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Molecular Identification and Phylogenetic Tree of Mycotoxigenic Fungi from Sorghum (*Sorghum bicolour*) In Niger Republic, Africa

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Sorghum is a member of the Poaceae grass family and is considered the fifth most important cereal in the world after wheat, maize, rice and barley and second in Africa after maize. Millions of dollars are lost annually in Africa due to fungal contamination of vital crops like sorghum, threatening food security and livelihoods. This study focused on the molecular identification of mycotoxigenic fungi in sorghum samples from Niger Republic. Samples were collected from stores and markets across the four major zones of Niger. The standard pour plate method was used for

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estimating and isolating potentially mycotoxigenic fungi, which were then identified based on morphological characteristics and confirmed using molecular techniques. BioEdit Sequence Alignment Editor version 7.2.5 was used for sequence editing. The edited sequences were compared to known sequences in a database using the NCBI BLAST search tool to identify the specific fungal species. The phylogenetic tree was constructed using Mega 11 and the UPGMA platform at default settings. The study revealed variations in the average fungal count between zones and between store and market samples within each zone. No statistically significant ($p<0.05$) difference was found in average fungal counts between zones for store samples. However, a statistically significant difference was observed in market samples, with Zone I recording significantly lower average counts (1.75x10^2 CFU/g) compared to other zones. The average fungal counts ranged from 8.50x10^2 to 19.0x10^2 CFU/g in store samples and 1.75x10^2 to 17.67x10^2 CFU/g in market samples. The most commonly isolated fungal genera, in descending order, were *Aspergillus niger*, *Aspergillus flavus*, *Mucor* sp., *Aspergillus fumigatus*, *Penicillium* sp., *Aspergillus glaucus, Trichoderma* sp., *Fusarium* sp., *Chrysosporium* sp., and *Curvularia* sp.. Molecular techniques confirmed the presence of *Penicillium glandicola*, *Aspergillus flavus*, *Aspergillus fumigatus*, and *Fusarium solani* and their phylogenetic relationships and origin were determined from their gene sequences. This study identified fungal contamination, particularly in stored sorghum, as a threat to food security due to reduced grain quality and potential health risks, highlighting the need for improved post-harvest practices to minimise fungal growth and ensure the safety of this vital food source. This study identified fungal contamination, particularly in stored sorghum, as a threat to food security due to reduced grain quality and potential health risks.

Keywords: Mycotoxigenic fungi; phylogenetic tree; sorghum; Molecular techniques.

1. INTRODUCTION

Sorghum bicolour (L.) Moench is an herbaceous cereal cultivated from seed and one of the most frequently contaminated grains with mycotoxigenic fungi. In Nigeria’s agribusiness, sorghum cultivation is now amongst the world’s leading positions, ranked at number two with an output of 6.9 million metric tons after the United States of America, the world’s number one, with an output of 9.0 million metric tons, according to the United States Department of Agriculture. It is one of the principal staple cereals in Sub-Saharan Africa (SSA). Sorghum is used as raw materials and ingredients for a variety of food products, which are consumed by all age groups, including infants (Adewunmi et al., 2021). Millions of dollars are lost annually in Africa due to fungal contamination of vital crops like sorghum, threatening food security and livelihoods (Udomkun et al., 2017; Gbashi et al., 2018). Sorghum is a member of the *Poaceae* grass family and is considered the fifth most important cereal in the world after wheat, maize, rice and barley and second in Africa after maize (Ssepuuya *et al.*, 2018; Terna *et al.*, 2019; Kazungu *et al.*, 2023). It is an economically important and staple food crop for over half a billion people in developing countries, mostly in arid and semi-arid regions where drought stress is a major limiting factor. Although sorghum is generally considered tolerant, drought stress still significantly hampers its productivity and nutritional quality across its major cultivation areas (Abreha et al., 2022). Its significance lies in its role as a staple food for millions living in semi-arid regions of Africa and Asia, where its nutritional value and resilience make it vital for food security especially relevant in today's changing climate (Mwadalu and Mwangi, 2013; Hariprasanna and Rakshit, 2016; Visarada and Aruna, 2019; Chadalavada *et al.*, 2021). Sorghum is cultivated globally for its incredibly diverse uses. From nourishing grains for sustenance to animal feed, alcoholic beverages, building materials, and even biofuels (Kazungu *et al.*, 2023).

