Original Research Article

Phylogenetic and Gene Expression of *Norsolorinic acid*analysisof Aflatoxigenic Strains and Quantification of Aflatoxin B_1Levels intoWhite Rice Grainsin Kenya

Abstract: The most important pathogens of global significance in food security are mycotoxigenic fungi.Aflatoxigenic strains contamination in rice grains can lead to a health risk for consumers.Under appropriate conditions, aflatoxigenic fungi can grow and subsequently produce aflatoxins.Aflatoxin poses a real threat to food safety and security and destroys approximately 25% of the world's food crops and every year 420,000 people die from consuming contaminated food causing an estimated loss of 33 million healthy life years. This study screened Norsoloric Acid gene involved in the biosynthesis pathway, gene expression profile of aflatoxigenic strains characterized from rice grains, and assessed aflatoxin B_1 levels in white rice grains.We combine the use of moleculartechniques and Enzyme-Linked Immunosorbent Assay to detect aflatoxin biosynthesis *aflD* gene and quantify gene expressionprofile, and analyzed aflatoxin B_1levels, respectively.

The results shown that all aflatoxigenic strains analyzed in this study are clustered within the same clade based on the *aflD* gene. The targeted *aflD* gene for expression was amplified in all aflatoxigenic strains with higher relative level in *Aspergillus flavus* but no relative expression shown in *Aspergillus fumigatus*. Enzyme-Linked Immunosorbent Assay showed that 43.1 % of the samples were found positive, with15.9 % being local rice and 27.2 % imported rice. Notably, 11.3 % of analyzed samples were above the recommended aflatoxin B_1 in rice grains established by European Union with aflatoxin concentration ranging between 0 µg/kg to 3.2 µg/kg.These results are indicative of exposure of the population to aflatoxin $B_1$and possible health hazard. Our study reveals high levels of aflatoxin B_1 contamination in bothimported and local rice grains in Kenya. This study highlights the species diversity of aflatoxigenic fungi that have the potential to contaminate different types foodstuffs.

Keywords: Rice grains; aflatoxigenic; gene expression; aflatoxin B_1; contamination; ELISA; exposure;survey.

1. Introduction

Microbial pathogens, particularly mycotoxigenic fungi, are a significant global menace to food safety and security[1, 2]. In tropical climate conditions, aflatoxigenic fungi of *Aspergillus*species easily contaminate small grains like wheat, barley, rye, rice, triticale, and corn [3,4]. Mycotoxins are among the microbial toxins of most concern to public health and represent a significantbarrier to the international trade of agricultural products and an obstacle in the face of global harmonization of regulatory standards, as discussed by[5]. At any stage of the foodstuffs production process, before and during harvesting, drying, transport, and storage, mycotoxigenic fungi can colonize grains crops and expose consumers to the risk of mycotoxin contamination directly through food consumption or indirectly through animal feed [6].The contamination of foodstuffs and commodities with mycotoxins is a recurrent problem in Sub-Saharan Africa (SSA). It is caused mainly by post-harvest infestation of *Aspergillus* species section *flavi* during storage, holding, processing, transportation,and marketing.Among all mycotoxins, AFs are known as the most poisonous and toxic to human beings,significantly impactingthe economic burden offoodstuffs and consumers[7, 8]. Aflatoxins (AFs) are secondary metabolites typically produced through certainaflatoxigenic species of *Aspergillus*, like *A. flavus*, *A. astellatus*, *A. nomius*,and *A. parasiticus* [9, 10].AFs are genotoxic, carcinogenic, teratogenic, mutagenic, hepatotoxic, and immunosuppressive [11].The most studied AFs among the main types are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2), while aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2) have received considerable attention.AFs were first discovered following a terrible livestock poisoning occurrence in England involving turkeys [12].Of all the AFs, AFB_1 is one of the most prevalentAFs, which represents a problem for consumers' health due to being hepatotoxic and a potent carcinogenic [13,14].The challenge of AFs stands prominent everywhere in the world and specifically in African countries, where contamination by aflatoxins is recorded in foodstuffs with an incidence of 50 %, for instance, with 1642 µg/kg infestation in white rice grains[15].Assessment of AFB_1 incidence in cereals is necessary to avoid and decrease the adversative health effects onthe population. AFs in rice grainscould be quantitatively determined by applying diverse investigative methods.Molecular techniques like PCR, Real-Time PCR, and phylogenetic analyses are commonly used to validate the morphological characterization of *Aspergillus* species section *Flavi*[5].

Comment [HC1]: According to research on the contamination of foodstuffs with aflatoxin mainly in the fields, we invite the author to add this parameter in the context part of the introduction!

