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Molecular Identification and Phylogenetic Analysis of Aflatoxigenic Aspergillus Species in Stored Maize from Maiduguri, Nigeria

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Abstract **Original Research Article**

Fungal contamination of stored maize poses important food-safety and post-harvest quality concerns, particularly in warm environments where Aspergillus and other toxigenic fungi can proliferate. This study investigated the fungal diversity of stored maize in Maiduguri, Nigeria, and confirmed selected isolates using internal transcribed spacer (ITS) sequencing. Maize samples were collected from six warehouses representing harvest years 2021–2024, and fungi were isolated using culture-based techniques and identified morphologically. Genomic DNA from representative isolates was extracted, subjected to ITS-PCR, sequenced by the Sanger method, and compared with reference sequences in the NCBI GenBank database using BLASTn. A total of 38 fungal isolates were recovered, comprising eight species, with Aspergillus spp. accounting for 76.31% of the isolates. A. niger was the most frequently recovered species (21.05%), followed by Scopulariopsis candida (18.42%) and A. oryzae (15.79%). Fungal loads varied considerably among warehouses, ranging from 1.5×10^4 to 9.8×10^4 CFU/g. ITS sequencing confirmed A. aflatoxiformans, A. fumigatus, Talaromyces islandicus, A. parvisclerotigenus, and A. flavus with 100% query coverage and 99.62–100% sequence identity. Phylogenetic analysis further supported the clustering of the isolates with closely related GenBank reference strains. The predominance of Aspergillus, including members associated with aflatoxin production, highlights the potential food-safety implications of inadequate maize storage. The findings demonstrate the usefulness of combining conventional mycology with ITS-based molecular identification for characterizing fungal contaminants and support improved storage hygiene and routine surveillance of stored maize in Maiduguri.

Keywords: stored maize; fungal contamination; Aspergillus; ITS sequencing; molecular identification; phylogeny; food safety.

Introduction

Maize (*Zea mays* L.) is a major cereal crop and an important staple for food and feed, particularly across sub-Saharan Africa. However, its quality and safety can be compromised by fungal contamination during production, harvesting, transportation, and storage, with poor post-harvest handling and inadequate storage conditions increasing the risk of fungal proliferation and mycotoxin accumulation (Awono *et al.*, 2025; Gachara *et al.*, 2024). In Nigeria, *Aspergillus*, *Fusarium*, and *Penicillium* are among the most frequently reported fungal contaminants of maize, with

Aspergillus generally predominating across several agroecological zones (Awono et al., 2025; Orole *et al.,* 2025). Their occurrence is of particular concern because toxigenic species can produce aflatoxins, fumonisins, and other mycotoxins associated with food spoilage, reduced nutritional quality, and significant human and animal health risks. Recent evidence from Nigeria indicates widespread mycotoxin contamination of maize, with aflatoxins and fumonisins representing major food-safety concerns, particularly under favourable climatic and storage conditions (Awono *et al.,* 2025; Scientific African, 2025).

Accurate identification of fungi associated with stored maize is therefore essential for understanding contamination patterns and assessing potential mycotoxin risks. Although conventional identification based on colony and microscopic characteristics remains useful, morphological similarity among closely related species can limit reliable species-level discrimination. Molecular approaches based on internal transcribed spacer (ITS) sequencing provide greater resolution and have become an established tool for fungal identification and phylogenetic assessment, although closely related taxa may sometimes require additional genetic markers for definitive discrimination (Chindamporn *et al.,* 2023). Recent studies in Nigeria have demonstrated the value of molecular approaches in resolving fungal communities associated with stored maize and confirming the predominance of *Aspergillus* species, including potentially toxigenic taxa (Peter *et al.,* 2025; Ibrahim *et al.,* 2024). Therefore, this study aimed to isolate and identify fungi associated with stored maize in Maiduguri, determine the diversity and prevalence of *Aspergillus* species, and assess their genetic relationships using ITS-based molecular sequencing.

Materials and Methods

Sample Collection

Maize grains (20 g each) were obtained from six warehouses (A–F) across Maiduguri Metropolis. Sampling covered four harvest years (2021–2024). Each sample was aseptically collected, sealed, and transported to the Mycology Laboratory, Federal University Dutse.