Sorghum is the fifth most important cereal crop globally and the dietary staple of around 500 million people. It is an important food crop in many parts of eastern and southern Africa. Sorghum is tolerant, relative to other major cereal crops, to adverse growing conditions. It is efficient in photosynthesis and in water and nutrient use. Sorghum was ranked number two by the Association for Strengthening Agricultural Research in Eastern and Central Africa (ASARECA) among the major cereal crops in terms of resilience, nutrition, and opportunity to enter the commercial markets (Mesfin & Girma, 2022; Ahmad Yahaya et al., 2022). The Niger Republic is a major sorghum producer and consumer. Sorghum consumption is substantial, in the country contributing significantly to household food security (FAO, 2015). According to Tonapi *et al.* (2020) Niger republic ranks 9th in the world for sorghum production with 2100 thousand metric tonnes trailing the USA (9271 thousand MT), Nigeria (6862 thousand MT), Sudan (4953 thousand MT), Ethiopia (4932 thousand MT), India (4800 thousand MT), Mexico (4531 thousand MT), Brazil (2273 thousand MT) and China (2194 thousand MT). In terms of domestic sorghum consumption, the Niger Republic ranks tenth (1,800 thousand metric tons) and eleventh in production (1,900 thousand metric tons) as of 2023 (Indexmundi, 2023a, b).

The safety and quality of sorghum during storage and transportation are often compromised by several biotic stresses, among which contamination with mycotoxigenic fungi is one of the most important (Prom, 2023). Improper drying and storage make sorghum grains an ideal substrate for mould growth (Kange *et al.*, 2015). These fungi not only compromise grain quality but also produce harmful mycotoxins that affect both human and animal health, posing a serious health risk (Udomkun et al., 2017). Crop contamination by fungal species leads to economic losses due to spoilage, rendering products unfit for consumption or unmarketable (Salem-Bekhe *et al.*, 2011). Africa faces a significant economic hurdle due to mycotoxin contamination. A lack of adequate post-harvest storage facilities is a major culprit, leading to an estimated $67 million in annual losses from rejected exports (Atanda *et al.*, 2013; Daniel *et al.*, 2016; Terna *et al.*, 2019).

Fungal contamination significantly impacts food security (Medina *et al.*, 2017; Ajmal *et al.*, 2022). Hence, this study focused on identifying and characterising the mycotoxigenic fungi present in sorghum from the Niger Republic. The study's findings hold significant potential to enhance seed quality and boost the market value of stored sorghum seeds in the study area.

2. MATERIALS AND METHODS

Area of Study: The Niger Republic has a wide range of climates, with four major climatic zones from north to south climatic zones (Fig. 1). The climate is Sahelian, with a long dry season lasting eight to ten months (October to May), a short rainy season lasting three or four months (June to September) and a wide variation in the number of rainy days from north to south, with annual rainfall ranging from less than 100 mm to 700-800 mm, dividing the country into four climatic zones. The Saharan zone in the north characterised by cumulative annual rainfall less than 150 mm, with rainy seasons lasting no more than one month a year. The average temperature is around 35°C, and the climate is desert climate. The Sahelo-Saharan zone, between isohyets of 150 and 300 mm, with a high level of pastoral activity. The Sahelian zone, where most of Niger's agricultural activity is concentrated, has with annual rainfall of between 300 and 600 mm, and the Sudanian zone, located south of parallel 13°10' North, with a cumulative rainfall in excess of 600 mm.

Sample collection: Sorghum samples were collected from stores and markets in the Niger Republic. All samples were collected in the dry season and transported in plastic. 500g of the samples were collected from the regions of Dosso, Maradi, Niamey, Tillabéri, Zinder and Agadez (Table 1).