The biosynthesis pathway of AFsinvolves numerous enzymes encoded in genes located in a cluster of 75 kb in size[16]. In *Aspergillus*, the order of these genes is highly preserved [17]. There are 30 genes positioned within the same order in entirely aflatoxigenic fungi of the *Flavi* section [18, 19]. The cluster is controlled in a coordinated way, and *norsolorinic acid*is the first stable precursor of AFs[20, 21], which is then converted into*averantin*in the central of the aflatoxin biosynthetic pathwayby a reductase encoded by the *aflD* gene [21, 22]. Indeed, a more profoundknowledge about the elements included in the regulatory path of the biosynthetic of aflatoxin gene clusters that activate in aflatoxigenic strainsis anessentialprerequisite for mitigating and minimizing AFs and preventing foodstuffs and commodities contamination in the future[23, 24].

Global consumption of rice has recently increased. Rice grains was consumed arround 490.27 million metric tons worldwide in the year of 2018-2019. The consumption of rice grains in Kenya is increasing at 12 % per yearafter wheat and maize with 4 % and 1 % respectively. Its local production is estimated to be between 100.000 and 120.000 metric tons per annum, while its consumption has increased from 400.000 to 410.000 tons per annum [25].The contamination of rice grains with AFs has been documented in several studies, with the findings showing that rice samples surveyed contain AFs [26, 27, 28].The European Union (EU) has set the maximum tolerable limits (MTL) for AFs in rice precisely, 4 µg/kg for total aflatoxins (B_1+B_2+G_1+G_2) and 2 µg/kg for AFB_1 [29].

Comment [HC2]: The articles cited here to support the contamination of rice in the world are certainly quite numerous, but date from 2016 and 2019, this article whose work was carried out in Burkina Faso is more recent: “Compaoré H, Samandoulougou S, Compaoré S.C, Bambara A, Ratongue H, Traoré L, Sawadogo-Lingani H. (2021b). Occurrence of Aflatoxins in rice intended for infant flour production in Ouagadougou, Burkina Faso. Journal of Advances in Microbiology, 21 (10): 55-66.”
We invite the author to integrate it

Manymethods are currently applied for the detection of AFcontamination, including thin-layer chromatography (TLC), enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography with a fluorescence detector (HPLC-FLD), or tandem mass spectrometry (HPLC-MS/MS)[30, 31]. TLC and ELISA are widely utilized to detectAFscontamination in various foods, offering higher sensitivity around 1–20 µg/kg [32, 33].

Due to the tropical climates with extreme ranges of temperature, humidity, and rainfall levels throughout the year, rice grains sold in the markets, retail markets, and millers in Kenyaareat an increased risk of contamination by AFs, posing a threat to food safety and security to the population and animal health.Aflatoxin poses a real threat to food safety and security and destroys approximately 25% of the world's food crops and every year 420,000 people die from consuming contaminated food causing an estimated loss of 33 million healthy life years. The most significant outbreaks of acute aflatoxicosis from highly contaminated food have been documented in Kenya. In July 2004, an outbreak of acute hepatotoxicity with 317 cases

and 150 deaths occurred as a result of AFs poisoning from ingestion of contaminated maize [34]. Another outbreak of aflatoxicosis occurred in 1981 from contaminated maize,with 500 acute illnesses and 200 deaths in Makueni County and other parts of Kenya[35]. From the above cases, it is clear that AFs food poisoning is a common occurrence in Kenyan cereals, a food commodity commonly used by many communities as staple food. However, no study has been performed in Kenya to assess the safety of rice grains for AFB1 and the risk associated with consuming this staple product. Therefore, our study aimed to screen aflatoxigenic strainsthrough Norsolorinic Acid (NOR)and quantify AFB_1 in rice grainssold in the marketsto evaluate the exposure levels from rice consumption and the associated liver cancer risk from this toxin.

2. Material and Methods

Sample Collection

Rise samples were collected randomly (1 kg) from local retail markets and millers in Mwea (59 samples) and at Jamhuri Market in Thika (39 samples) (Kenya) and labelled appropriately. A total of 98 samples (local rice produced in Mwea and imported rice originating from Biriyani, India, Pakistan, and Thailand) were taken in line with the alternative sampling plan for the Official Control of Mycotoxins in Food [36]. Representative samples were put in sealed plastic ziplock bags and transported to the Molecular Biology and Biotechnology Laboratory at Pan African University Institute for Basic Sciences, Technology and Innovation (PAUSTI).

Isolation of Fungi

All samples of rice grains collected were analyzed usingthe direct plating approach defined by Pitt and Hocking [37]. Rice grains were surfacesterilized by dipping in dilutedcommercialbleach (2.5% sodium hypochlorite) for 1 min before rinsing thrice in sterile distilled water.For every sample, 20 pieces (rice grains suspected to be infected by fungi) were positioned aseptically on a layer of moistened filter paper in the Petri dishes. The Petri dishes were incubated upright at 25 ℃ for 3-5 days in darkness and ventilated for 12 h onthe 3rd day. Isolation of fungi from the suspected grains was performed using Ulster's method[38]. Fungi growing on distinctive seeds were isolated from emerging colonies on modified *rose Bengal* chloramphenicol *agar (MRBA)* [39]. The cultural characteristics described in taxonomickeys by Klich were used to identify *Aspergillus* section *Flavi*[40].Pure cultures

were performed for subsequent studies using two different media, malt extract agar (MEA) and potato dextrose agar (PDA), according to Pitt and Hocking [39]and [41]for seven days.

