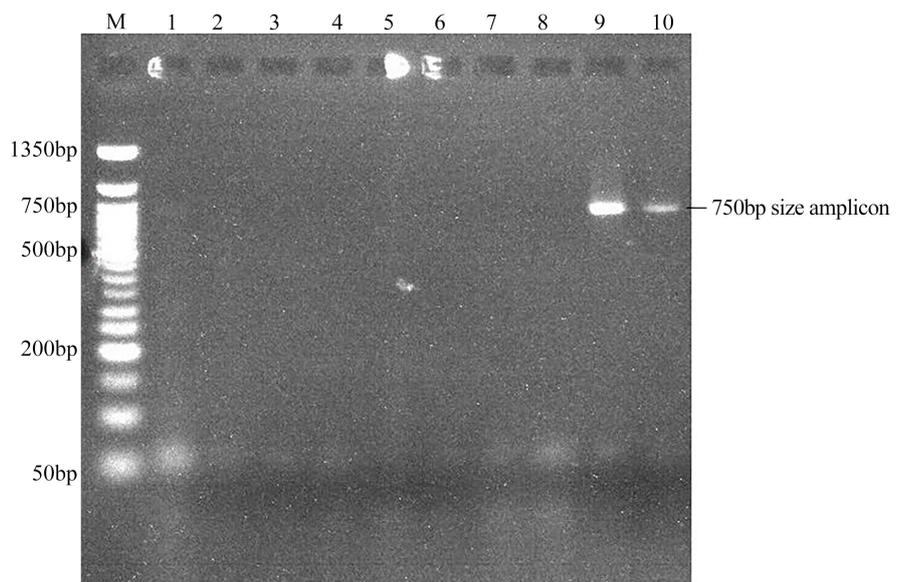
The gel picture for the amplification of AFLD structural gene at 750 bp is shown in **Figure 3**. The lane M = 50 bp molecular weight. Out of the 10 fungal strains loaded in the gel, only two (2) were amplified showing 20% of the strains having aflatoxin D structural gene.

Figure 4 shows the gel amplification of AFLQ gene at 450 bp. The lane M = 50 bp molecular weight maker. Out of the 10 fungal strains loaded in the agarose comb, seven (7) (70%) of the strains showed amplification at 450 bp and showed possession of AFLQ structural genes. The *Aspergillus flavus* (from Oju), *Aspergillus flavus* (from Buruku), *Aspergillus flavus* (from Guma), *Aspergillus flavus* (from Vandeikya), *Aspergillus aculeatus* (from Otukpo), *Aspergillus flavus* (from Ushongo) and *Aspergillus aculeatus* (from Okpokwu) show the presence of the AFLQ gene at 450 bp; while *Aspergillus aculeatinus* (from Gboko), *Aspergillus niger* (from Ogbadigbo) and *Aspergillus flavus* (from Makurdi) show no presence of the gene AFLQ at 450 bp.

Genetic relatedness of the isolates studied using the method of Maximum Likelihood showed close phylogenetic relationship amongst the *Aspergillus* species (**Figure 5**).

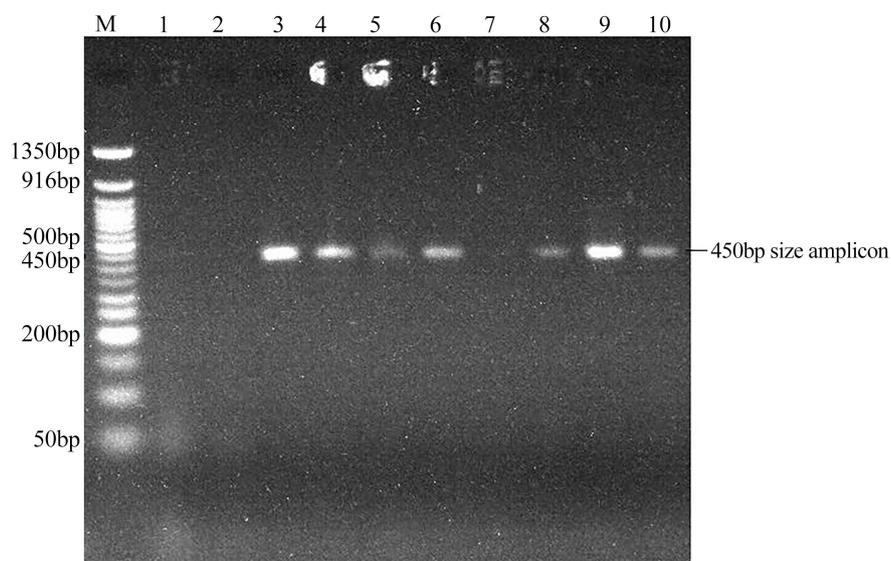
4. Discussion

Study on the occurrence, molecular characterization and phylogenetic relationship of *Aspergillus* species isolated from garri points to a potential food safety problem arising from the consumption should the product be contaminated with aflatoxigenic or invasive species. In the present study, out of one thousand six hundred and forty-six *Aspergillus* isolates, the isolates with related morphological features were selected together. Out of these grouping, ten (10) fungi



Gel image showing amplification of AFLD gene at 750bp
Lane M = 50bp molecular weight ladder

Figure 3. Gel image showing amplification of AFLD gene at 750 bp. Lane M = 50 bp molecular weight ladder.



