Research Article

Isolation, Characterization and Genetic Diversity of *Aspergillus flavus* in Animal Feed

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Abstract | *Aspergillus flavus* is known for producing aflatoxins (AFs), which are harmful mycotoxins that can spoil food crops and represent significant health risks to animals and humans. The aim of this study was to analyze fifty samples of livestock and poultry feed to identify mycotoxigenic fungi at both morphological and molecular levels, focusing on their toxigenic potential and genetic diversity. Out of the samples tested, six were confirmed as *Aspergillus flavus* using internal transcribed spacer (ITS) specific primers, accounting for approximately 12 % of the total detected microorganisms. Morphological and molecular analyses revealed that all strains exhibited 97-100 % similarity with a reference strain and were significant producers of B-type AFs. The data showed that all samples except one (S1) contained total aflatoxin levels below 20 µg/kg, considered safe for animal consumption according to the European Union (EU), United States Food and Drug Administration (US FDA), and World Health Organization (WHO) guidelines. To assess the genetic variability among *A. flavus* strains, twelve inter simple sequence repeats (ISSR) primers and seven Sequence Related Amplified Polymorphism (SRAP) primer combinations were utilized, producing scorable and reproducible banding patterns with about 52 % polymorphism. Various genetic diversity parameters, including polymorphic information content (PIC), effective multiplex ratio (EMR), marker index (MI), and resolving power (RP) were evaluated to determine effectiveness of the primers in distinguishing the genetic variations among the *A. flavus* strains. As particularly valuable markers, the results indicated that ISSR-13, SRAP-1, and SRAP-6 exhibited higher PIC, RP, and MI values, thereby proving to be more informative for identifying the genetic variants.

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Introduction

Mycotoxins are toxic secondary metabolites produced by fungi that contaminate several products of agricultural and food substances and represent a serious risk to the humans and animals health (Alshammari, 2023). The United Nations Food and Agriculture Organization estimated that

approximately 25 % of the global crop production is yearly contaminated by mycotoxins (Zhang *et al*., 2022). Mycotoxin-contaminated products can lead to acute and chronic toxicity (Popescu *et al*., 2023), resulting in high production costs, decreased animal performance, and reduced profitability (Kolawole *et al*. 2024).

The toxigenic fungi play critical roles in food processing and quality, with mycotoxin production highly dependent on several ecological factors such as moisture, temperature, aeration, light, pH, substrate type, and animal species (Elkenany and Awad, 2021). In particular, the climatic conditions in African and Southeast Asian countries significantly influence AF contamination in the various agricultural products (Jallow *et al*., 2021). At the same time, global warming may introduce AF risks to previously unaffected regions, including Europe (Leggieri *et al*., 2021). AFs are the primary mycotoxins produced by various *Aspergillus* species, particularly those in the Flavi section (Alameri *et al*., 2023). These toxins are commonly found in food and feed and are among the most potent natural carcinogens, leading to serious public health and economic issues for farmers and consumers worldwide (Makhlouf *et al*., 2019).

The most common and harmful types of AFs include AFB1, AFB2, AFG1, AFG2, AFM1, and AFM2. Specifically, *A. flavus* produces AFB1 and AFB2, while *A. parasiticus* generates AFG1 and AFG2 (Pandey *et al*., 2019). Conversely, AFM1 is a metabolite of AFB1 hydroxylation formed in the livers of dairy cows that consume contaminated feed (Min *et al*., 2021). Consequently, the AFB and AFG types are commonly found in food and feed crops, while AFM2 and AFM1 (B1 metabolite) are primarily detected in animal byproducts such as milk and dairy products (Schamann *et al*., 2022). The mycotoxigenic fungus *A. flavus* is responsible for the widespread contamination of key crops; with AFB1 being the most dangerous and carcinogenic natural chemical compound. According to Makhlouf *et al*. (2019), chronic exposure to AFB1 is a leading cause of hepatocarcinoma with AFB1-contaminated foods linked to the greatest number of deaths and disability-adjusted life years (DALYs).

Given its ability to produce carcinogenic mycotoxins and infect immunocompromised individuals, *A. flavus* is of a particular concern. The Flavi section contains 35 species grouped into eight series (Djenontin *et al*., 2023); some of these species are morphologically indistinguishable as they all share morphological traits. However, data from whole-genome sequencing has revealed metabolic and genetic differences among these species (Kjaerbølling *et al*., 2020). Therefore, studying the genetic diversity of *A. flavus* is crucial for understanding its evolutionary characteristics and developing control and prevention strategies (Zhang *et al*., 2022).

Advancements in molecular biotechnology have led to the widespread use of DNA marker technologies to study genetic variation in the biological populations. Multiple techniques such as DNA sequencing, RFLP, AFLP, RAPD, SSR, ISSR, and SRAP are now commonly used (Abastabar *et al*., 2022). The ITS region of nuclear DNA is frequently sequenced to detect fungal taxonomy at both the species and intra-species levels (Chinnasamy *et al*., 2023). SRAP is a co-dominant marker system that amplifies genome-wide fragments efficiently, combining simplicity and reliability (Awaly and Ahmed, 2020). The ISSR technique uses a single primer to amplify regions between microsatellites without requiring prior DNA sequence knowledge. It is a highly reproducible, efficient, rapid, informative, and widely used economical technique. Both ISSR and SRAP are used for assessing genetic variation, genome mapping, fingerprinting, construction of linkage maps, and phylogenetic trees of a wide range of plant-pathogenic fungi (Salim *et al*., 2019).

