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Molecular characterization of aflatoxin producing fungi associated with maize in selected areas of Morogoro municipality, Tanzania

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Abstract

Aspergillus species remain to be a doughty species for the safety of agricultural crops by colonizing and contaminating them via mycotoxin production, particularly aflatoxin. This study aimed at conventionally screening and molecularly detect and characterize Aflatoxin producing fungi particularly *Aspergillus* spp from Maize selling markets and maize grain milling machines in Morogoro urban and Mvomero district. Thirty maize grain samples were collected from Morogoro Municipality and Mvomero district in Tanzania. The laboratory analysis was done using the blotter technique, where maize grain samples were subjected to a blotter test and the resultant growing mycelia were transferred to Potato Dextrose Agar (PDA). Thereafter, colony morphology, colour and texture for fungal identification was observed. Macro and microconidia, seriate and septate were used for initial identification of fungi and finally confirmed by a molecular technique through extracting fungal Deoxyribonucleic acid (DNA) from mycelia followed by subjecting them to Polymerase Chain Reaction (PCR) by amplifying the Internal Transcribed Spacer (ITS) region finalizing with DNA sequencing through sanger sequencing method. The results of conventional screening showed that the majority of the maize grain samples were contaminated with *Fusarium* spp. 36.7% (11/30), followed by *Penicillium* spp. 30.0% (9/30), *Aspergillus* spp. 20.0% (6/30) and *Rhizopus* spp. 13.3% (4/30). Sequence analyses of *Aspergillus* species revealed that out of 6 *Aspergillus* isolates, 3 were *Aspergillus* spp namely; *Aspergillus flavus, Aspergillus niger* and *Aspergillus wentii* while 1 was *Talaromyces flavus.* These results confirm the presence of potential aflatoxin-producing fungi that may colonize the maize. In conclusion, the presence of diverse fungal genera, including aflatoxin-producing *Aspergillus* species, highlights the need for broader screening by expanding the sample size while considering the spatial and temporal characteristics to plan for fungal contamination mitigation measure in future.

Key words: *Aflatoxin; Aspergillus flavus; Deoxyribonucleic acid (DNA); Maize; Polymerase Chain Reaction (PCR)*

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Introduction

In Tanzania, *Zea mays* (maize) is a staple food consumed widely thus, screening of aflatoxin producing fungi and assess maize contamination status is crucial for development and implementation of strategic mitigation measures to address adverse health effects in the community. Recently, Polymerase Chain Reaction (PCR)-based methods have emerged as major tool for detection of aflatoxin-producing fungi in foods (Bintvihok *et al.,* 2016).

Aflatoxin-producing fungi are derived from the genus *Aspergillus* capable of producing potentially hazardous, toxic and carcinogenic secondary metabolite known as aflatoxin (Mahuku *et al.*, 2023). The aflatoxin contamination of food crops such as maize, poses a big threat to public health, trade and livelihood in Tanzania (Kamala *et al.*, 2016). The most important aflatoxin producers are *Aspergillus flavus* and *Aspergillus parasiticus* (Boni *et al.*, 2021; Mahuku *et al.*, 2023). Ability to produce aflatoxin is highly conserved in some species such as *A. parasiticus* but highly variable for other *Aspergillus* species. *A. parasiticus* seems to have more than 95% strains, capable of producing aflatoxin while toxigenicity of *A. flavus* varies broadly depending on the geographic origin, substrate acted upon and strains (Temu, 2016). Toxigenic *A. flavus* strains produce various aflatoxins including aflatoxin B1 (AFB1), aflatoxin B2 (AFB2) and often cyclopiazonic acid (CPA); in which AFB1 being the most important in food safety. Likewise, *A. parasiticus* strains produce aflatoxins G1 (AFG1) and G2 (AFG2) (Gallo *et al.*, 2012).

Fungal infection and colonization is well recognized in agricultural crops particularly maize and groundnuts (Karaca *et al.*, 2017), although other crops such as cotton, dried cassava, coffee, chili tree nuts and legumes are also reported to be contaminated by aflatoxin (Boni *et al.*, 2021). Aflatoxin production plays significant role in yield loss and it is influenced by stresses such as drought (Debnath *et al.*, 2012; Nji and Babalola, 2022). Previous studies have shown that among various types of aflatoxin produced, six out of eighteen, namely B1, B2, G1, G2, M1, and M2, have been identified and are considered most important (Wacoo *et al.*, 2014).