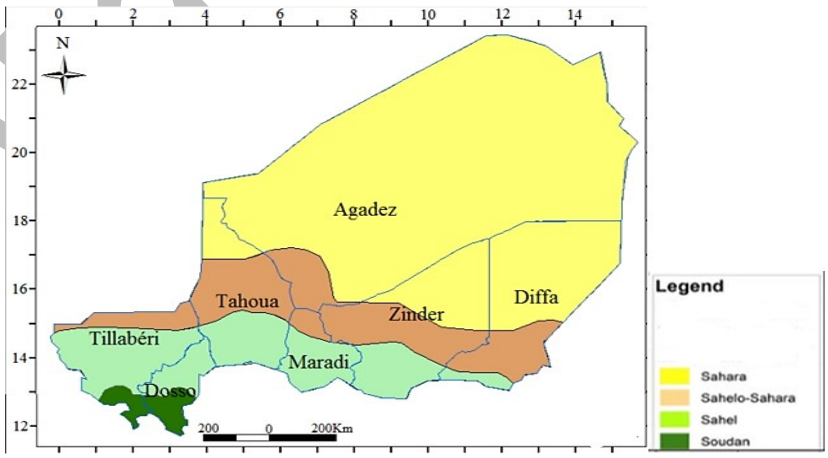

Fig. 1. Agroclimatic zones of Niger

Table 1. Sorghum Samples Collected from Markets and Stores in Niger

Zone	Store	Market	
I Soudan	4	4	
II Sahel	12	12	
III sahelo Sahara	12	12	
IV Sahara	4	4	
Total	32	32	64

Isolation and estimation of fungi in Sorghum: A Fungal count was carried out using the pour plate technique. 1g of each of samples was taken and added into a test tube containing 9ml of sterile water, and a serial 10-fold serial dilution was made until a dilution of 10^{-2} was obtained. 1 ml of the 10^{-2} dilution of each sample was aseptically transferred into sterile petri dishes, and molten potato dextrose agar medium was poured into the respective petri dishes and allowed to solidify before incubating at 27±2 °C for 48-72 hours (Gaddeyya *et al.*, 2012; Al-Mohanna, 2016). The plates were then screened for the presence of discrete colonies after an incubation period, and the actual numbers of fungi are estimated in colony-forming units per gram (CFU/g). The fungal concentration was expressed in colony forming unit (CFU) per gram of sample (equation 1).

$$cfu/_{g} = \frac{Number\ of\ fungal\ colonies\ x\ Reciprocal\ of\ Dilution\ factor}{Volume\ plated} \quad (1)$$

Characteristically distinct colonies of fungi obtained after incubation were subcultured onto fresh agar plates to obtain pure cultures. Pure fungal isolates obtained were then stored on appropriate agar slants for identification and further analysis.

Identification of fungal isolates: Fungal isolates were identified based on cultural morphology on agar plates and Microscopy. The technique of Oyeleke and Manga (2008) was adopted for the identification of the isolated fungi using lactophenol cotton blue stain. The identification was achieved by placing a drop of the stain on a clean grease grease-free glass slide. With the aid of an inoculating needle, a small portion of the aerial mycelia from the representative fungi cultures was removed and placed in a drop of lactophenol. The mycelium was well spread on the slide with the needle, and a cover slip was gently placed over it. The slide was then mounted and viewed under the light microscope with ×10 and ×40 objective lenses. The morphological characteristics and appearance of the fungal organisms seen were identified in accordance with Campbell and Johnson (2013).