Contamination of livestock and poultry feed by fungi may occur during shipping, storage, and marketplaces. Fungal contamination, particularly by *Aspergillus* species in the Flavi section, is a global issue affecting poultry and livestock feed. Therefore, the objectives of this study were to isolate and identify *A. flavus* from different livestock and poultry feed samples using morphological and ITS molecular techniques. Furthermore, quantitative and qualitative AF analyses of the isolated *A. flavus* strains were evaluated using the liquid chromatography-tandem mass spectrometry analysis (LC-MS-MS) technique. Finally, the genetic biodiversity and relationships among the *Aspergillus* isolates was established by combining data from ISSR and SRAP molecular markers.

Materials and Methods

Samples collection

Twenty-five samples of each of livestock and poultry feeds were purchased from 25 different shops in Giza Governorate, Egypt. Approximately 450 g of each feed type were randomly collected from each shop. The samples were stored in sterile plastic bags at room temperature until used.

Isolation and morphological identification

To isolate the mycotoxigenic fungi, 5 g of each sample were mixed with 45 ml of peptone buffer and shaken vigorously. Ten microliters of each suspension were aseptically inoculated in quintuplicate onto Rose Bengal agar plate (Lab M, Neogen Company, UK). The plates were incubated in darkness at 25 ˚C for 5-7 d. After purification using a single spore colony technique, identification of the obtained *A. flavus* isolates was based on macroscopic morphological characteristics descriptions, including colony color, growth, texture, size, and other characteristics according to Samson *et al.* (2007). Microscopic features such as presence of vesicles, conidia, phialides, matulae, and conidiophores were also examined. All isolates were preserved at –20 ˚C in rose bengal broth mixed with 20 % glycerol.

Liquid chromatography-tandem mass spectrometry analysis (LC-MS-MS)

The quantitative and qualitative analyses of aflatoxins produced by *A. flavus* isolates were carried out using the LC-MS-MS technique as described by Nualkaw *et al.* (2020). In this technique, chromatographic separation of the analyte was performed on tandem mass spectrometry applied bio systems AB Sciex 4000 Q trap coupled with HPLC 1200 series Agilent Technologies and Column C18 Eclipse XDB (5µm, 4.6 × 150 mm). Mobile phase A consisted of an aqueous solution with 0.1 % formic acid (v/v), while mobile phase B composed of 0.1 % formic acid (v/v) in Acetonitrile. Mass spectrometer parameters were optimized using an electrospray ionization source in the positive ionization mode.

Molecular identification of the fungal isolates

DNA yield and purity was assessed using a NanoDrop spectrophotometer (NanoDrop 2000, ThermoFisher Scientific, Germany) and agarose gel electrophoresis (Bio-Rad, USA. Six *A. flavus* isolates were selected based on their morphological characteristics, re-cultured in rose bengal broth that consisted of mycological peptone 5.0, dextrose 10.0, Dipotassium phosphate 1.0, Magnesium sulphate 0.5, Rose Bengal 0.05, agar 12.0, and dist. water 1 l, pH 7.2 (0.2), and then incubated at 25 ˚C. After 48 h, the developing mycelia were harvested and pulverized with liquid nitrogen. The cells were harvested by centrifugation at 12000 g for 5 min. After washing the pellets for three times using 0.85 % saline solution, genomic DNA was extracted using the GeneJET Genomic DNA purification Kit (Thermo Fisher Scientific, Lithuania). The yields and purity of DNA were evaluated by NanoDrop (NanoDrop 2000, ThermoFisher Scientific, Germany) and agarose gel electrophoresis (Bio-Rad, USA). The ITS region was amplified using two primers: *ITS1* (5′-TCC GTA GGT GAA CCT GCG G-3′) and *ITS4* (5′-TCC TCC GCT TAT TGA TAT GC-3′) (Luo and Mitchell, 2002). The polymerase chain reaction (PCR) amplification program was conducted using a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems). PCR products were purified using a gel extraction kit and sequenced by Macrogen, Inc. (Seoul, South Korea) on an ABI 370 × 1 DNA sequencer (Applied Biosystems, USA). Sequence analysis was performed using the BLAST V2.0 application (http://www.ncbi.nlm.nih.gov/BLAST/).

The genotypic diversity evaluation

The genetic diversity of *A. flavus* isolates was evaluated using SRAP and ISSR molecular markers.

Sequence-related amplified polymorphism (SRAP) analysis: *Aspergillus flavus* isolates were subjected to SRAP analysis using seven primer combinations, made from forward primers (“Me”) targeting GC-rich exon regions and reverse primers (“Em”) targeting AT-rich intron regions (Table 1). The amplification was conducted using a Biorad thermocycler (USA) as per the protocol adapted by Zhang *et al.* (2022) with slight modifications. SRAP analysis was performed using 20 µl reaction volume that contained: 1 µ (40 ng) DNA, 10X PCR reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 1 µl from forward and reverse primers (2 µM/ µl of primers) and 7 µl dist. H_2O. PCR reactions were conducted under the following conditions: the initial step of 5 min. at 94 ˚C, then 5 cycles of 1 min. at 94 ˚C, 1 min. at 35 ˚C, 1 min. at 72 ˚C followed by 35 cycles comprised 1 min. at 94 ˚C, 1 min. at 50 ˚C, 2 min. at 72 ˚C, a final extension for 10 min. at 72 ˚C, and then cooling down to 15 ˚C.