The methods for detection and quantification of aflatoxin produced by fungi are very expensive and rarely done, especially in developing countries like Tanzania. These highly advanced methods include; thin-layer chromatography, high performance liquid chromatography (Boni *et al.*, 2021), gas-liquid chromatography, and gas chromatography/mass spectrometry. Other much simpler and faster but expensive are immunological methods have the disadvantage of "one substrate-one assay" setup. These methods include; the immunoaffinity column chromatography, the radioimmunoassay, and ELISA (Bintvihok *et al.*, 2016). The conventional methods of identifying and detecting fungi in foods is common in our setting and could provide indication of colonization of aflatoxin producing fungi in pre and post-harvest food products. However, the outcomes from this technique which rely on morphological characteristics can be highly variable because it depends on the media and culture conditions being used (Bintvihok *et al.*, 2016). These methods are time consuming, labor intensive and require the expertise of mycologists.

Macro and micro morphology study of aflatoxin producing fungi on agar plate and microscopy respectively have been always done (Mtega *et al.*, 2020; Agape *et al.*, 2021), but studies on screening and or characterization of these food contaminating and aflatoxin producing fungi are rarely if not at all done in our setting. This study therefore purposely aimed at utilizing molecular techniques for screening of aflatoxin-producing fungi associated with maize grains in the selected areas in Tanzania to identify actual fungal species in the study area. This study was designed to accurately characterize fungal species infecting maize grains in selected regions of Tanzania, employing molecular diagnostic techniques to specifically detect and profile aflatoxin-producing fungi.

Materials and methods

Study Area

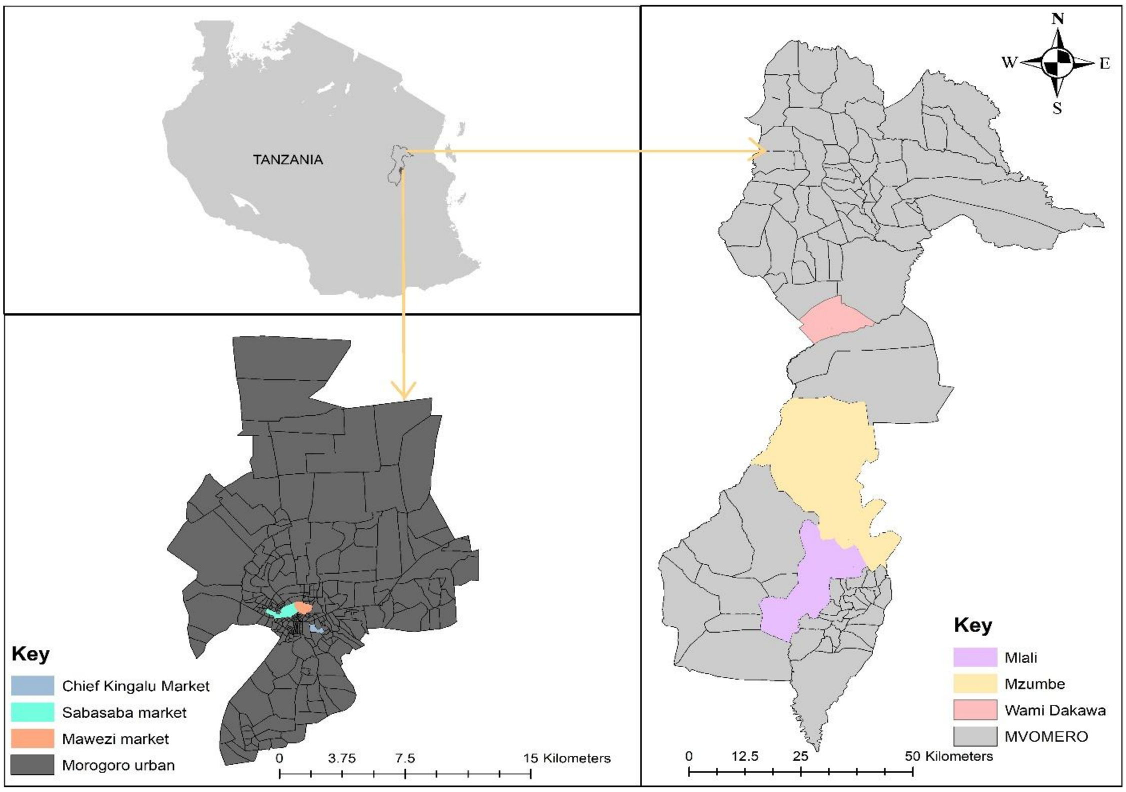
The study was conducted in Morogoro town, Mlali, Mzumbe and Dakawa in Morogoro region in Tanzania mainland, involving areas with dissimilar agro-ecological features to identify high risk areas (Fig 1). Morogoro urban lies between 6° 49′S and 37°39′E at the foot of the Uluguru Mountains whose topography includes mountainous terrain, hills, and plains, characterized by a hot, humid tropical climate with temperatures ranging from 16°C to 33°C and 821- 1505 mm annual rainfall. The area sits at altitude of approximately 509 meters above the sea level and has a distinct wet season from November to April. Mlali village lies at approximately 7° 5′ 51′′ S and 37° 23′ 47′′ E. The area is a flat river plain that is depended on for irrigation and used all year round for vegetable production. Maize farming always rotated with tomato, eggplants and sweet pepper is practiced by farmers. The area is characterized by various soil types, including fertile alluvial soil in lower-lying areas and oxisols in hilly areas with moderately high temperatures all year round. It receives rain with a long rain (March to May) and short rain (November to January).