Molecular identification of fungal isolates: The ITS (Internal Transcribed Spacer) region sequencing method was employed to confirm the identity of isolated fungal species. The fungal genomic DNA was extracted using the protocol stated by Dellaporta *et al.* (1983). PCR technique was used to amplify the ITS region of the fungal DNA using the primers ITS1 (5’ TCC GTA GGT GAA CCT GCG G 3’) and ITS4 (5’ TCC TCC GCT TAT TGA TAT GC 3’). The reaction mixture included 10 µl of 5x GoTaq reaction buffer, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP mix, 1 µl of each primer, 0.3 units of Taq DNA polymerase (Promega, USA), and 8 µl DNA template, made up to 42 µl with sterile distilled water. PCR was carried out in a GeneAmp 9700 PCR System Thermal cycler (Applied Biosystem Inc., USA). The amplified DNA fragments were visualised using gel electrophoresis to confirm successful amplification, and the purified fragments were sequenced on an ABI 3500xl Genetic Analyser (Applied Biosystems) using the BigDye Terminator v3.1 cycle sequencing kit, following the manufacturer’s instructions. BioEdit Sequence Alignment Editor version 7.2.5 was used for sequence editing. The edited sequences were compared to known sequences in a database using the NCBI BLAST search tool to identify the specific fungal species (Altschul *et al.*, 1997; Al-Hindi *et al.*, 2018). The phylogenetic tree was constructed using the Mega 11 and UPGMA platform at default settings.

3. RESULTS

3.1 Isolation and Morphological Identification

Table 2 shows the average fungal count of sorghum samples collected from stores and markets across four zones in Niger. Variations were observed in the average fungal count between zones and between store and market samples within each zone. There was no statistically significant difference in average fungal counts between zones for store sorghum samples. However statistically significant difference was observed in fungal counts between zones for market sorghum samples, with zone I recording significantly lower average fungal counts (1.75×10^2 CFU/g) compared to other zones. There was also no statistically significant difference in average fungal counts between store and market sorghum samples across zones. The average fungal counts ranged from 8.50×10^2 to 19.0×10^2 CFU/g in store samples and 1.75×10^2 to 17.67×10^2 CFU/g in market samples across zones.

Fungal isolates were identified based on their macroscopic and microscopic characteristics. The fungal species isolated include *Trichoderma sp., Aspergillus niger. Aspergillus fumigatus, Aspergillus flavus, Aspergillus glaucus, Chrysosporium* sp., *Penicillium* sp., *Fusarium* sp. *Curvularia* sp. and *Mucor* sp... The morphological and microscopic characteristic of *Penicillium* and *Aspergillus* is presented in Fig. 2.

Table 2. Average Fungal Count of Sorghum Samples Collected in Niger

Zone	Store ($\times10^2$ CFU/g)	Market ($\times10^2$ CFU/g)
I	8.50 ± 5.19^{ae}	1.75 ± 0.75^{ae}
II	19.00 ± 5.23^{ae}	17.67 ± 4.47^{be}
III	14.83 ± 3.14^{ae}	14.83 ± 2.42^{abe}
IV	10.25 ± 3.01^{ae}	10.00 ± 2.65^{abe}

Values are presented as mean ± standard error of mean (SEM). Values with different superscripts are significantly different at $p < 0.05$. Superscripts a,b compare zone by zone, while superscript e compares store and market for each zone