Isolation and Purification of Fungi

One gram of homogenized maize sample was suspended in sterile Ringer's solution and serially diluted to 10^{-9}. Aliquots (0.1 mL) from the 10^{-3} dilution were spread onto potato dextrose agar (PDA) plates and incubated at **25 °C for 5–7 days.** Distinct colonies were selected based on their macroscopic characteristics and repeatedly subcultured onto Sabouraud dextrose agar (SDA) until pure cultures were obtained. Pure isolates were examined microscopically using lactophenol cotton blue mounts, and identification was based on colony morphology and microscopic features using established taxonomic keys. (Thierry *et al.,* 2025; Peter *et al.,* 2025).

DNA Extraction and PCR Amplification

Genomic DNA was extracted from ground fungal mycelia using a modified cetyltrimethylammonium bromide (CTAB) protocol, in which approximately 100 mg of mycelial material was incubated in extraction buffer containing Tris-HCl, CTAB, NaCl, EDTA, β-mercaptoethanol, and proteinase K at 65 °C for 30 min, followed by chloroform extraction and isopropanol precipitation, and DNA quality was assessed spectrophotometrically using the A260/A280 ratio (Dar *et al.,* 2025).

The fungal internal transcribed spacer (ITS) region was amplified in a 25-µL PCR mixture containing 12.5 µL of PCR Master Mix, 1 µL each of ITS1 and ITS4 primers, 5 µL of template DNA, and nuclease-free water, using an initial denaturation at 95 °C for 5 min, followed by 32 cycles of 95 °C for 30 s, 58 °C for 30 s, and 75 °C for 45 s, with a final extension at 72 °C for 7 min; ITS1/ITS4 remains a widely used universal primer pair for fungal identification and typically amplifies approximately 500–600 bp of the ITS region (CDC, 2024; Dar *et al.,* 2025).

Gel Electrophoresis and Sequencing

PCR amplicons were resolved on 1% agarose gel containing ethidium bromide and visualized under UV illumination using a molecular-weight marker to confirm the expected 500–600 bp fragments. Amplicons showing clear bands were purified and commercially sequenced by Inqaba Biotec, South Africa using Sanger sequencing. The resulting sequences were edited, aligned, and compared with reference fungal sequences in the NCBI GenBank database using BLASTn to determine species identity based on sequence similarity and query coverage (Altschul et al., 1990; Chindamporn *et al.*, 2023). The ITS region was used as the primary molecular marker because of its established utility for fungal identification and DNA barcoding (Abarenkov *et al.*, 2023).

Phylogenetic Analysis

Sequences were edited and aligned using MEGA X. Neighbor-joining phylogenetic trees were constructed with 1000-bootstrap replications. Reference sequences were retrieved from GenBank based on highest homology.

Results

Distribution of Fungal Isolates

Thirty-eight fungal isolates were recovered across all years (Table 1). *Aspergillus* species constituted 76.31% of total isolates. *A. niger* (21.05%) was most frequent, followed by *S. candida* (18.42%) and *A. oryzae* (15.79%).

Table 1. Frequency of Fungal Species in Stored Maize (2021–2024)

Species	No. of Isolates	% Occurrence
Aspergillus niger	8	21.05
A. flavus	5	13.16
A. fumigatus	5	13.16
A. oryzae	6	15.79
A. parvisclerotigenus	3	7.89
A. aflatoxiformans	2	5.26
Scopulariopsis candida	7	18.42
Talaromyces islandicus	2	5.26

The predominance of *A. niger* parallels findings elsewhere in Nigeria (Orole & Chongs, 2024; Frisvad *et al.,* 2024) and suggests its ecological advantage under low water activity. However, *A. flavus* and its relatives (*A. aflatoxiformans*, *A. parvisclerotigenus*) remain most concerning due to their aflatoxin-producing capacity.