Table 1: *Names and sequences used for SRAP analysis of Aspergillus flavus isolates.*

Forward name	Sequence primer	Reverse name	Sequence primer
Me1	5′-TGAGTCCAAACCGGATA-3′	Em1	5′-GACTGCGTACGAATTAAT-3′
Me2	5′-TGAGTCCAAACCGGAGC-3′	Em2	5′-GACTGCGTACGAATTTGC-3′
Me3	5′-TGAGTCCAAACCGGAAT-3′	Em3	5′-GACTGCGTACGAATTGAC-3′
Me4	5′-TGAGTCCAAACCGGACC-3′	Em5	5′-GACTGCGTACGAATTAAC-3′

Inter simple sequence repeat (ISSR) analysis: The PCRs were carried out using twelve ISSR primers, where their sequences are shown in Table 2. ISSR-PCR was performed according to the method conducted by Ibrahim *et al.* (2019). PCR amplification was carried out in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems). A reaction volume of 20 µl containing 1 µl DNA template (20 ng/ µl), 10 µl master mix (Biotecke Corporation), 1 µl primer (100 ng/ µl), and 8 µl dist. water was used. The PCR condition was one cycle of 5 min. at 94 ˚C (initial denaturation) followed by 37 cycles of denaturation at 94 ˚C for 1 min., annealing at 52-56 ˚C for 1 min., extension at 72 ˚C for 2 min., and followed by a final extension at 72 ˚C for 10 min.

Phylogenetic diversity analysis

To explore the evolutionary history of *A. flavus* isolates, a phylogenetic tree was constructed using the neighbor-joining (NJ) method based on ITS sequences, utilizing MEGA 5.2 software (Molecular Evolution Genetic Analysis version 5.2) (Kumar *et al.*, 2008; Tamura *et al.*, 2011). A maximum composite likelihood method was employed for this analysis.

Table 2: *Names and sequences used for ISSR analysis of Aspergillus flavus isolates.*

Primer	Sequence
ISSR- 01	5'-AGAGAGAGAGAGAGAGC-3'
ISSR- 02	5'-AGAGAGAGAGAGAGAGG-3'
ISSR- 03	5'-ACACACACACACACACT-
ISSR- 05	5'-GTGTGTGTGTGTGTGTG-3'
ISSR- 06	5'-CGCGATAGATAGATAGATA-3'
ISSR- 07	5'-GACGATAGATAGATAGATA-3'
ISSR- 08	5'-AGACAGACAGACAGACGC-3'
ISSR- 09	5'-GATAGATAGATAGATAGC-3'
ISSR- 11	5'-ACACACACACACACACA-3'
ISSR- 12	5'-ACACACACACACACACC-3'
ISSR- 13	5'-AGAGAGAGAGAGAGAGT-3'
ISSR- 14	5'-CTCCTCCTCCTCCTCTT-3

Analysis of markers data

A binary data matrix was generated by visually scoring amplified fragments with the same gel mobility as 1 for presence or 0 for absence for all samples. The final data sets included both polymorphic and monomorphic bands. Genetic diversity parameters such as polymorphism information content (PIC), information index (I), effective multiplex ratio (EMR), and resolving power (Rp) were calculated following the methods described by Chesnokov and Artemyeva (2015). Dice's similarity matrix coefficients were computed among genotypes using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA). The PAST software (Version 1.91) computer program (Hammer *et al.*, 2001) was used to construct the dendrogram or phylogenetic trees, according to the Euclidean similarity index depending on Dice's coefficients similarity matrix.

Statistical analysis

A standard deviation (SD) ± mean was used to express the data. Graph Pad Prism software 7.0 was used to statistically assess the isolates' differences using two-way analysis of variance (ANOVA) and Tukey's multiple comparison test, with a significance level of $p < 0.05$ (Tukey, 1949).

Results

Morphological identification of the isolated fungi

About fifty-one fungal isolates were obtained from the livestock and poultry feed samples. Six out of these fifty-one isolates (approximately 12 %) exhibited the typical morphological and microscopic characteristics of *A. flavus*, including a yellowish-green colony with colorless or yellowish on the reverse side. The isolates featured smooth globose conidia, and long and rough conidiophores in the distal region; with heads that were typically radiating and biseriate, occasionally forming columns in the aerial mycelium. The diameter of the conidiophores was about 4.5-6 µm. These morphological results are illustrated in Figure 1.

Table 3: *The closest hits and accession numbers of the Aspergillus flavus isolates and their aflatoxins concentration.*

Isolate no.	Source of isolation	Closest hits	Gene identity %	Genbank accession	AFB1 (µg/ kg)	AFB2 (µg/ kg)	Total AFB (µg/ kg)
S1	Poultry feed	*A. flavus* strain IBB_17	100 %	MH793837.1	19.49	1.53	21.02
S11	Animal feed	*A. flavus* strain ND52	98.81 %	MG659646.1	13.73	2.31	16.04
S4	Poultry feed	*A. flavus* strain v312-74	98.29 %	OR418503.1	4.77	0.47	5.24
S7	Poultry feed	*A. flavus* isolate FZM1	97.51 %	OR905958.1	6.43	3.33	9.76
S6	Poultry feed	*A. flavus* isolate KC491416	99.62 %	LN812958.1	7.37	0.55	7.92
S6(2)	Poultry feed	*A. flavus* isolate ATT	99.24 %	OR149208.1	9.59	1.23	10.82