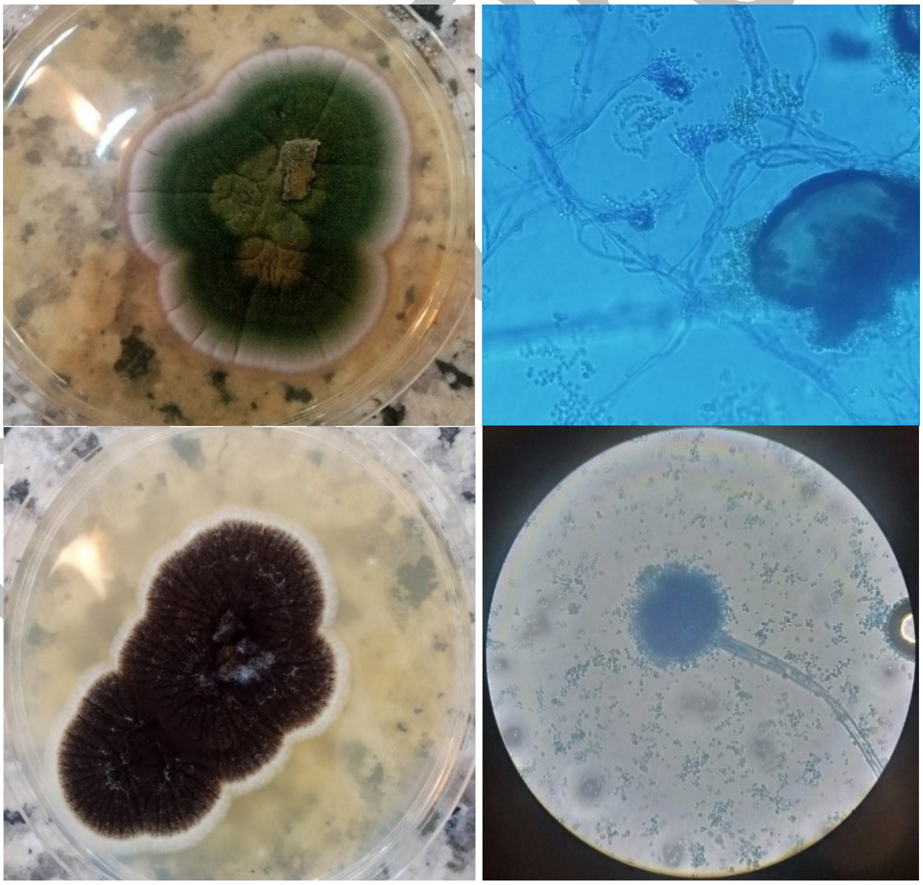

Fig. 2. Morphological and Microscopic characteristics of *Penicillium* and *Aspergillus*

3.2 Frequency of Occurrence of Fungi Species in Sorghum

Fig. 3 shows the frequency of fungi species isolated from the analysed sorghum samples. *Aspergillus niger* was the dominant fungus, with 36.7% occurrence. Other frequently isolated fungi species include *Aspergillus flavus* (24.4%), *Mucor* sp (13.3%), and *Aspergillus fumigatus* (10.9%). *Penicillium* sp. was also detected at a moderate level (9.4%). Notably, *Aspergillus glaucus, Trichoderma* sp., *Fusarium* sp., *Chrysosporium* sp., and *Curvularia* sp. were found at lower frequencies (all ≤ 1.6%).

3.3 Molecular Identification of Mycotoxigenic Fungal Isolates

The identity of mycotoxigenic isolates was further confirmed using the ITS region sequencing analysis. The BLAST results of the fungal DNA sequence correspond to the similarity between the sequence queried and the biological sequences within the NCBI database. From the results, the predicted organisms were *Penicillium glandicola, Aspergillus flavus, Fusarium solani* and *Aspergillus fumigatus* (Table 3). The agarose gel electrograph indicating the ITS target region amplification of the test isolates is shown in Fig. 4. The phylogenetic relationships and origin of