Genomic DNA Extraction

Four*Aspergillus*strains (*A. flavus*, *A. parasiticus,A. nomius*and *A. fumigatus*) were identifiedfor this study.The genomic DNA was extracted from seven-day-oldculture grown on mediaaccording to [42] method with modification.A total of 50-100 mg of fungal mycelium was scraped and placed in a 2 mL Eppendorf tube. The tubes were frozen in liquid nitrogen and vortexed vigorously for 30 minutes with steel beads to crush the mycelial wall and release the DNA. A volume of 500 µL of Lysis Buffer (100 mM Tris-HCl pH 8, 1 mM EDTA, 100 mM NaCl, 10 mM B-mercaptoethanol, and 1 % *Sodium dodecyl sulfate*), 5 µL of RNase A, and 1 µL of Proteinase K were added. Tubes were then incubated at 65 °C for 45 minutes in buffer. Thereafter, 270 µL of Sodium/Potassium acetate (3M) was added, and samples were well-mixed and centrifuged at 13,000 rpm for 10 minutes. After centrifugation, the supernatant (700 µL) was transferred to a fresh tube, and an equal volume of chloroform: isoamylalcohol (24:1) was added and mixed;samples were placed on a bench for 5 minutes, followed by centrifugation at 13,000 rpm for 10 minutes. The supernatant (700 µL) was transferred into new tubes, and 80 µL of Sodium/Potassium acetate (3M) and 587 µL of ice-cold isopropanol were added and then mixed well by inverting the tubes and then incubated at -20 °C overnight. After that, the samples were centrifuged at 13,000 rpm for 30 minutes, and the supernatant was discarded carefully. The DNA pellets were washed with 1mL of 70 % ethanol and centrifuged at 13,000 rpm for 10 minutes. The DNA pellets were air-dried, dissolved in 50 µL of Tris-EDTA (TE) buffer, and stored at -20 °C until use.

Set	Primer Name	Sequences (5′–3′)	Length of PCR Product (bp)
aflD		F-ACCGCTACGCCGGCACTCTCGGCAC R-GTTGGCCGCCAGCTTCGACACTCCG	400

Table 1. **Oligonucleotide Primer Sets Used for the Polymerase Chain Reaction (PCR)**

Abbreviations in the sequences: **F**, forward; **R**, reverse.

Sequencing and Phylogenetic Analysis

The *aflD*gene PCR products were purified using Qiagenkit for purification, and the products of*aflD*gene primers were sent to Macrogen Europe for sequencing analyses. After getting the forward and reverse primer sequences of each strain, sequences were edited with MEGA7 [43]and Geneious software (http://www.geneious.com).The data were contrasted in GenBank using the BLASTn algorithm. Additionally, strain information was evaluated using the reference strains of NCBI (http://www.ncbi.nlm.nih.gov).Using the maximum likelihood method based on the Tamura-Nei model [44], the phylogenetic relationship was found.

RNA Extraction

The fourstrainsidentified earlier were screened for gene expression to quantify the potential aflatoxigenic producers by amplifying the norsolorinic acid (NOR), *aflD* (nor-1) gene involved in the aflatoxin biosynthetic pathway.RNA's isolation was done using Rneasy Mini kit (Qiagen) following the manufacturer's instructions with brief modification. Briefly, 50 mg of fungal mycelium were scraped and frozen in liquid nitrogen and then ground into fine powder by vortexing in steel beads. The powder was transferred in a 2 mL Eppendorf tube, a suitable volume of Buffer RLT for a working solution was added, then centrifuged for 3 minutes at a maximum speed to disrupt and homogenize the lysate. After centrifugation, the supernatant was removed to a new tube. Additionally, 70 % of ethanol was added to the lysate and mixed well by pipetting. A volume of 700 µL of the mixed sample was transferred to a Rneasy Mini spin column placed in a 2ml collection tube and centrifuged for 20 seconds at ≥ 8000 x g, the flow-through discarded. Thereafter, 700 µL Buffer RW1 wasadded to the Rneasy spin column, centrifuged for 20 seconds at ≥ 8000 x g, and discarded the flow-through. After that, a volume of 500 µL RPE wasadded to the Rneasy spin column and centrifuged, and the flow-through was discarded. This step was repeated (twice). Thereafter, the Rneasy spin column was positioned in a new 1.5 ml collection tube, 50 µL of RNase-free water was added to the spin column membrane and centrifuged for 1 minute at ≥ 8000 x g to elute the RNA.The concentration and purity of RNA were measured using a Nano-drop Spectrophotometer (Model PCR Max Lambda, Thermo Fisher Scientific, Waltham, MA, USA), and the quality of all extracted RNA was measured by taking their absorbance at 260 nm and 280 nm.