Gel image showing amplification of AFLQ gene at 450bp. Lane M = 50bp molecular weight ladder

Figure 4. Gel image for the amplification of AFLQ gene at 450 bp. Lane = 50 bp molecular weight ladder.

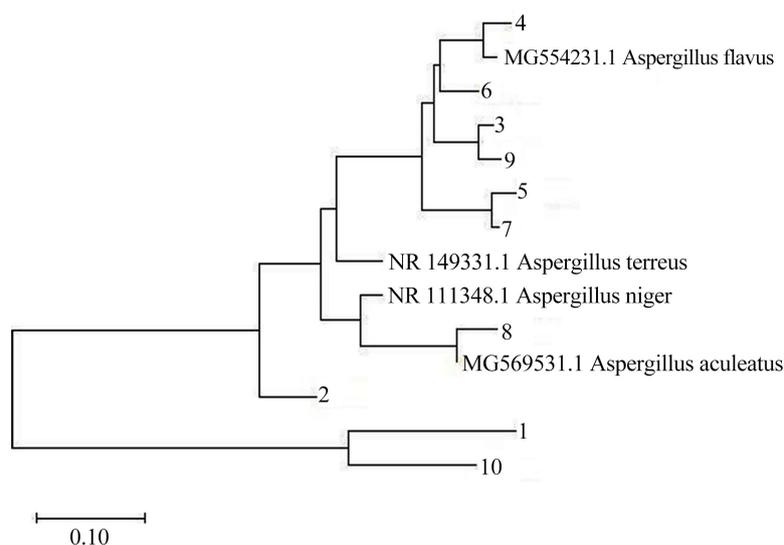


Figure 5. Phylogenetic Relationship of the *Aspergillus* species. KEY: 1: *Aspergillus aculeatinus*; 2: *Aspergillus niger*; 3: *Aspergillus flavus*; 4: *Aspergillus flavus*; 5: *Aspergillus flavus*; 6: *Aspergillus flavus*; 7: *Aspergillus flavus*; 8: *Aspergillus aculeatus*; 9: *Aspergillus flavus*; 10: *Aspergillus aculeatus*.

(representing each group) were studied for aflatoxigenic producing potentials using molecular approaches.

The occurrence of *Aspergillus* species isolated from garri sold in Benue State, North Central, Nigeria showed the presence of *A. flavus*, *A. fumigatus*, *A. niger*, *A. aculeatinus*, and *A. aculeatus*. *Aspergillus niger* obtained from white garri was the highest species load obtained in the present study pointing to the ubiquitous habit of *A. niger*. *Aspergillus fumigatus* that causes invasive aspergillosis [8] was the second highest isolate number. This was followed by *A. flavus* known to

produce aflatoxins that cause a lot of damages to humans and their livestock [9] [10] [11] [12]. Previous studies in Bayelsa, Kogi, and Benue States, all in Nigeria, have also confirmed the isolation of *Aspergillus* species, though none of these studies characterized the organisms to the species levels [36] [37] [41]. Of all the isolated species of the present study, *A. flavus* was most notable fungus in aflatoxin production. World Health Organization, WHO [42] estimated that 5% - 28% of liver cancer cases are caused by aflatoxin. *Aspergillus fumigatus* is the predominant *Aspergillus* responsible for the causation of invasive aspergillosis as it has been the most common species recovered from these cases of invasive aspergillosis. The next most commonly recovered species are *A. flavus*, *A. niger* and *A. terreus* [7].

The *Aspergillus aculeatinus* and *A. aculeatus* were the least isolated species in the present study. According to Gautier *et al.* [43], these groups of fungi were not known previously, and the cultural and traditional methods of characterization were not able to categorize and differentiate them from the other *Aspergillus* strains, but have been successfully characterized by use of DNA sequence. They belong to the group of “cryptic” *Aspergillus* species which were not known previously. Gautier *et al.* [43] had noted that, these organisms have been a major cause of life-threatening infections, especially in individuals whose immunities have been compromised. These organisms belong to the Nigri section of the *Aculeatus* clad, and included *Aspergillus aculeatinus*, *A. aculeatus*, *A. indologenus*, *A. japonicus*, *A. uvarum* [43]. They cause aspergillosis, with various clinical presentations such as aspergilloma also known as fungus ball of the lung, asthma exacerbations and severe asthma with fungal sensitization, allergic bronchopulmonary aspergillosis, cutaneous and wound infections, osteoarticular infections, chronic pulmonary aspergillosis, chronic invasive and granulomatous sinusitis, *Aspergillus* bronchitis, invasive pulmonary aspergillosis, and disseminated aspergillosis [6] [7]. These organisms have been reported somewhere to be very resistant to antifungal agents [44].