Table 2. Mean Fungal Load of the isolates recovered from stored maize in the Warehouses

Warehouses	Mean Load (CFU/g)
Warehouse A	$3.2 \pm 0.8 \times 10^4$
Warehouse B	$1.5 \pm 0.6 \times 10^4$
Warehouse C	$3.9 \pm 1.1 \times 10^4$
Warehouse D	$2.7 \pm 1.2 \times 10^4$
Warehouse E	$1.9 \pm 0.9 \times 10^4$
Warehouse F	$9.8 \pm 2.1 \times 10^4$

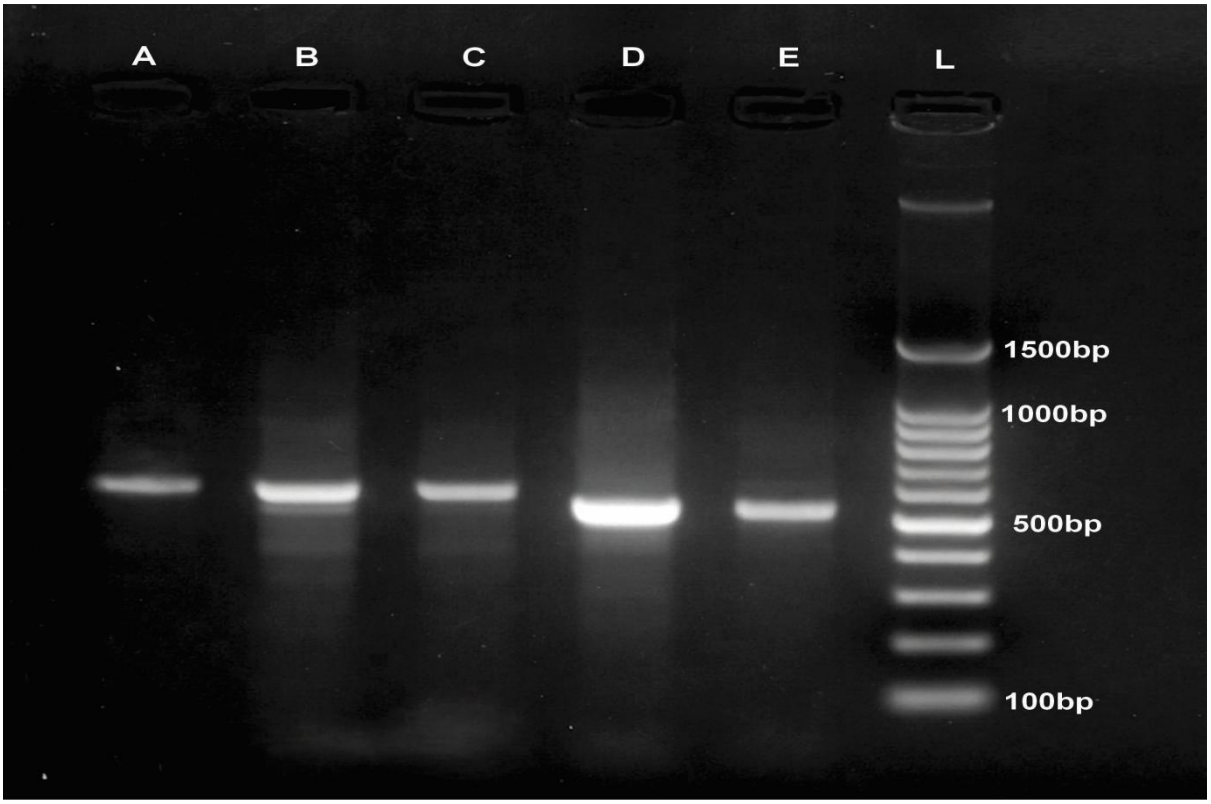
The results demonstrated marked variation in mean fungal loads among the six warehouses, indicating differences in storage conditions. Warehouse F recorded the highest fungal load ($9.8 \pm 2.1 \times 10^4$ CFU/g), whereas Warehouses B ($1.5 \pm 0.6 \times 10^4$ CFU/g) and E ($1.9 \pm 0.9 \times 10^4$ CFU/g) recorded the lowest. Warehouses A ($3.2 \pm 0.8 \times 10^4$ CFU/g), C ($3.9 \pm 1.1 \times 10^4$ CFU/g), and D ($2.7 \pm 1.2 \times 10^4$ CFU/g) showed intermediate fungal loads, with Warehouse C exhibiting the highest within this group. The relatively large standard deviation in Warehouse F ($\pm 2.1 \times 10^4$ CFU/g) further suggests greater spatial or batch-to-batch variability in fungal contamination, potentially reflecting inconsistent moisture, temperature, aeration, or handling conditions within the facility.