cDNA Synthesis

Reverse transcriptase was done using a cDNA synthesis kit. cDNA was prepared using 5X Hot Firepol® EvaGreen® HRM Mix (ROX) (Solis BioDyne) following the manufacturer's

instructions using random hexamers. Briefly, a master mix consisting of 1 µl of FIREScript RT, 0.5 µl of dNTP MIX (20 mM of each), 1 µl random hexamers (100 µM), 2 µl of 10x RT Reaction Buffer with DTT, 0.5 µl RiboGrip RNase Inhibitor, 5 µl of Nuclease-free H_2O was made and a 10 µl volume of total RNA was added to the master mix. Confirmation of successful cDNA synthesis was done using conventional PCR with amplification of primers for qPCR in Proflex PCR System with the following parameters: First Denaturation 95 °C for 3 minutes, second Denaturation 95 °C for 30 seconds, Primer annealing 58 °C for 45 seconds and Elongation 72 °C for 7 minutes. Then, after PCR reactions, the PCR product was confirmed by gel electrophoresis. The cDNA was then kept at -20 °C until use.

Table 2. **Primer Sets Used for the Real-Time Polymerase Chain Reaction (qRT-PCR)**

Set	Primer Name	Sequence (5'->3')	Product Length (bp)
aflD	**Forward primer**	AAT CAC AGT CCG AGG GTT CTT G	165
	Reverse primer	TGA TCA TAC CCG AGC ACA GAT	
β-tubulin	**Forward primer**	AAG ATC AAC GAG GAC GGC AC	222
	Reverse primer	CTC GAA CGA ACG ACG ACC A	

Gene Expression Profile Analysis

The gene expression in the aflatoxin biosynthetic pathway was assessed in triplicates for all aflatoxigenic strains identified. The housekeeping gene (*β-tubulin*) was used as a control during the experimentation. Two primers were designed using Genius Prime software and NCBI Gene bank for the gene of interest,*aflD* and *Beta tubulin*, ahousekeeping gene Table 1.Quantifying relative gene expression by real-time quantitative PCR was conducted using the comparative of $2^{\Delta\Delta CT}$ method. For gene expression analysis, *aflD* gene, which is in control for the conversation of *norsolorinic acid* (*NOR*) to *averantin* (*AVN*) in the centreof the aflatoxinbiosynthetic pathway[45], was chosen.

Universal qPCR kit (5x HOT FIREPol® EvaGreen® qPCR Mix Plus) was utilized in running qPCR in qTOWER^3 - Real-Time PCR Thermal Cyclers (Analytik JenaAG, Konrad-Zuse-str. 1 07745 Jena. Germany) 96-well real-time qPCR following the manufacturer's instruction. A total volume of 20 µL, including 4 µL of 5x HOT FIREPol, 13 µL of Nuclease-free H_2O, 0.5

µL of each oligonucleotide primer sets above for qPCR and 2 µL cDNA was performed in the system. The qPCR cycle parameters were set as: initial denaturation at 95 ºC for 2 minutes, denaturation at 95 ºC for 15 s, annealing plus elongation at 57 ºC for 30 s melting curve analysis (60 -95 ºC) for 10 s. Relative expression was determined using the $2^{\Delta\Delta CT}$ method[46, 47].

Quantitative Determination of AFB_1 levels by ELISA

Local and imported white rice grains were randomly sampled from local retail markets and millers in Mwea and at Jamhuri Market in Thika, Kenya, and appropriately labelled. A total of forty-four different samples comprising homegrown rice in Mwea and imported rice originating from Biriyani, India, Pakistan, and Thailand, respectively, were taken following the alternative collection plan for the Official Control of Mycotoxins in Food (OCMF) [36]. Representative samples were then sealed in plastic ziplock bags, transferred to the Laboratory of Molecular Biology and Biotechnology at Pan African University Institute for Basic Sciences, Technology and Innovation, and prepared for subsequent ELISA analyses according to OCMF.

Samples Preparation and ELISA Test Procedure

The quantitative analysis of AFB_1 from samples collected was performed based on a competitive enzyme immunoassay using RIDASCREEN® Aflatoxin B_1 30/15 test kit (Art. No: R1211, R-Biopharm, Darmstadt, Germany). Samples collected were powdered using a moulinex blender, 5 g of the represented sample were transferred in sealed plastic bags (zip lock) and then stored at 4˚C [48] until use. All samples were examined according to the instructions ofthe manufacturer in duplicates. Samples preparation for ELISA test were performed according to the technique described by R-Biopharm GmbH[49]. Briefly, to perform ELISA test rice grains was ground in fine powder, then 5 g of rice ground was transferred in to 50 ml of falcon tube, twenty-five milliliter methanol: water (70:30) was added in the falcon then after the samples were shaken vigorously for 3 minutes with shaker. The obtained extract was filtered through a filter paper and diluted with distilled water (1:1). At a minimum, 50 µL of the diluted filtrate was used per well in the test. Test procedure was done according to RIDASCREEN® Aflatoxin B_1 30/15 (Art No.: 1211) test kit manual, a volume of 50 µL of the standard solution and prepared sample was added to the wells of micro titer plate in duplicate. Then, 50 µL of the enzyme conjugate was added, and this step was directly followed by adding on top 50 ml of the antibody solution to each well, then