Mzumbe is situated within the geographical coordinates of approximately 6°50′ to 7° 00′S and 37° 30′ to 37° 40′ E. The area covers an altitude ranging from 500 to 600 meters above the sea level. The area is characterized by hilly terrain and moderate temperatures (27.8°C to 32.6°C) and is dominated by metamorphic rocks types like gneiss and granulites. It receives annual rainfall ranging from 821mm - 1050 mm, primarily long season between November and May and short season from July to August.

Dakawa, located in the Mvomero District of the Morogoro region, with specific geo- climatic coordinates of approximately 6°26′S and 37°32′E. The area is situated within the low-lying Wami-Mkata plains, with a characteristic lowland agro-ecological zone, generally positioned at an altitude between 150 to 450 meters above the sea level. The area experiences a tropical savannah climate with consistency high temperature throughout the year, with averages ranging from 18°C to 30°C. It receives a bi-modal rainfall season, with long season from March to May and short season from November to January. The area has sandy clay loam soil at Dakawa irrigation scheme suitable for rice and maize production.

Figure 1

A map showing Sample collection sites in Morogoro municipality and Mvomero district in Morogoro region



Study Design

Maize grains samples were collected from six sites namely Mawenzi, Kingalu and Sabasaba markets in Morogoro Urban district and Mlali, Mzumbe and Dakawa in Mvomero district. Sample size was estimated using a standard formula for determining prevalence when the true contamination level is unknown was applied (Equation **1**). An assumed prevalence of 50% (p = 0.5) was used, together with a precision level of 20% and a 95% confidence interval. The actual sample size required was 180 maize grain samples for all sites, however, to attain a minimum requirement, approximately 30 samples were collected due to insufficient funding.

To ensure equal representation across the study area, five maize grain samples from five sellers were randomly collected from each of the six sampling sites (Mlali, Mzumbe, Dakawa, Kingalu, Sabasaba, and Mawenzi). Samples were packaged in individual ziploc bags, giving a total of 30 samples for laboratory analysis.