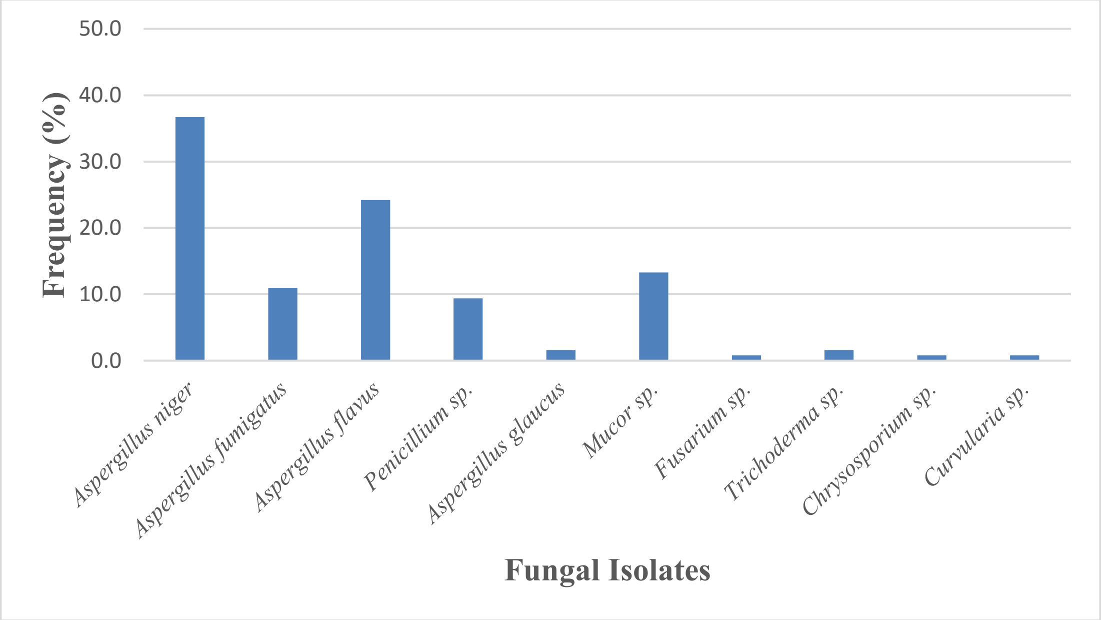

Fig. 3. Frequency of occurrence of mycotoxigenic fungi

Table 3. Identification and BLAST Analysis of Fungal Isolates

Sample ID	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
6	*Aspergillus flavus*	1061	1061	95%	0.0	98.36%	PP860870
5	*Penicillium glandicola*	1175	1175	100%	0.0	100%	PP860872
10	*Aspergillus fumigatus*	736	736	92%	0.0	89.91%	PP860869
12	*Fusarium solani*	1022	1022	93%	0.0	98.14%	PP860871

Table 4. PAIRWISE genetic distance showing the relationship between isolates

	S6	S5	S10	S12
S6				
S5	0.25781			
S10	0.00160	0.22845		
S12	0.22306	0.00322	0.22843	

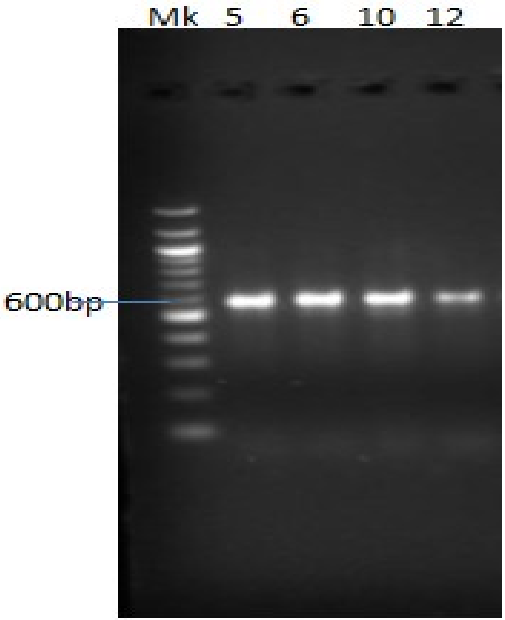

Fig. 4. A photographic image of the agarose gel indicting the amplification of the ITS region of the test fungal isolates