Table 3. NanoDrop confirmatory result of the DNA extracted from the maize isolates

S/No	Sample	N.A concentration (ng/µl)	DNA purity (260/280)	N.A purity (260/230)
1	A	3534.6	2.17	2.26
2	B	107.4	2.03	2.42
3	C	450.2	2.17	2.42
4	D	116.1	2.05	2.09
5	E	3533.8	2.16	2.24

The NanoDrop results indicate that DNA was successfully extracted from all five maize fungal isolates, although the concentrations varied substantially. Samples A and E yielded the highest DNA concentrations (3534.6 and 3533.8 ng/µL, respectively), while sample B had the lowest concentration (107.4 ng/µL); samples C and D yielded 450.2 and 116.1 ng/µL,

respectively. The 260/280 ratios ranged from 2.03 to 2.17, which are within or slightly above the commonly acceptable range for relatively pure DNA, suggesting minimal protein contamination. Similarly, the 260/230 ratios (2.09–2.42) indicate generally good DNA purity with limited contamination from salts, carbohydrates, or other organic compounds. Generally, the results confirm that the extracted DNA was of adequate concentration and purity for downstream molecular analyses such as PCR amplification and sequencing.



Key: L= Ladder A= *Aspergillus aflatoxiformans* B= *Aspergillus fumigatus* C= *Talaromyces islandicus* D= *Aspergillus parvisclerotigenus* E= *Aspergillus flavus* isolate

Plate 1. The Agarose Gel Image of the PCR Products of the DNA Extracted from Fungal Isolate Obtained from the Maize Samples.

The agarose gel electrophoresis profile showed distinct DNA bands for all five fungal isolates (A–E), with the DNA ladder (L) serving as the molecular size reference. Clear bands were observed for *Aspergillus aflatoxiformans* (A), *A. fumigatus* (B), *Talaromyces islandicus* (C), *A. parvisclerotigenus* (D), and *A. flavus* (E), indicating successful amplification of the target DNA region. The presence of discrete, well-defined bands with limited smearing suggests that the PCR products were of satisfactory quality and suitable for subsequent molecular identification and sequencing. Overall, the gel profile confirmed successful PCR amplification of the fungal isolates and supported their further characterization by sequence analysis.

Table 4. Molecular Identity of ITS-Amplified Fungal Isolates

ID	Identified Species	% Coverage	% Identity	GenBank Accession
A	*A. aflatoxiformans* 087-A2	100	99.62	MG662405.1
B	*A. fumigatus* RS_20	100	100	MK267099.1
C	*T. islandicus* W.Kh-B.I	100	100	MH727227.1
D	*A. parvisclerotigenus* Maci262	100	100	MG745384.1
E	*A. flavus* R97190	100	100	MK542007.1

The ITS-based molecular identification confirmed the identity of all five fungal isolates with high sequence similarity to reference strains in GenBank. All isolates showed 100% query coverage, while sequence identity ranged from 99.62% to 100%, indicating strong agreement between the obtained ITS sequences and their corresponding reference sequences. *Aspergillus fumigatus*, *Talaromyces islandicus*, *A. parvisclerotigenus*, and *A. flavus* showed complete (100%) sequence identity, whereas *A. aflatoxiformans* showed 99.62% identity. The corresponding GenBank accession numbers provided additional molecular evidence for species-level identification. These findings demonstrate that ITS-PCR coupled with sequence comparison was effective for confirming the morphological identification of the fungal isolates.