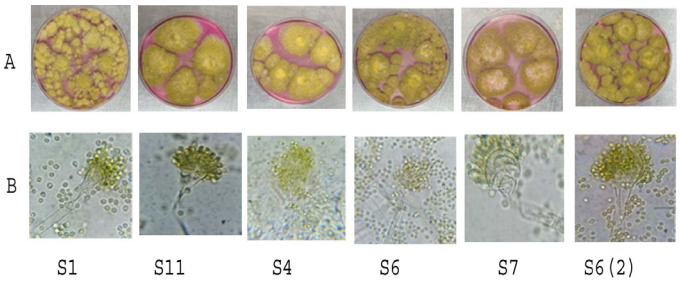

Figure 1: *The major morphological of A. flavus isolates.* **A:** *Colonies on Rose Bengal agar at 25 ˚C typically consisted of a dense felt of yellow-green conidiophores, reaching a diameter of 3-5 cm in 5-7 d.* **B:** *Microscopic morphological characteristics: Conidial heads usually radiate, turning from yellow-green to dark yellow-green and then breaking into multiple loose columns. Hyaline and coarsely roughened conidiophores that can reach a length of 1.0 µm (with some isolates reaching 2.5 µm). Vesicles, 25-45 µm in diameter, which were globose to sub-globose.*

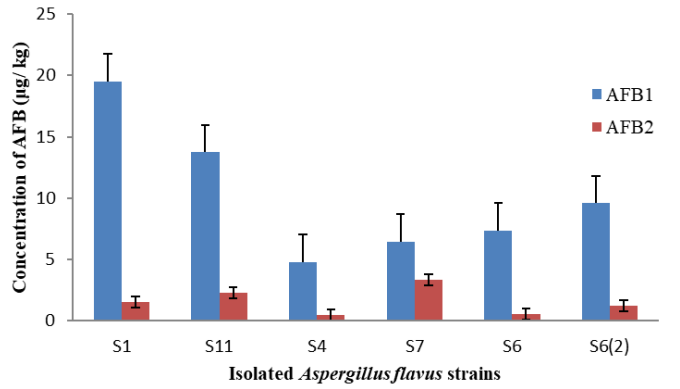

Figure 2: *Aflatoxin B (AFB) concentrations detected in the six Aspergillus flavus strains using the liquid chromatography-tandem mass spectrometry (LC-MS-MS) analysis. Error bars represent the standard deviation (±SD).*

Evaluation of toxigenic potential of the Aspergillus flavus strains

To assess the risk associated with the presence of *A. flavus* strains in feed, the potential of these isolated fungal strains to produce various toxins was evaluated using the LC-MS-MS system (Table 3 and Figure 2). All strains expressed significantly higher toxigenic potential for AFB1 compared to AFB2 ($p< 0.05$). Table 4 highlights significant differences in the ability of the isolated strains to produce the two types of AFB, with no significant differences observed between replicates of the same strain. The S1 strain produced the highest toxicity for AFB1 (19.49 µg/ kg) followed by the S11 strain (13.73 µg/ kg). In contrast, the S7 strain produced high toxicity for AFB2 (3.33 µg/ kg) followed by the S11 strain (2.31 µg/ kg), The S4 strain showed the lowest significant toxigenic potential for both AFB1 and AFB2, recording 4.77 µg/ kg and 0.47 µg/ kg, respectively.

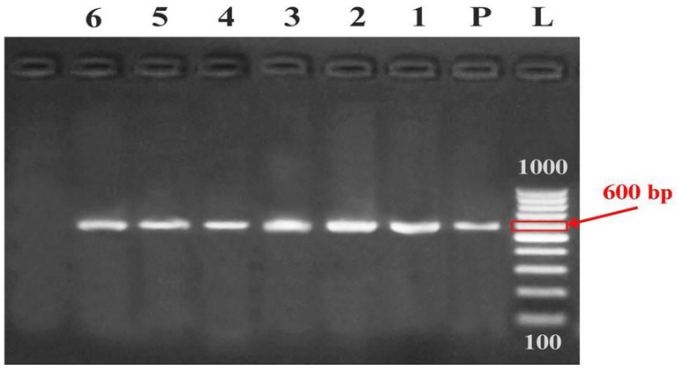

Figure 3: *ITS region amplification of the six isolated Aspergillus flavus strains (lanes 1: S1, 2: S11, 3: S4, 4: S7, 5: S6-1, and 6: S6-2) demonstrating a single band at 600 bp of a 100 bp Ladder marker (L) and positive control (P).*

Table 4: *The combined analysis of variance (ANOVA) showing potential of the isolated toxigenic strains for aflatoxins production.*

S.V	d.f	AFB1 (MS)	AFB2 (MS)	$F_{0.05}$
Replicates	2	0.11^{ns}	0.046^{ns}	4.10
Strains	5	90.55^{**}	19.95^{**}	3.35
Error	10	0.043	0.044	
Total	17			

*Where; S.V.: source of variance, d.f.: degree of freedoms, MS: mean square, ns: non-significant at 0.05 %, **: indicates high significance*

Molecular identification of the Aspergillus flavus isolates

The molecular identity of the six isolated fungi was confirmed using ITS 1 and ITS4 primers that produced a unique band at 600 pb for each isolate (Figure 3). Each band was subsequently sequenced.