The identification of mold by conventional clinical laboratory methods use microscopic and macroscopic morphological features [23]. This method has its attendant limitations. For instance, due to the unusual poor sporulation and anti-fungal resistance phenotypes, clinical isolates formerly identified as *A. fumigatus* have been re-identified using multi-locus sequence analysis. “Cryptic” species identification has been made possible by DNA-sequencing [24]. Polymerase chain reaction (PCR) a molecular technique was adopted in the present study and ITS primers used. Internal Transcribed Spacer (ITS) used as primer sequences in the present study, has been proposed to be adopted as the primary fungal barcode marker having highest probability of successful identification for the broadest range of fungi [25]. Internal Transcribed Spacer (ITS) combines the highest resolving power for discriminating closely related species with a high PCR and sequencing success rate across wide range of fungi [25].

Detection of aflatoxin specific structural and regulatory genes including AFLD,

AFLP and AFLQ by polymerase chain reaction was used for the differentiation of aflatoxin producing and non-aflatoxin producing isolates. *Aspergillus flavus* from Ushongo and *A. aculeatus* from Okpokwu amplified AFLD gene. There was a clear amplification of AFLP by *A. flavus*. The amplification of AFLP structural gene at 650 bp by the strains of *Aspergillus flavus* from the study area shows their potential aflatoxin B1 biosynthetic ability. *Aspergillus flavus* expressed the aflatoxin specific genes AFLP, AFLD and AFLQ. Aflatoxin D gene (*aflD*) codes a norsolorinic acid ketoreductase which converts 1'-keto group of norsolorinic acid to 1'-hydroxyl group of averantin [19]. The *aflP* codes for the enzyme O-methyltransferase A which converts sterigmatocystin (ST) to O-methylsterigmatocystin (OMST) [20] [21] [22]. The *aflQ* codes for Oxidoreductase which converts O-methylsterigmatocystin (OMST) to AFB1 and AFG1, dihydro-Omethylsterigmatocystin (DHOMST) to AFB2 and AFG2 [20] [21] [22]. The expression of genes AFLP, AFLD and AFLQ in the present study is consistent with the reports of Scherm *et al.* [17] and Sohrabi and Taghizadeh [16] in the molecular detection of aflatoxigenic molds. The use of PCR amplification of genes involved in aflatoxin biosynthesis had been previously used to differentiate aflatoxigenic from non-aflatoxigenic molds [15] [16] [18]. Scherm *et al.* [17] had previously demonstrated the use of reverse transcription polymerase chain reaction for the differentiation of aflatoxin and non-aflatoxin producing isolates of *Aspergillus flavus* and *A. parasiticus*. Using specific primers based on conserved sequences of structural genes *aflD*, *aflG*, *aflH*, *aflI*, *aflK*, *aflM*, *aflO*, *aflP*, and *aflQ* and regulatory genes *aflR* and *aflS*, needed for the biosynthesis of aflatoxin B1, Scherm *et al.* [17] noted the expression profile of three genes *aflP*, *aflD*, and *aflO*. These genes (*aflP*, *aflD*, and *aflO*) were consistently correlated with the capacity of the organisms' ability to produce aflatoxin B1 or not.

The phylogenetic tree plotted between the isolated organisms and reference sequences from the NCBI was done, and the outcome showed a close relationship between the isolates and their most identical sequence in the NCBI database. This tree formed three major clades with most of the isolates clustering with *Aspergillus flavus* in the first cluster. *Aspergillus flavus* from Buruku LGA formed the closest cluster with the reference sequence of *Aspergillus flavus* MG554231 and *Aspergillus flavus* from Vandeikya LGA was closely related to this cluster having a 0.09 genetic distance apart. The cluster was closely related to a branch formed between *A. flavus* from Oju LGA and *A. flavus* from Ushongo, of which both of them have a 0.03 distance apart. Again, a member of the cluster is the most distant group that comprise a cluster between *Aspergillus flavus* from Guma and *Aspergillus flavus* from Makurdi LGA that also has a 0.03 genetic distance apart. Another clade is *Aspergillus aculeatus* which clustered with the reference sequence of MG569531 of *Aspergillus aculeatus*, while the *A. niger* stands out as an outgroup of these clades. However, the most distant group is the group consisting of a cluster between *Aspergillus aculeatus* from Gboko LGA and *A. aculeatus* from Okpokwu, and this group recorded a 0.28 genetic distance apart.

5. Conclusion

DNA sequence analysis showed that *Aspergillus* species isolated were *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. aculeatinus*, and *A. aculeatus* with *A. niger* being the most abundant. Aflatoxin specific structural genes including AFLD, AFLP and AFLQ which code for enzymes that perform key functions in aflatoxin B1 biosynthetic pathway were expressed by *Aspergillus flavus*. Synthesis of aflatoxin in garri, a ready-to-eat food product could have negative health implications to consumers. Phylogenetic tree showed a close relationship among the isolates.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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