The maize grain samples were aseptically collected in ziploc bags using sterile forceps, properly labelled and sent to the microbiology laboratory in the Department of Crop Science and Horticulture at Sokoine University of Agriculture (SUA), Morogoro.

$$n = \frac{Z^2 \times p(1-p)}{d^2} \text{--------------------------------} \ (1)$$

Where:
$Z=1.96$ for 95% confidence
$p=0.5$
$d=0.2$ (precision chosen to match feasible sample size)

Fungal Isolation, Microscopic Identification, Media and Culture conditions

Fungi were firstly isolated by blotter method, in which the maize grains were placed in the wetted blotter paper in the petri dish, under the laminar flow hood in sterile conditions, which were then incubated at 23°C for about 3 to 5 days. The grown fungal mycelia on the blotter paper were examined microscopically by wet method using lactophenol cotton blue as stain, with the aid of cellotape method, observed under different magnifications. These grown fungal mycelia were inoculated on the prepared Potato Dextrose Agar (PDA) by using inoculating needle both at the periphery and center of the sterile petri dish. Finally, the inoculated media plates were sealed by cellotape and incubated at 23˚C for 3-5 days. **S**ub-culturing was done to obtain pure cultures of the *Aspergillus species.* The media plate with well grown pure cultures of *Aspergillus species* were selected for DNA extraction.

DNA Extraction from fungal mycelia

The fungal DNA were extracted following Mahuku protocol (Mahuku, 2004). The plate with pure culture of *Aspergillus species* was taken to a laminar flow hood, fresh mycelia (150mg) was transferred using sterile straight wire to a sterilized 1.5-mL Eppendorf (micro centrifuge) tube containing 300µL of Tris-Ethylenediaminetriacetate Saline (TES) extraction buffer (0.2 M Tris-HCl [pH 8], 10 mM EDTA [pH 8], 0.5 M NaCl, 1% SDS and acid-washed, sterilized 0.5 mm glass-beads). The transferred mycelia were macerated for 2 minutes with a hand-held disposable homogenizer that fit the 1.5 µL micro centrifuge tube. The sample was then mixed for 30 secs through vortexing followed by addition of 200 µL of TES extraction buffer containing proteinase K (final concentration of 50 µg/mL). The content was thoroughly mixed by vortexing then placed in a water bath at 65˚C for 30 minutes. Then one-half volume (250µL) of 7.5 M ammonium acetate was added and incubated at approximately 5˚C in the refrigerator for 10 minutes, the content then centrifuged for 15 min at 20,800 g. After centrifugation the supernatant was transferred to a new tube followed by addition of an equal volume (500 µL) of ice-cold isopropanol, then incubated at -20˚C for 1hour. The content was then centrifuged again for 10 min at 20800g to pellet the DNA. The supernatant was discarded and DNA pellet was washed with 800µl of cold 70% ethanol, the tube turned upside-down on clean sterile paper for 10-15 min to air-dry the DNA. The DNA was eluted from pellet with twice-repeated extractions with 250µL of 1× TE buffer (10mM Tris-HCl [pH 8], 1mM EDTA), each time centrifuging to avoid collecting pelleted polysaccharides.

The DNA solution obtained was transferred to a 1.5µL micro centrifuge tube followed by addition of 5µL of RNase (20mg/mL), and incubated at 37˚C for 60 min. The DNA was recovered and air-dried as described above. Finaly, the dried DNA pellet was dissolved in 100µL of 1× TE buffer. To verify the DNA quality and integrity, 2µL of each DNA sample mixed with 2µL of loading dye on a parafilm and 2µL of ladder were loaded on wells of prepared 0.8% agarose gel stained with ethidium bromide, using 5× Tris Acetate EDTA (TAE) buffer and allowed to run at 100V for 30 minutes. The gel image was viewed using OTTO gel documentation system (Japan) and then normalized for further studies.

PCR Optimization

Master Mix preparation

A master mix was set for 20µl reaction volume, containing PCR reagents including DNA polymerase, dNTPs, $MgCl_2$, buffer, primers (both forward and reverse primers) and molecular biology water was prepared following the volume proportions as shown in Table 1.

Table 1

Constituents of Master Mix by volume for PCR set up

ITEM	Reaction/volume (µL)	Number of samples (11)
Master Mix	12.5	137.5
Forward primer (ITS-1)	1	11
Reverse primer (ITS-4)	1	11
Molecular biology water	5.5	60.5
DNA	1	11
Total volume	20	225

Nine DNA samples were used and a negative control with addition of volume of single sample for correction of pipetting error, thus total volume for master mix correspond to eleven DNA samples as shown in Table 1. Thus, master mix was prepared, distributed in the separate labelled PCR strip followed by addition of optimal volume of DNA samples in each strip. The PCR strips closed and transferred to PCR room for running (Mancini *et al.*, 2016).