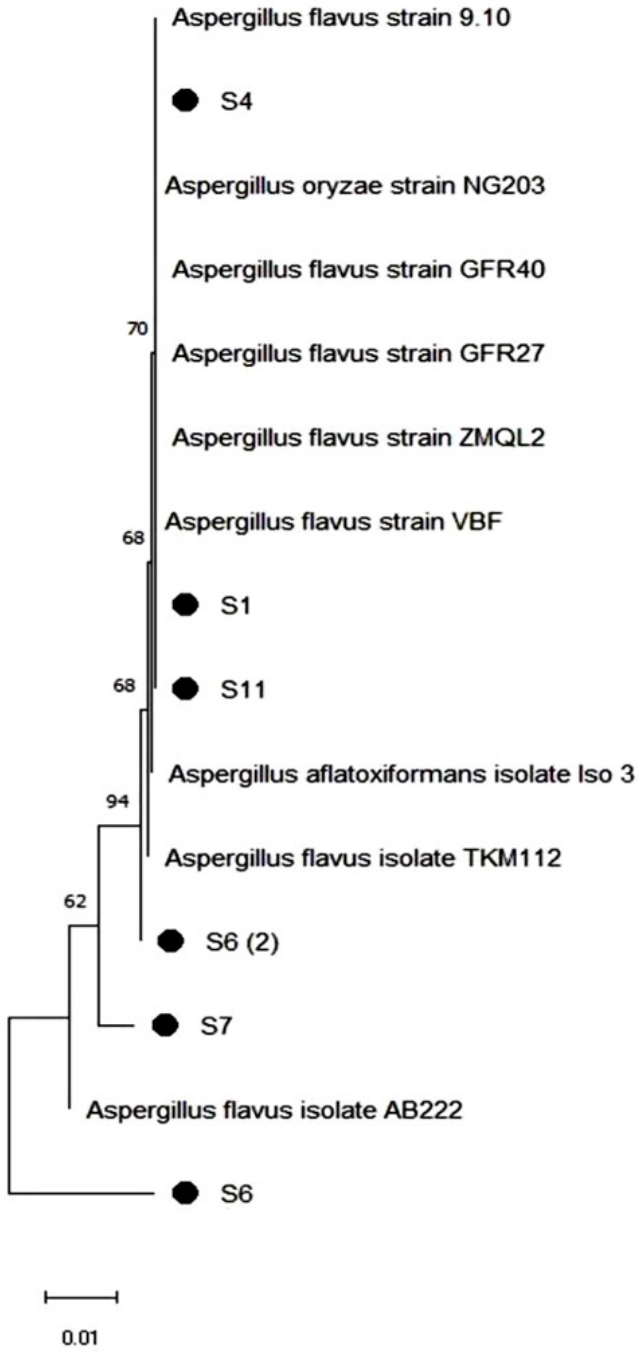

Figure 4: *The NJ phylogenetic tree of six Aspergillus flavus strains with the closest hits in GenBank.*

Comparing the ITS sequences of the six isolates with the database on the BLAST website revealed 97-100 % similarity, confirming their identity as *A. flavus* (Table 3). Neighbor-joining (NJ) analysis of the evolutionary relationships based on the ITS region sequences indicated that the isolated fungi were closely related to *A. flavus*. Furthermore, isolates belonging to the same species were grouped into the same clade or sub-clade (Figure 4). The NJ phylogenetic tree was divided into two major clades; the second clade containing the S6 isolate. The first major clade was further divided into two sub-clades A and B. Sub-clade A was branched into groups I and II containing the S7 isolate. Group 1 was further splited into two sub-groups A and B, where sub-group B included the S6 (2) isolate while sub-group A contained the S1, S4, and S11 isolates.

Genotypic divergences and fingerprints of Aspergillus flavus strains

Twelve ISSR and seven SRAP primer combinations showed high stability, producing sharp bands for further analysis using DNA samples from six strains of *A. flavus* (Figures 5 and 6, respectively). Analysis of PCR products revealed that 72 out of 139 ISSR total bands and 49 out of 96 SRAP total bands were highly polymorphic, representing 52.6 % and 52.3 % polymorphism, respectively. The band sizes ranged from 1568 to 89 bp for ISSR and 840 to 100 bp for SRAP (Tables 5 and 6). The genetic variation parameters were calculated for both ISSR and SRAP primers.

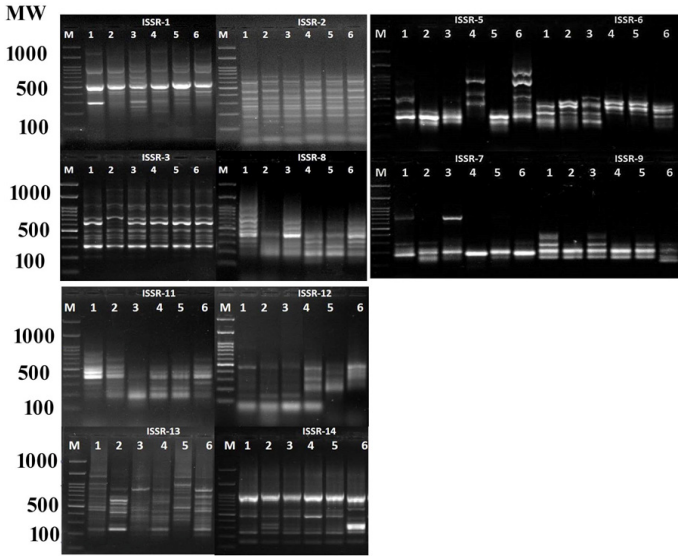

Figure 5: *ISSR marker amplification of the six isolated Aspergillus flavus strains (lanes 1-S1, 2-S11, 3-S4, 4-S7, 5-S6-1, and 6-S6-2) and M- 100 bp Ladder marker.*