kindly mixed and raised for 30 minutes at room temperature (20-25 ºC). Later, liquid was discarded from wells by beating them upside down dynamically against the absorbent paper; followed by cleaning the wells twice with a washing buffer (250 µL). After the cleaning step, a solution of 100 ml of substrate/chromogen was added to all wells, mixed gently, and incubated for 15 minutes in the room temperature (20-25 ºC). Lastly, 100 ml of the stop solution was added intoall wells and the absorbance was measured at 450 nm in ELISA plate reader (ELX800 UV, Biotek, USA).

Absorbance Determination

The absorbance values of the standards, and samples tested were calculated according to the manufacture's instruction. Briefly, absorbance of the standards, and samples measured were divided by the absorbance value of the first standard (zero standard) and multiplied by 100:

$$\% \text{ absorbance} = \frac{\text{absorbance standard/sample}}{\text{absorbance zero standard}} X\, 100$$

The zero standard is created equal to 100 % and the absorbance values were stated in percentages. The calculated values of all standards were typed into a system of coordinates on semilogarithmic graph paper against the concentration (µg/kg) of aflatoxin B_1. The aflatoxin B_1 concentration in µg/Kg matching to the absorbance of each sample established was read from the standardization curve correspondingly to the test preparation record for aflatoxin B_1.

Data Analysis

The data were entered in Microsoft Excel Data Analysis for statistical analysis. Phylogenetictrees based on Maximum Likelihood were constructed based on the best model using MEGA7.Graphical representation of the data was displayed in Graph Pad prism version 7.0.Analysis of variance (ANOVA) was used to compare the means and Tukey's HSD test ($P \leq 0.05$) used for mean separations, IBM® SPSS® Statistics 25.0 was used to determine the difference between levels of aflatoxin B_1 in rice grains and maximum limits levels permitted in Kenya and European Union. The difference between the aflatoxin mean in local and imported rice samples was compared by independent t-test. $P<0.05$ was considered as a significance.

3. Results

Comment [HC3]: In the results section there are no images of the fungal strains isolated from rice, we invite the author to insert macroscopic or microscopic aspects of rice fungi isolates in PDA or MEA medium!

The Maximum Composite Likelihood (MCL) tree was constructed forthe *aflD*gene sequence to describe the phylogenetic relationships among the aflatoxigenic strains, as shown in Figure 1. The tree with the highest log likelihood (-681.12) is shown. Sequencing and phylogenetic analysis of the *aflD* gene revealed that Aspergillus flavus strains clustered together with strains of *A. parasiticus*, *A. nomius*, and *A. fumigatus* (Figure 1). Reference strains of *A. flavus*, *A. parasiticus*, and *A. nomius* were grouped within the same clade. However, *A. fumigatus* (4A5) did not form a distinct cluster with strains of *A. parasiticus* and *A. nomius*. Overall, the aflatoxigenic strains analyzed in this study clustered within the same clade based on the *aflD* gene, which plays a critical role in converting norsolorinic acid to averantin during the intermediate stages of the aflatoxin biosynthetic pathway, rather than based on their source of isolation.

Comment [HC4]: We invite the author to add the nomenclature of related reference strains from Gen Bank to the figure 1 of the phylogenetic tree (replace the codes). This will facilitate understanding of the grouping of rice fungal strains around the *Aspergillus* section *Flavi* strains.