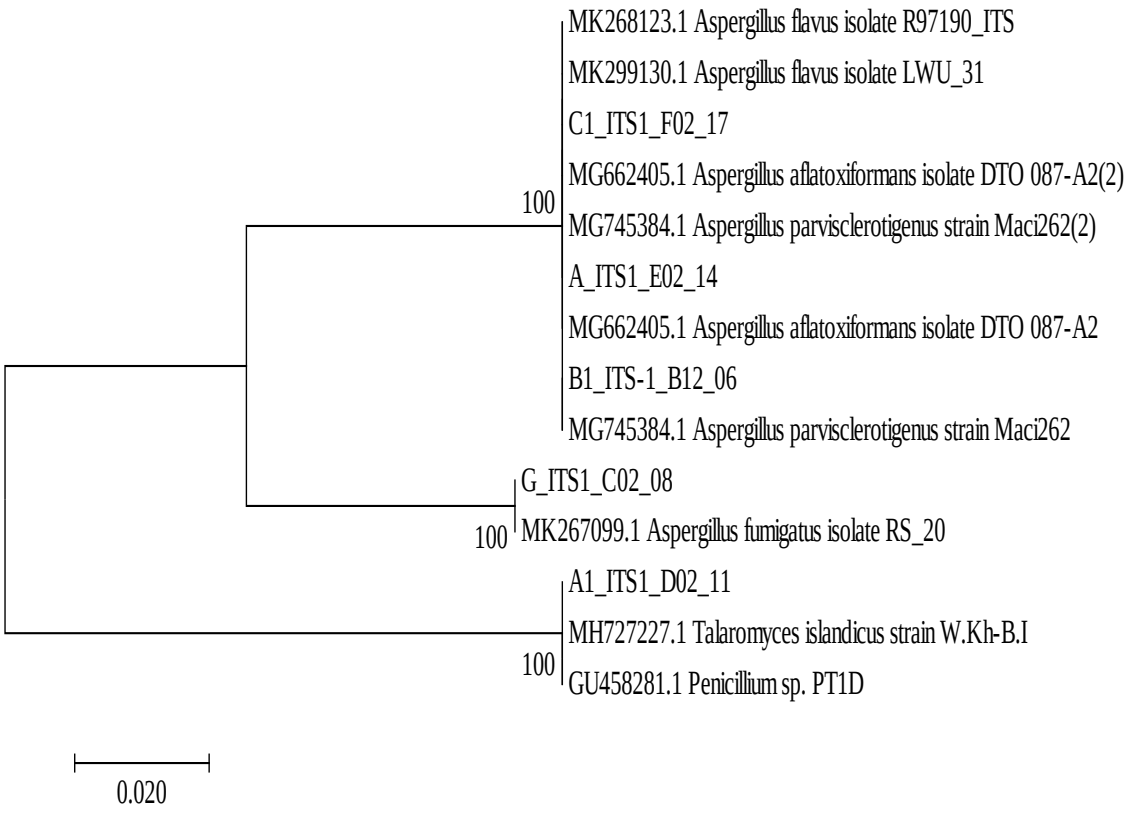


Figure 1: Phylogenetic tree of fungi associated with aflatoxin contaminated maize together with their closest representative available in GeneBank

The phylogenetic tree generated from ITS sequence data illustrates the evolutionary relationships among the fungal isolates obtained from maize samples (Figure4.1). *Aspergillus flavus, A. aflatoxiformans*, and *A. parvisclerotigenus* clustered within the same clade, showing 100% sequence similarity with corresponding GeneBank reference strains. *Talaromyces islandicus* grouped closely with *Penicillium* spp. in a separate clade, indicating close genetic relatedness. In contrast, *Aspergillus fumigatus* formed a distinct lineage, reflecting a more distant phylogenetic relationship with the other isolates.

Discussion

The recovery of 38 fungal isolates representing eight species demonstrates considerable fungal diversity in the stored maize examined in Maiduguri. *Aspergillus* spp. accounted for 76.31% of all isolates, with *A. niger* being the most frequently recovered species, followed by *Scopulariopsis candida* and *A. oryzae*. The predominance of *Aspergillus* is consistent with recent studies showing that this genus is well adapted to stored cereal environments and frequently dominates maize mycobiota under warm storage conditions. Daba *et al.* (2024) reported substantial occurrence of *Aspergillus* and *A. flavus* in stored maize, with fungal development influenced by storage duration and initial grain moisture. Similarly, a recent Nigerian study found *Aspergillus* section Flavi to be the predominant fungal group in maize, confirming the continuing importance of this genus in Nigerian grain systems (Bashir *et al.*, 2025). The relatively high occurrence of *A. niger* in the present study may reflect its ability to tolerate relatively dry storage environments, although the absence of direct measurements of moisture content and water activity limits attribution to a specific environmental factor.