PCR Optimization and gel electrophoresis

For each of the PCR reaction mix contained 50ng/µL of each gDNA sample, 1×buffer (10 mm Tris-HCl pH 8.0, 1mm EDTA pH 8.0), 0.25 mm dNTPs, 2.5 mm $MgCl_2$, 0.1 mm of each of forward primer ITS-1, (5′- TCC GTA GGT GAA CCT TGC GG 3′) and reverse primer ITS-4, (5′- TCC TCC GCT TAT TGA TAT GC 3′) (Temu, 2016; Algammal *et al.*, 2021;) and 0.5 U Taq polymerase. PCR condition was; Initial denaturation at 94˚C for 1 minute followed by 34 cycles of denaturation at 94˚C for 30 seconds, annealing at 54.2˚C for 45 seconds, extension at 68˚C for 1 minute and final extension at 68˚C for 5 minutes (Table 2). PCR products (amplicons) were visualized on 1% agarose gel electrophoresis stained with ethidium bromide, submerged in 1× TAE buffer and ran for 1:30 hours at 120V. Gel documentation system was used to view the gel and results were recorded.

Table 2

Polymerase Chain Reaction (PCR) conditions

Activity	**Temperature (˚C)**	**Time (sec)**
Initial denaturation	94	60
Denaturation	94	30
Annealing	54.2	45
Elongation	68	60
Final elongation	68	300
Number of cycles		34×

DNA sequencing and multi-alignment analyses for identification of potential aflatoxin producers by using 5.8S ITS rRNA Sequences

Four DNA samples were sent to Macrogen-Europe, for nucleotide sequencing. The DNA nucleotide sequencing of 5.8S –ITS rRNA gene was done by using Sanger sequencing method using ITS1 and ITS 4 primers (Reverse and Forward) targeting regions ranging in size from 565 to 613bp to be used as a marker for identification of the *Aspergillus* species. Nucleotide sequence cleaning and analysis was done using Sequencher 5.4.6 software. Nucleotide basic alignment search tool (BLASTn) was used to compare the similarity and identity of *Aspergillus* species obtained from the samples in relation to those available in the National Centre for Biotechnological Information (NCBI). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version X (Kumar *et al.*, 2018). Multi-aligment was performed by ClustalW(Fountain *et al.*, 2014) using the sequences of several *Aspergillus* species belonging to Section Flavi (*A. minisclerotigenes, A. fumigatus, A. oryzae, A. niger, A.wentii, and A. flavus)*. Phylogenetic trees were prepared by the Maximum Likelihood method (Aj *et al.*, 2020). All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Bootstrap values were calculated from 1000 of the bootstrap procedure using programs within MEGA X package which refers to tests of the reliability of an inferred tree.

The evolutionary history was inferred by using the Maximum Likelihood method based on the general time reversible model (Kumar *et al.*, 2018). The tree with the highest log likelihood (-1142.36) is shown in Figure 5. The percentage of trees in which the associated taxa clustered together is shown next to 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 Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018)

Statistical analysis

This study was a descriptive investigation that used molecular techniques to screen maize grains for aflatoxin-producing fungi. Frequencies and percentages were used to summarize the distribution of fungal genera isolated from the samples. The data were analyzed using Microsoft Excel software.

Results

Macro and micro morphology analysis

Analysis of different fungi genera isolated from maize grain samples for cultural and morphological characteristics showed that, there was variation in the colony colour, margins and texture. The morphological characteristics of fungi including *Aspergillus* spp, *Penicillium* spp, *Fusarium* spp *and Rhizopus* spp are presented in Fig 2a & 2b.