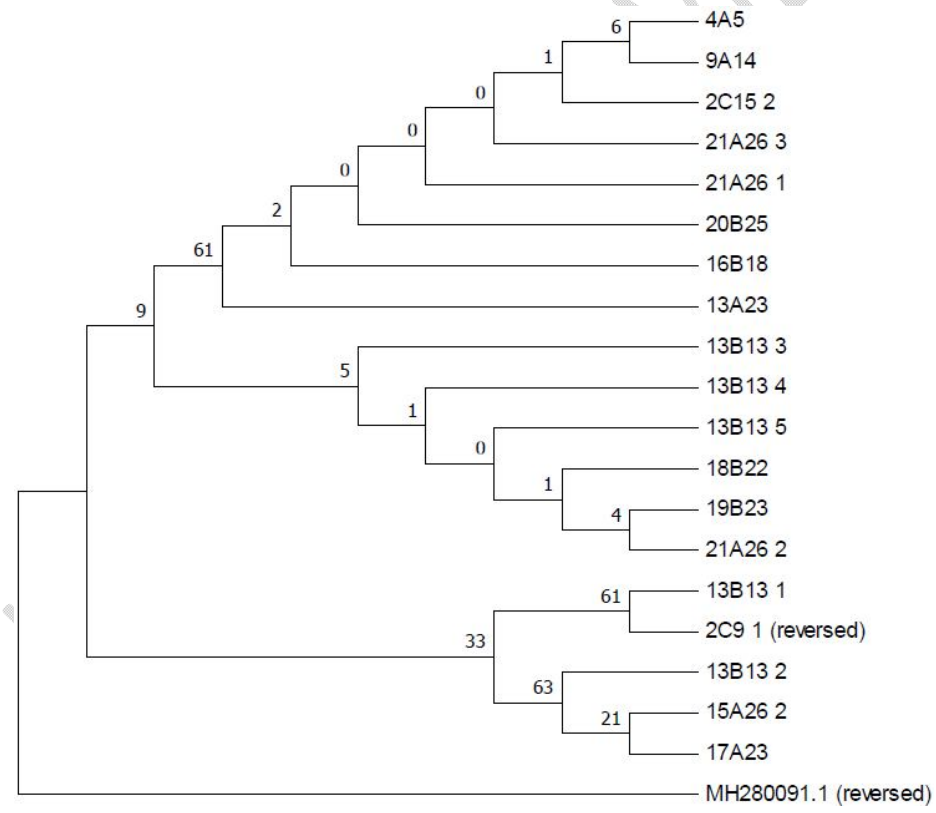

Figure 1. Molecular Phylogenetic analysis by Maximum Likelihood method showing the phylogenetic relationships among the aflatoxigenicstrains based on *aflD*gene sequence.The percentage of trees in which the associated taxa clustered is shown above the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and

BioNJ algorithms to a matrix of pairwise distances estimated using the MCL approach and then selecting the topology with superior log likelihood value

Comment [HC5]: Figure title is too long, we invite the author to move the details into the text !

Analysis of Aflatoxin Gene Expression

Four Aspergillus species were analyzed for the expression of the *aflD* gene, a key gene in the biosynthetic pathway of aflatoxin production. Notably, the relatively high expression levels of the *aflD* gene were observed in three strains: *A. flavus, A. parasiticus,* and *A. nomius.* Figure 2-A presents the mean expression levels of the *aflD* gene across these aflatoxigenic strains, with *A. flavus* showing a significantly higher mean expression, as determined by real-time PCR quantification. Figure 2- B illustrates a boxplot analysis of the relative *aflD* expression levels for all tested strains, highlighting variability among the species. Amplification curves for the *aflD* gene, as shown in Figure 2- C, confirm the specificity of the reaction by plotting fluorescence intensity against cycle number. To ensure the reaction's specificity, melting curve analysis was performed, plotting fluorescence against temperature (Figure 2- D).

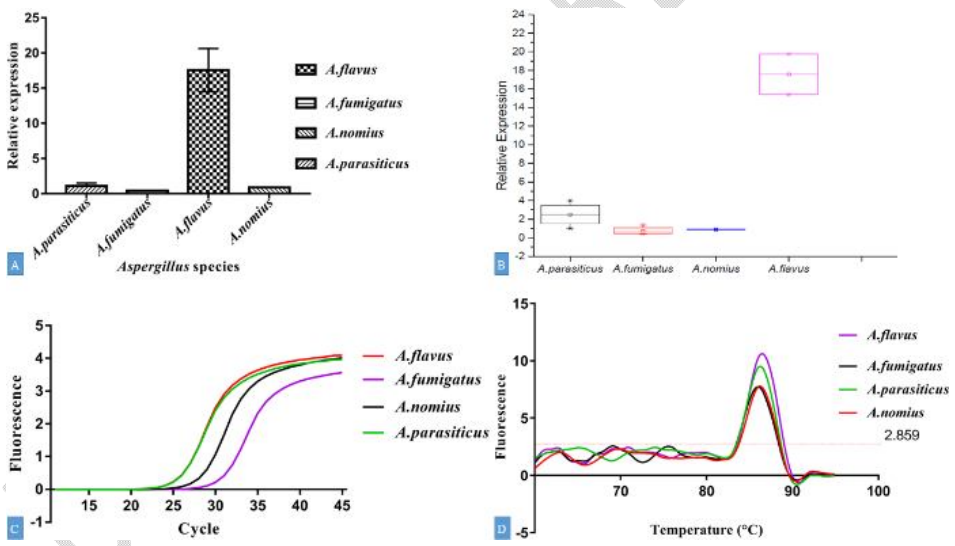

Figure 2. Real-time PCR amplifications of *aflD* gene targeted in different aflatoxigenic strains: A, Graphical representation of the relative gene expression levels of the *norsolorinic acid* gene *nor-1*; B, Boxplot analysis of the relative expression of *aflD* gene; C, Relative fluorescence against cycle number; D, Melting curve analysis against temperature.