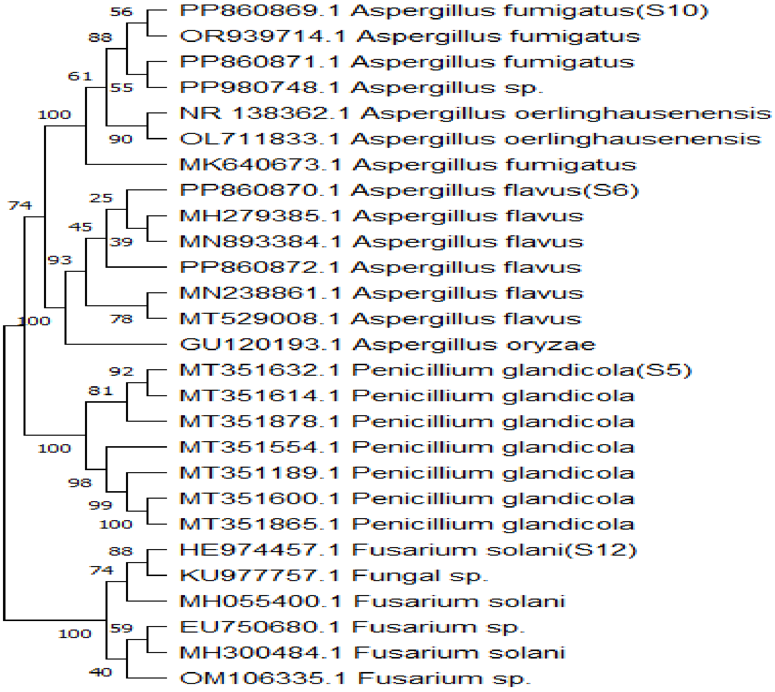

Fig. 5. Phylogenetic Tree of the fungal isolates based on its Gene Sequences

the test isolates were investigated using a phylogenetic tree (Fig. 5) constructed from their gene sequences obtained from the NCBI database. The phylogenetic tree was constructed with the neighbour joining method, using maximum likelihood parameters and a distance-based method.

4. DISCUSSION

In Africa, mycotoxins are one of the five major food safety concerns, and cereals are the most important sources of these toxins (Tola and Kebede, 2016; Adeyeye *et al*., 2022). Thus, threatens these vital food sources. This study assessed the fungal contamination levels and identified mycotoxigenic fungal species present in sorghum samples collected from stores and markets across four zones in the Niger Republic. There was no statistically significant difference in average fungal counts between zones for store sorghum samples. similarly, there was also no statistically significant difference in average fungal counts between store and market sorghum samples across zones. However statistically significant difference was observed in fungal counts between zones for market sorghum samples. The average fungal counts ranging from 8.50×10^2 to 19.0×10^2 CFU/g in store samples and 1.75×10^2 to 17.67×10^2 CFU/g in market samples across zones were lower than those reported by Garba *et al.* (2017) in Nigeria. The study recorded fungal count ranging from 1.0×10^4 to 9.5×10^6 CFU/g in sorghum grains sampled from the field, market and different storage facilities in the six agro-ecological zones of Nigeria. This observed difference could be attributed to variations in climate, storage duration, or sampling methods.

Our study identified various fungi species with the potential of producing mycotoxins in both store and market sorghum samples, highlighting the vulnerability of sorghum to fungal contamination in Niger. These fungal species were present at varying frequencies, with *Aspergillus niger* being the dominant fungus, with 36.7% occurrence. Other frequently isolated species included *Aspergillus flavus*, *Mucor* sp., *Aspergillus fumigatus* and *Penicillium* sp.. The diversity of fungal species identified in this study aligns with findings from Orimoloye *et al.* (2018) and Terna *et al.* (2019), who both documented a wide range of fungal contaminants at varying frequencies in cereals in Africa. Terna *et al.* (2019) identified *Aspergillus, Alternaria, Mucor, Penicillium, Aureobasidium, Pythium, Fusarium, Colletotrichum, Chrysosporium, Chrysonilia, and Curvularia* species in Stored Sorghum Seeds in Lafia, Nasarawa State, Nigeria, with *Alternaria* species (53.01%) being the most occurring. Another study on fungi associated with stored sorghum grains in Southwest Nigeria by Orimoloye (2018) identified *Aspergillus, Fusarium, Colletotrichum, Penicillium, Macrophomina* and *Rhizopus* as common fungi species associated with sorghum grains, with *Aspergillus* species being the most predominant fungi with the highest incidence.