Of particular concern was the recovery of *A. flavus, A. aflatoxiformans*, and *A. parvisclerotigenus*, because members of *Aspergillus* section Flavi include important aflatoxin-producing taxa. Recent molecular characterization of stored cereal isolates confirmed the occurrence of *A. aflatoxiformans* and *A. flavus* among grain-associated *Aspergillus* populations (El-Dawy *et al.*, 2024). Likewise, Katati *et al.* (2024) demonstrated substantial diversity within section Flavi associated with maize and emphasized that species composition can influence aflatoxin contamination. However, the detection of these species in the present study should not by itself be interpreted as evidence that every isolate produced aflatoxin, since aflatoxin production is strain-dependent and requires direct toxin analysis or characterization of relevant biosynthetic genes. This distinction is particularly important because *A. oryzae*, although closely related to *A. flavus*, is generally regarded as a domesticated and predominantly non-aflatoxigenic lineage (Sharma *et al.*, 2025). Therefore, the present findings indicate potential aflatoxin risk, rather than directly demonstrating aflatoxin production by the recovered isolates.

The marked variation in fungal load among warehouses further suggests that storage conditions may have contributed to differences in fungal proliferation. Warehouse F recorded the highest mean load ($9.8 \pm 2.1 \times 10^4$ CFU/g), whereas Warehouse B had the lowest ($1.5 \pm 0.6 \times 10^4$ CFU/g). Although specific environmental parameters were not measured, differences in grain moisture, temperature, aeration, handling practices, and duration of storage could plausibly account for this variation. Experimental evidence shows that fungal development in stored maize is strongly affected by storage duration and initial moisture content, while storage temperature can substantially influence *A. flavus* growth and aflatoxin accumulation (Daba *et al.*, 2024; Shi *et al.*, 2023). Similarly, Atnafu *et al.* (2025) demonstrated that storage systems and duration significantly affect toxigenic fungal incidence and mycotoxin contamination, with improved storage systems reducing fungal development. The relatively

high load recorded in Warehouse F therefore indicates a need for closer monitoring of storage hygiene and grain environmental conditions.

The molecular results provided strong confirmation of the selected fungal identifications. The extracted DNA showed adequate purity, PCR produced distinct amplicons, and ITS sequencing generated 100% query coverage with 99.62–100% sequence identity to corresponding GenBank reference sequences. These high similarity values provide strong support for the species assignments and demonstrate the usefulness of molecular analysis in complementing morphological identification. The phylogenetic pattern, in which the *A. flavus*, *A. aflatoxiformans*, and *A. parvisclerotigenus* isolates grouped within the *Aspergillus* lineage while *T. islandicus* formed a separate lineage, was consistent with their taxonomic relationships. Nevertheless, ITS should be interpreted as a primary identification marker rather than an infallible discriminator among closely related *Aspergillus* taxa. Recent work on section Flavi has demonstrated substantial cryptic diversity and has used additional markers such as calmodulin for improved species resolution (Katati *et al.*, 2024; Sharma *et al.*, 2025). Thus, multilocus sequencing involving markers such as calmodulin (CaM) or β-tubulin (BenA) would provide stronger confirmation where closely related *Aspergillus* species are involved.

Overall, the combined culture-based, spectrophotometric, PCR, BLAST and phylogenetic results provide consistent evidence that stored maize in Maiduguri harbours diverse fungal populations, with *Aspergillus* being the dominant group and several potentially toxigenic species present. The findings are important because recent evidence from North-West Nigeria has demonstrated widespread occurrence of aflatoxins and other mycotoxins in maize and identified section Flavi as a major fungal group of concern (Bashir *et al.*, 2025). Improved drying, storage hygiene, moisture control, regular fungal surveillance and molecular confirmation of suspicious isolates should therefore form part of an integrated strategy for reducing fungal and mycotoxin risks in stored maize.

Conclusion

This study demonstrated substantial fungal diversity in stored maize from Maiduguri, with *Aspergillus* spp. predominating among the 38 isolates recovered. The occurrence of *A. flavus*, *A. aflatoxiformans*, and *A. parvisclerotigenus* is of particular food-safety importance because members of this group include recognized aflatoxin-producing fungi. Fungal loads varied considerably among warehouses, suggesting that differences in storage conditions may influence fungal proliferation. ITS-PCR and sequencing successfully confirmed five representative isolates with 99.62–100% sequence identity and provided molecular support for their taxonomic placement. The findings highlight the value of combining conventional mycology with molecular identification for surveillance of stored-maize fungi. Improved storage management, particularly control of grain moisture and storage conditions, together with routine monitoring of potentially toxigenic *Aspergillus* species, is recommended to minimize fungal and associated mycotoxin risks in maize.

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