Quantitative Determination of Aflatoxin B_1 Levels in Rice Grains

Forty-four (44) samples, including local and imported rice grains (25 and 19, respectively) were analyzed for aflatoxin B_1 content (quantitatively) using an ELISA kit. Figure 3, presents the standard curve of aflatoxin B_1 generated from the absorbance values of the standards and samples in the system.An exponential correlation (Figure 3) was obtained by plotting the percentage of absorbance (y) and concentration (x) of aflatoxin B_1 (R^2 = 0.96061) of the standard calibration samples supplied by the manufacturer (RIDASCREEN® Aflatoxin B_1 30/15). Thedetermination of the aflatoxin B_1 concentration (µg/kg) corresponding to the absorbance of each sample was recited from the calibration curve according to the test preparation record. The detection limit was 1 µg/kg, and the recovery rate of 100 %. The recovery rate of less than 100 % shows more than 1 µg/kg of aflatoxin B_1. Values that exceeded the legal threshold of 2.0 µg/kg were considered unfit for consumption.

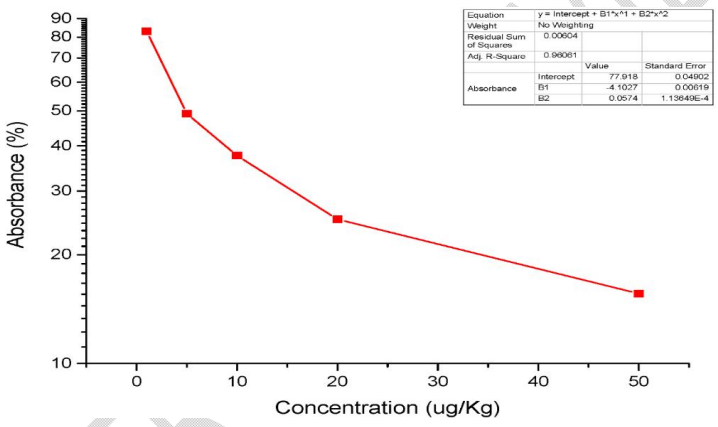

Figure 3. Standard curve of aflatoxin B_1 (R^2 = 0.96061).

The result revealed that 19 samples of surveyed rice grainshad been contaminated by aflatoxin B_1, as shown in Table 1. Quantitative analysis reveals that 43.1 % of inspectedsamples were found positive,of which 31.8 % were under permittedlevels. Nevertheless, 11.3 % were surpassing the accepted levels (table 3). The result of the present study demonstrates that the concentration of aflatoxin B_1 in tested rice grain samples extended from 2 µg/kg to 3.2 µg/kg using ELISA assay. The AFB_1 concentration average in the imported rice grains was higher than the findings in the local rice grains. However, no significant difference was observed in the AFB_1concentration mean in samples collected ($P < 0.05$).

Table 3. Occurrence and level of aflatoxin B_1 in rice grains

Source of Sample	№ of Samples	Sample Status		Concentration of Aflatoxin B_1(µg/Kg)			Permitted levels
		Positive	Negative	0	<2	>2	
Local	25 (56.8%)	**07** (15.9%)	18 (40.9%)	17 (38.6%)	**06** (13.6%)	**02** (4.5%)	<2µg/Kg (Commission Regulation No. 1881/2006)
Imported	**19 (43.1%)**	**12** (27.2%)	07 (15.9%)	08 (18.2%)	**08** (18.2%)	**03** (6.8%)	
Total	44 (99.999%)	**19** (43.1%)	25 (56.8%)	25 (56.8%)	**14** (31.8%)	**05** (11.3%)	

4. Discussion

In this study, genomic DNA for the norsolorinic acid gene region of aflatoxigenic strains, which is involved in the middle of the aflatoxin biosynthetic pathway, was amplified and sequenced. A comparison of *aflD* gene region sequences with aflatoxigenic strain sequences deposited in the GenBank showed a high similarity inthe aflatoxigenic strains isolated. Alignment of selected isolates showed a high evolutionary divergence. A close relationship was observed between *A. flavus, A. parasiticus and A. nomius* isolates, while *A. fumigatus* was distantly related to the *A. parasiticus* and *A. nomius* isolates. The isolates of *A. flavus* strains showed close evolutionary relationships, with all isolates clustering (Figure 4). The conservation in *A. flavus*, *A. parasiticus* and *A. nomius* may be related to the functional importance of the *aflD* gene,as the first stable precursor in the aflatoxin biosynthetic pathway, encodes an enzyme responsible for catalyzing the conversion of norsolorinic acid, the initial stable intermediate, into averantin across all aflatoxigenic strains[50; 51].