The evolutionary relationship among various fungal taxa is illustrated in Fig. 1. The phylogenetic tree focuses particularly on three fungal genera, including *Aspergillus*, *Penicillium*, and *Fusarium*. The construction of the tree was based on genetic similarity and displayed the values of bootstrap at the node of each taxon and clade, respectively, as it supports the branch point of each node statistically. Certain clusters within and between the three genera were observed to exhibit the highest bootstrap values of up to 100%. This shows strong support for these evolutionary relationships. There is strong support for these evolutionary relationships within the genus of *Penicillium* because they exhibit the highest bootstrap values, reaching up to 100%. However, the bootstrap values are low within certain clusters, showing a weak evolutionary relationship. Few species within the genus of *Aspergillus* show the lowest bootstrap values, such as 25% and 39%. This implies that there is relatively weaker support for these groupings. The differences in bootstrap value show varying levels of confidence in the clusters. Clades with high bootstrap support are well-defined, while branches with lower support are less certain. This helps in distinguishing closely related groups of organisms from those that are less closely related.

Environmental factors and host-specific profiles have a strong impact on the occurrence of specific genera of fungal and invariably on the types of mycotoxins they accumulate. In countries like Niger, which has a mixture of hot and dry climates, the southern and northern parts of the country experience a tropical climate and a desert climate respectively. High to optimum temperature, rainfall, and humidity favour the growth of several genera of fungi, which include: *Aspergillus*, *Penicillium*, and *Fusarium*. The three genera of fungi, *Aspergillus*, *Penicillium*, and *Fusarium,* are typically found in warmer regions of the country where sorghum is produced (Akello *et al.,* 2021; Mohammed *et al.,* 2022). All the fungi are associated with mycotoxin production, and there is a high risk because of certain strains of *Aspergillus flavus* (Ceniti *et al*., 2021; Shabeer *et al*., 2022). This is a major contributor to Aflatoxin B1, which is deadly compared to the other genera (Owumi *et al*., 2023). Clusters of Aflatoxin biosynthetic gene activity and expression could be high in the sorghum (Szabó *et* al., 2024) because of the presence of three mycotoxin-producing fungal genera in the sorghum samples from Niger. In addition, specific plant compounds interfere with the pathways and hence stop these isolates from producing aflatoxins. Currently, certain plant extracts are used as active ingredients in aflatoxin biocontrol products in sub-Saharan Africa, effectively reducing aflatoxin contamination when applied before and after harvest (Ortega-Beltran & Bandyopadhyay, 2021). Since the four isolates of this study are members of aflatoxin-producing fungi, there could be the possibility of high aflatoxin contamination in the sorghum from Niger. To confirm the amplified *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. isolates, and to identify the isolates that the ITS primer could amplify. Nucleotide BLAST of the partially sequenced genes confirmed the isolates to be *Aspergillus fumigatus, Aspergillus flavus, Penicillium glandicola* and *Fusarium solani.* These entire organisms in one way or another produce mycotoxins, hence the people of Niger are at risk to their safety in terms of cereal food. The observed high contamination levels, particularly in some store samples, threaten food security by reducing grain quality and posing potential health risks to consumers. Given the essential role of sorghum in ensuring food security in semi-arid regions, these findings suggest the urgent need for improvements in post-harvest handling, storage practices and monitoring systems to minimise fungal contamination and ensure the safety of sorghum, a vital food source in Niger.

5. CONCLUSION

This study identified fungal contamination, particularly in stored sorghum, as a threat to food security due to reduced grain quality and potential health risks. *Aspergillus niger* was the dominant fungus identified, followed by others with mycotoxin-producing potential, including *Aspergillus flavus*, *Mucor* sp., *Aspergillus fumigatus*, and *Penicillium* sp.. The average fungal counts in stored and market samples ranged from $8.50x10^2$ to $19.0x10^2$ CFU/g and $1.75x10^2$ to $17.67x10^2$ CFU/g, respectively. Although these counts are lower than those reported in other regions, such as Nigeria, the presence of these mycotoxigenic fungi in sorghum still poses a significant threat to food safety and security. Their presence necessitates improvements in post-harvest practices to minimise fungal growth and ensure the safety of this vital food source.

As part of the ongoing study, mycotoxin analyses using high-performance liquid chromatography (HPLC) are underway to quantify contamination levels and enhance our understanding of food safety risks associated with fungal contamination in sorghum. These results will be crucial for developing mitigation strategies to ensure the safety of sorghum in Niger.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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