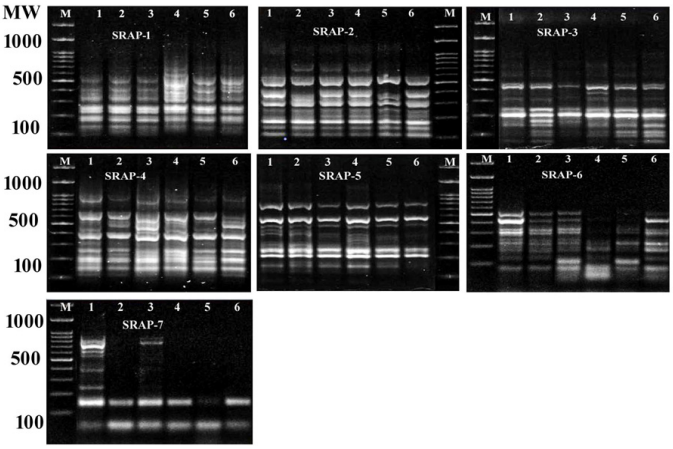

Figure 6: *SRAP marker amplification of the six isolated Aspergillus flavus strains (lanes 1-S1, 2-S11, 3-S4, 4-S7, 5-S6-1, and 6-S6-2) and M- 100 bp Ladder marker.*

Table 5: *PCR amplicons obtained from ISSR markers in Aspergillus flavus isolates.*

Number	Primer name	TBN	MBN	PBN	P (%)	EMR	PIC	MI	RP	BSR
1	ISSR-1	17	5	12	71	7.25	0.27	1.92	19.00	1568- 210
2	ISSR-2	14	12	2	14	12.28	0.06	0.68	24.33	725-119
3	ISSR-3	15	11	4	27	12.14	0.10	1.25	23.67	717-209
4	ISSR-5	9	1	8	89	3.25	0.35	1.14	9.67	1046-175
5	ISSR-6	8	3	5	63	5.53	0.24	1.34	13.00	497-175
6	ISSR-7	8	3	5	63	4.08	0.27	1.11	10.33	698-116
7	ISSR-8	13	5	8	62	6.83	0.23	1.58	16.67	349-141
8	ISSR-9	9	5	4	44	5.50	0.19	1.02	12.67	980-139
9	ISSR-11	9	5	4	44	6.42	0.17	1.07	14.33	750-201
10	ISSR-12	10	5	5	50	6.17	0.23	1.44	14.67	478-89
11	ISSR-13	15	2	13	87	6.78	0.34	2.31	16.33	1258-190
12	ISSR-14	12	10	2	17	10.81	0.07	0.71	22.33	919-153
	Total	139	67	72						
	Mean				52.6	7.25	0.21	1.3	16.42	

Where; TBN: total band number, MBN: monomorphic band number, PBN: polymorphic band number, P (%): polymorphism percentage, EMR: effective multiplex ratio, PIC: polymorphism information content, MI: Marker index, RP: resolving power, BSR: band range size.

Table 6: *PCR amplicons obtained from SRAP markers in Aspergillus flavus isolates.*

No.	Primer name	TBN	MBN	PBN	P (%)	EMR	PIC	MI	RP	BSR
1	SRAP-1 (Me1+Em1)	16	11	5	31	12.14	0.11	1.34	21.67	650-130
2	SRAP-2 (Me1+Em3)	12	8	4	33	9.25	0.15	1.41	20.33	670-120
3	SRAP-3 (Me2+Em3)	17	8	9	53	11.08	0.21	2.28	17.67	840-130
4	SRAP-4 (Me2+Em5)	12	5	7	58	8.58	0.15	1.31	19.00	460-110
5	SRAP-5 (Me3+Em2)	17	9	8	47	11.47	0.20	2.29	22.00	750-110
6	SRAP-6 (Me3+Em3)	13	3	10	77	6.36	0.33	2.09	17.00	490-100
7	SRAP-7 (Me4+Em3)	9	3	6	67	3.33	0.22	0.74	8.67	790-110
	Total	96	47	49						
	Mean				52.3	8.89	0.2	1.64	18	

Where; TBN: total band number, MBN: monomorphic band number, PBN: polymorphic band number, P (%): polymorphism percentage, EMR: effective multiplex ratio, PIC: polymorphism information content, MI: Marker index, RP: resolving power, BSR: band range size.

These parameters provided valuable insights into the diversity and variability within the isolated *A. flavus* strains. The obtained data showed that the mean values of PIC for ISSR and SRAP were 0.21 and 0.2, for EMR: 7.25 and 8.89, for MI: 1.3 and 1.64, and for RP: 16.42 and 18, respectively. Among the primers, ISSR-2 and ISSR-14 had the lowest number of polymorphic bands (PBN) at 2 bands representing 14 % and 17 % polymorphism, respectively. These primers also had the lowest PIC (0.06 and 0.7, respectively) and MI (0.68 and 0.71, respectively), but they had the highest EMR (12.28 and 10.81, respectively) and RP (24.33 and 22.33, respectively). In contrast, ISSR-13 produced the highest PBN of 13 bands, representing 87 %, while ISSR-5 had the highest percentage of polymorphism at 89 % out of 8 PBN. These primers also recorded the highest PIC (0.34 and 0.35, respectively) and MI (2.31) for ISSR-13, while ISSR-5 had the lowest EMR (3.25) and RP (9.67). Similarly, the SRAP-6 produced the highest PBN 10 bands, representing the highest polymorphic bands percentage at 77 % and the highest PIC was 0.33. The SRAP-2 generated the lowest PBN 4 bands representing 33 %, while the SRAP-1 recorded the lowest polymorphic bands percentage at 31 % out of 5 PBN. These primers also had the lowest PIC (0.11 and 0.15, respectively), while SRAP-1 had the highest EMR (12.14); however, SRAP-7 displayed the lowest EMR (3.33), MI (0.74), and RP (8.67). Evaluation of the genotype-specific markers for the six isolated strains of *A. flavus* was presented in Tables 7 and 8. A total 26 specific markers were identified for ISSR and