Gene expression analysesshowed variations, with three of four aflatoxigenic strains tested for *aflD*gene expression. We detected significantlyhigher gene expression stages of *aflD* in *A. flavus* than *A. parasiticus* and *A. nomius* strains. This may be linked to the aflatoxin toxicity in *A. flavus*,as reported by[52, 53]. *A. parasiticus* showed a weak expression level, followed by *A. nomius*. However, the detection of aflD in these strains confirms that they can equally be potential aflatoxin producers. Nevertheless, in contrast to these 3 strains in which relative gene expression levels were detected, we did not detect *aflD* in *A. fumigatus*. The relative fluorescence showed more concentration of RNA in 3 strains for each single curve observed (Figure 2-C). The amplification curves for all strains were specific and showeda single peak by melting curve analysis, as shown in Figure 2-D.

Further, we quantified aflatoxin B_1levels in rice grains of 44 samples taken from local retail markets and millers. 43.1 % of tested samples were positive for aflatoxin B_1, with 15.9 % of contamination for local rice grains and 27.2 % of pollution in imported rice. Seven of 25 rice

samples homegrown from Mwea contained aflatoxin B_1,ofwhich 13.6 % were below and 4.5 % above the permitted level. This observation might suggest alack of suitable grain management or procurement of low-quality rice grains from growers who do not observe appropriate post-harvest techniques.This is in line with the researchled in Turkey on the level and incidence of mycotoxin in rice[54]. Quantification of aflatoxin B_1 by ELISA assay showed a higher significance incidence in imported rice samples; 27.2 % of imported rice samples werecontaminated with aflatoxin B_1 in which 6.8 % of those samples have greater levels than the 2.0 µg/kg limit established by EU. These observations agree with others[55; 56;57] who surveyedthe incidence of aflatoxin B_1in rice grains. The highest proportion of contamination from imported rice could be explained by the storage, shipment and transport conditions along the way up to the destination point. Our findingsconcurwiththose reported by Nurshad [58], who investigated the incidence of aflatoxins in rice worldwide and communal health appearances from 1990 to 2015. In our study, the results displayed that the concentration of AFB_1 in rice grains analyzed ranges from < 2.0 µg/kg to 3.2 µg/kg. These results concur withthose described by Sangare-Tigori *et al.*[56], Compaoré *et al.* 2021and Anthony *et al.*[60] in West Africa (Ivory Coast, Burkina Faso and Nigeria), and by Madbouly *et al.*[61] in Egypt,which surveyed some mycotoxins and specially AFB_1 in rice grains with the range of <1.5-10.0 µg/kg, and 4.1-309.0 µg/kg, and with the limits of detection 11.0 µg/kg respectively.Here, we have established evidence of the occurrence of aflatoxinB_1in African staple cereal, highlighting the need for new guidelines and standards to safeguard the security of consumers of rice grains. The countries in Sub-Saharan Africa must endeavour to establish policies that can guide regular empirical food quality assessments, as this would enhance and guarantee a healthy population with better standards of living and sustained development for prosperity. Acceptance of assimilated strategies across different points of the value chain to avoid human exposure to aflatoxins in consumed rice grains is important.

Comment [HC6]: Aflatoxin contamination could well occur in the field before harvest, please mention this !

Comment [HC7]: We added the work carried out in Burkina Faso to reinforce the author's analysis on the contamination of rice with aflatoxins in West Africa!

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Conclusion

Based on molecular techniques, our analyses revealed the three aflatoxigenic strains*A. flavus*,*A. parasiticus*, and *A. nomius*are closely clustered within the same clade, suggesting the *aflD* gene plays a crucial role in regulating the biosynthetic pathway of aflatoxin production in aflatoxigenic species. The concentration of AFB1 in the analyzed samples ranged from 0 µg/kg to 3.2 µg/kg, with 43.1% of samples containing detectable levels. Notably, 11.3% of these samples exceeded the maximum tolerable limit for aflatoxins in rice, as set by the European Union (EU), which is 2 µg/kg for AFB1. These findings highlight the potential

exposure of humans, animals, and livestock to aflatoxin B1, posing significant health risks and leading to indirect economic impacts on society. This underscores the importance of educating the public on proper good cultural practices, grain handling-practices, especially during and after harvesting. Good practices pre- and post-harvest cereal respect as well as the development of grain protection techniques and technologies are necessary to protect consumers againstaflatoxindangers.Additionally, the study affirms the efficiency of ELISA method in detecting mycotoxins in cereals, offering advantages such as simplicity and reduced time requirements. Continuous monitoring of foodstuffs and agricultural products is essential to mitigate the risks associated with aflatoxin contamination. Such surveillance can help prevent critical issues in the marketing, distributing, and consuming contaminated food products.

Key Contribution: Aflatoxin contamination of cereals poses a serious concernfor human health worldwide. This article highlights aflatoxigenic strains, gene expression profile and aflatoxin B_1 levels in rice grains to ensurefood safety and security.

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