Table 7: *Aspergillus flavus strains and their specific ISSR markers.*

Genotypes	Positive unique marker	Negative unique marker	Total marker
S1	(ISSR-8) 682-980, (ISSR-11) 750	(ISSR-8) 186	4
S11	(ISSR-13) 342	(ISSR-1) 283, (ISSR-13) 310-418	4
S4	(ISSR-1) 569-253-228, (ISSR-2) 384, (ISSR-7) 349	(ISSR-6) 329, (ISSR-11) 251, (ISSR-13) 650	8
S7	(ISSR-1) 210, (ISSR-8) 139	(ISSR-5) 270-217, (ISSR-13) 465	5
S6	(ISSR-1) 324	-	1
S6(2)	(ISSR-5) 1046, (ISSR-9) 141	(ISSR-6) 398, (ISSR-14) 502	4
Total			26

Table 8: *Aspergillus flavus strains and their specific SRAP markers.*

Genotypes	Positive unique marker	Negative unique marker	Total marker
S1	(SRAP-7) 550-560, 590-700	(SRAP-4) 130	5
S11	(SRAP-4) 180	-	1
S4	(SRAP-3) 430	-	1
S7	(SRAP-1) 560, (SRAP-4) 360	(SRAP-6) 220-320	4
S6	-	(SRAP-3) 190	1
S6(2)	-	(SRAP-3) 560, (SRAP-5) 160-350	3
Total			15

15 for SRAP. Among the ISSR markers, the S1 strain exhibited the highest number with 5 specific markers, followed by the S7 strain with 4, the S6 strain with 3, and the other strains with 1 each. The data obtained from SRAP analysis showed that the S4 strain recorded the highest number (8) followed by the S7 strain (5) and then (4) by the other strains, except for the S6 strain that revealed a single specific marker.

Table 9: *Dice's coefficients similarity matrix of the pooled ISSR-SRAP primers among the six isolated Aspergillus flavus strains.*

	S1	S11	S4	S7	S6-1	S6-2
S1	1.00					
S11	0.85	1.00				
S4	0.83	0.83	1.00			
S7	0.81	0.84	0.82	1.00		
S6-1	0.81	0.84	0.86	0.86	1.00	
S6-2	0.86	0.86	0.80	0.83	0.84	1.00

Genetic relationships and phylogenetic trees of the isolated strains

Genetic variation and relationships among the six isolated *A. flavus* strains was assessed using Dice's similarity matrix coefficients. The similarity matrix of pooled ISSR-SRAP (Table 9) indicated that the S6(2) strain exhibited the highest similarity 86 % with strains S1 and S11, while revealing the lowest similarity 80 % with strain S4. A phylogenetic tree was constructed using UPGMA analysis of pooled ISSR-SRAP data for the six *A. flavus* strains (Figure 7). The cluster analysis was separated into two main clades. The first main clade was further divided into two sub-clades. The first sub-clade showed a close relationship between strains S4 and S6, while the second sub-clade included S7. The second main clade also had been splited into two sub-clades, the first sub-clade showing a close relationship between strains S11 and S6 (2) while the second sub-clade contained strain S1.

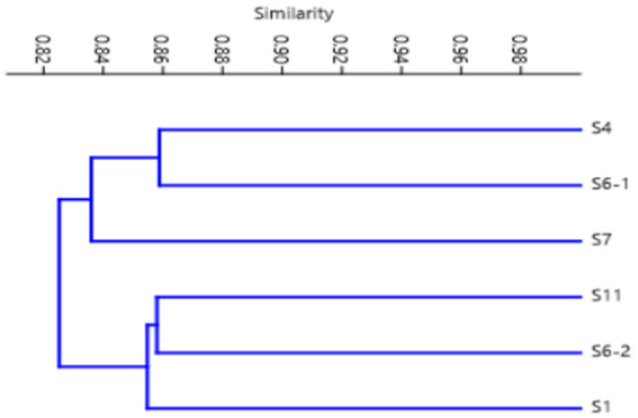

Figure 7: *Phylogenetic tree of the six Aspergillus flavus strains based on pooled ISSR-SRAP markers.*

Discussion

Fungi that grow on feed and food crops can

produce mycotoxins as toxic secondary metabolites, significantly impacting human health. Mycotoxins in feed can enter the food chain through contaminated animal products such as milk and meat, leading to various health issues depending on the type and concentration of the existing mycotoxin. In addition, mycotoxins can decrease feed intake and weight gain in animals, resulting in lower levels of essential nutrients in animal-derived food products (Popescu *et al*., 2023). Globally, *A. flavus* is the most dangerous fungal species that contaminates food and feeds and produces AFs (Tai *et al*., 2020). This study aimed to isolate, characterize, and identify mycotoxigenic fungi in feed, successfully isolating six *A. flavus* strains from different livestock and poultry feed samples. All the fungal strains were isolated from poultry feeds except for the S11 strain that obtained from animal feed. This could be attributed to high maize content in poultry feeds, often associated with the presence of toxigenic fungi during harvest, which pose contamination risks throughout post-harvest stages which may extend for more than a year. Maize is a primary source of *A. flavus* and its associated AFs in the temperate regions (Nsabiyumva *et al*., 2023). According to Reddy and Salle (2011), strains of *A. flavus* and *A. parasiticus* are responsible for 81.2 % of maize feed contamination. Morphological characteristics are still commonly used to identify *Aspergillus* species and classify fungal isolates or sections, facilitating further identification using various techniques (Krulj *et al*., 2020). Therefore, the current macroscopic analysis of the six isolated fungi revealed typical *A. flavus* morphological characteristics. Regardless of similarities in morphological features, variations among the fungal isolates were observed in terms of color shade, colony structure, conidiophores, and the potential for sclerotia formation upon cultivating of these isolates under similar conditions. The traditional microbiological identification techniques did not fully provide the exact and clearly defined categorization of *A. flavus*, thus a combination of conventional methods and molecular markers is preferable for accurately identifying the isolates' species, as the application of molecular approaches is required for a dependable and accurate identification of the tested fungal isolates. The ITS sequence serves as a universal barcode for fungal species identification (Krulj *et al*., 2020). In this study, ITS sequencing confirmed the identity of the isolated fungi, showing 97-100 % similarity with *A. flavus* reference strains, indicating that all six isolates were *A. flavus* strains and major producers of AFB (Table 3). Aflatoxin is classified as group 1 carcinogen for both animals and humans by the International Agency for Research on Cancer (IARC) (Krulj *et al*., 2020). Thus, several governments, including the European Union Commission (EUC), United States Food and Drug Administration (US-FDA), and World Health Organization (WHO), imposed legal restrictions on the maximum amounts of mycotoxins that can be present in food and feed, due to their negative impacts on human and animal health. The current results revealed that out of the six samples contaminated with *A. flavi*, only one (S1) exceeded the limit of 20 µg/ kg total AF levels that are allowed in feed by the EUC, Food and Drug Administration (FDA (Guerre, 2016), and WHO/ US-FDA (Jonathan *et al*., 2024), recording 21.02 µg/ kg, where the difference was insignificant (p= 0.5). The other isolates contained total AF levels below this threshold (20 µg/ kg). These results agree with those of Al-Hindi *et al*. (2017), who reported that 15.4 % of *A. flavus* isolates generated AFB1 Rin values ranging from 1.6 to 12.4 µg/ l. Similarly, Salim *et al*., (2019) reported that six out of twenty-five *A. flavus* isolates produced AFs; with a contamination percentage up to 24.0 %. Understanding the genetic diversity, molecular genetics, and metabolic profiles of the mycotoxigenic fungi is crucial for managing mycotoxins contamination in the various crops. Analyzing both inter- and intraspecific genetic variability of *A. flavus* through molecular markers is essential for effective control and preventative measures (Alshammari, 2023). This study employed SRAP and ISSR markers to analyze genetic variability among the obtained *A. flavus* strains, utilizing twelve ISSR primers and seven SRAP primer's combinations, resulting in scorable and reproducible banding patterns with approximately 52 % polymorphism. Genetic diversity parameters, including PIC, EMR, MI, and RP were used to assess the markers effectiveness in determining variability among the isolated fungal strains. Our findings indicated that ISSR-13, SRAP-1, and SRAP-6 were particularly informative markers exhibiting higher PIC, RP, and MI values. PIC is a measure of the market's ability to discriminate, which is equivalent to genetic diversity, because it depends on the number of known alleles and their frequency distribution. The maximum PIC value was 0.5 for the dominant markers. Furthermore, MI is an indicator of the overall usefulness of the maker system, a statistical measure whose value increases with the primer quality. Meanwhile, the resolving power (Rp) is a measure of

a primer combination's capacity to identify variations among a large number of genotypes (Chesnokov and Artemyeva, 2015). Furthermore, the present strains' genetic similarity coefficient of these markers was less than 1, which indicated that SRAP and ISSR could distinguish among the isolated *A. flavus* strains. Furthermore, UPGMA analysis separated the six isolated *A. flavus* strains into two clades.

Conclusions and Recommendations

Only a small percentage of the feed samples (six out of fifty samples representing approximately 12 %) was contaminated with *A. flavi* as confirmed by molecular identification and phylogenetic analysis, which aligned with the morphological identification. The obtained results indicated that, except for sample S1, the isolated samples contained total AF levels below 20 µg/ kg, considered safe for animal consumption. Nevertheless, biocontrol must be enhanced by other treatments strategies such as raising awareness, harvesting at a suitable time, quick drying of grains, suitable storage buildings, sorting, processing, and pre- and post-harvest pest control. Moreover, this study highlighted the effectiveness of ISSR and SRAP markers in estimating genetic variability among the isolated *A. flavus* strains, producing scorable and reproducible banding patterns with approximately 52 % polymorphism. Results of the genetic diversity parameters analyses, including PIC, EMR, MI, and RP confirmed that these markers are suitable for evaluating genetic variability within *A. flavus* strains.

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None.

Novelty Statement

This study enhances the understanding of Aspergillus flavus, a key mycotoxin producer, through the analysis of fifty livestock and poultry feed samples for mycotoxigenic fungi at both morphological and molecular levels. These findings offer valuable insights into the genetic dynamics of A. flavus, underscoring the necessity for continuous monitoring to address aflatoxin contamination risks in agriculture.

Author's Contribution

Conceptualization: REAM, DSA and DA.
Investigation: DA and GE.
Validation of results: REAM, DSA, SBA, and GE.
Writing original draft: DA and SBA, REAM, SBA.
Writing review & editing: REAM.

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Conflict of interest
The authors declare that they have no conflicts of interest.

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