Figure 2a

Macroscopic examination of the fungi culture on Potato Dextrose Agar (PDA) Aspergillus spp *(E), Penicillium* spp *(F), Fusarium* spp *and Rhizopus* spp (Coinfection) *(G) and Fusarium* spp *(H)*

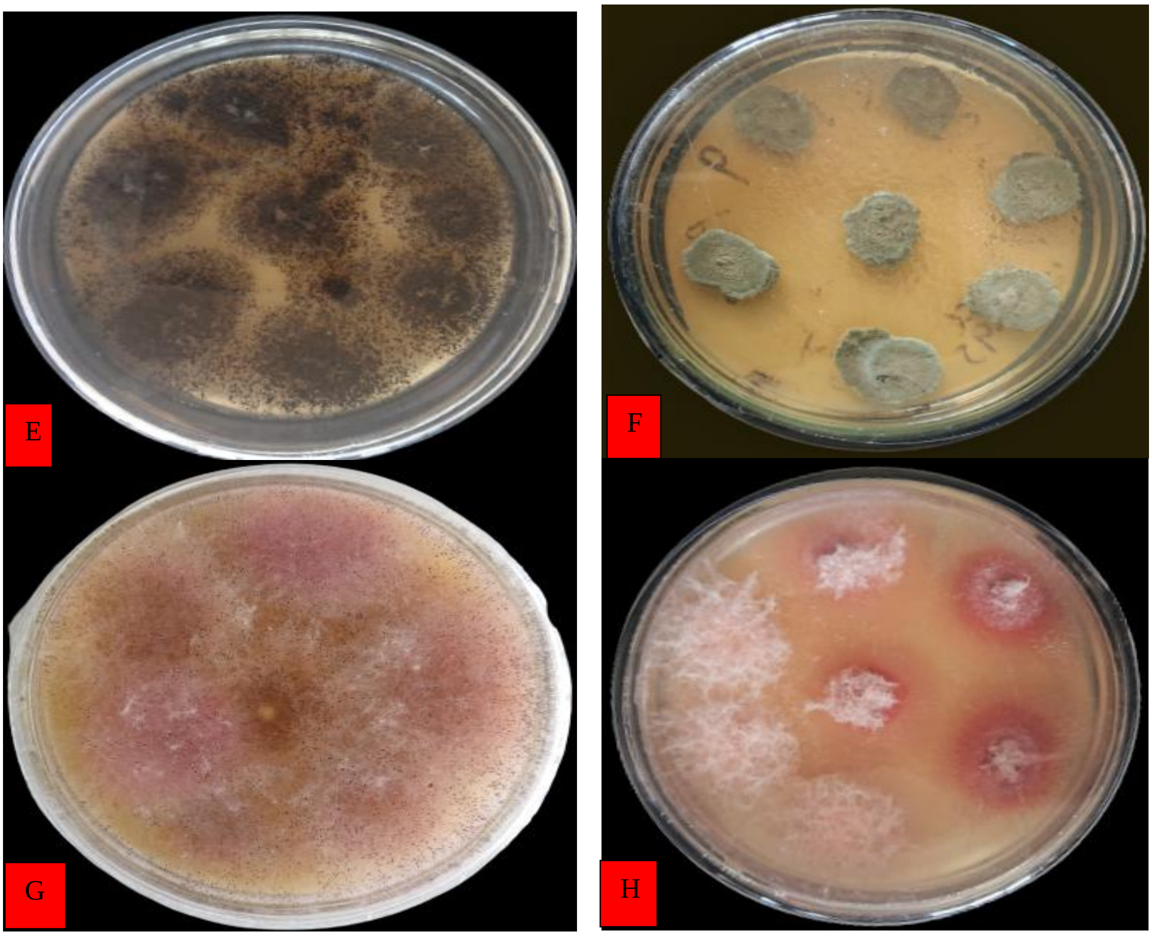
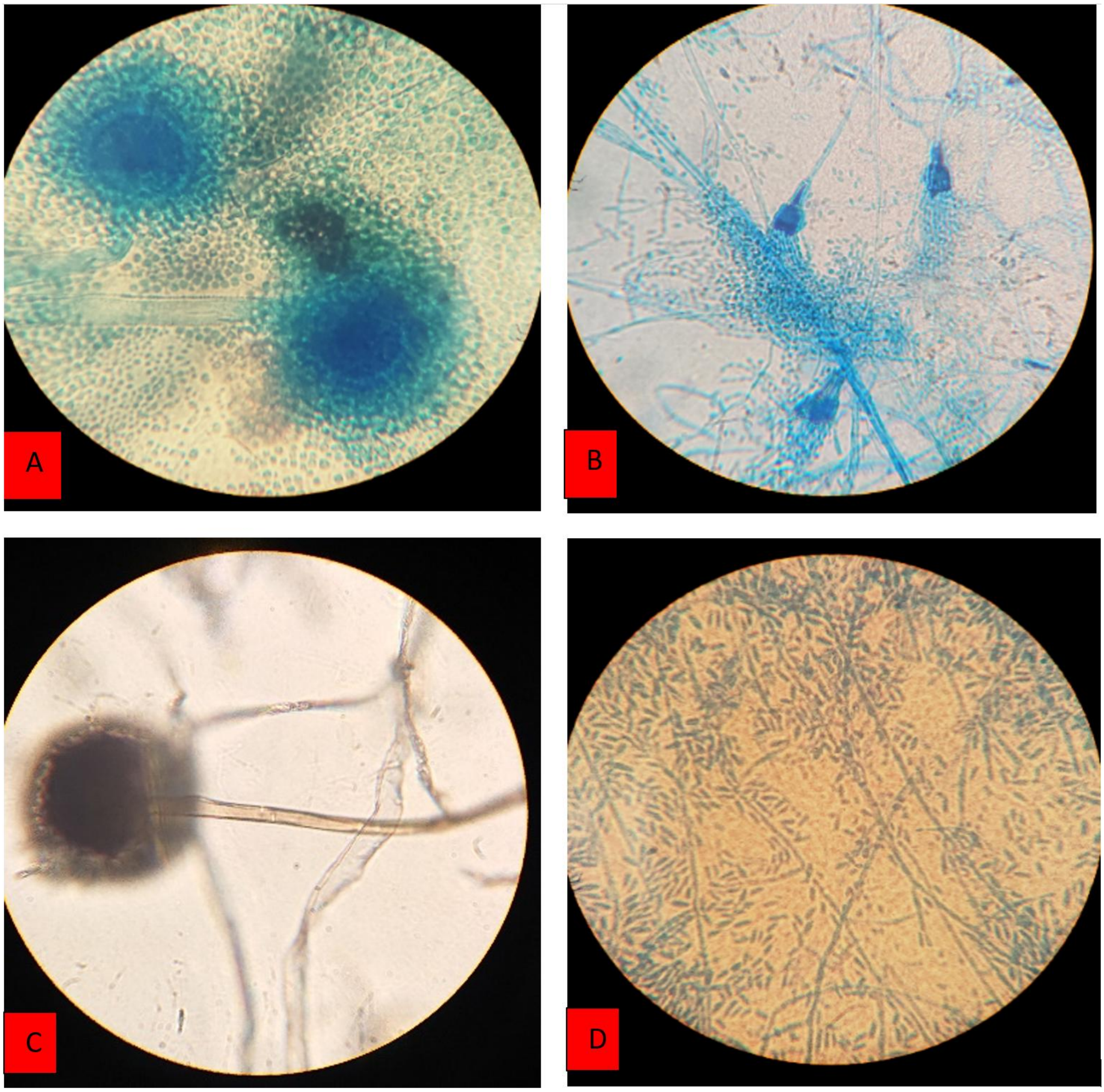


Figure 2b

Microscopic examination of fungi Aspergillus spp *(A), Penicillium* spp *(B), Rhizopus* spp *(C) and Fusarium* spp *(D)*



Occurrence of different fungi based on maize grains sampling sites

A total of 30 maize grain samples were first isolated through blotter method (Fig. 3). Three *Aspergillus* species namely, *A. niger, A. flavus, A. wentii* were found on the samples analyzed. Also, *Penicillium* spp, *Fusarium* spp as well as *Rhizopus* spp were found to colonize maize grains. More than one genus was found in 70% (N =30) of the samples. Among the isolated fungal genera, ***Fusarium* spp.** showed the highest frequency (36.7%), followed by ***Penicillium* spp.** (30.0%), ***Aspergillus* spp.** (20.0%), and ***Rhizopus* spp.** (13.3%)., as depicted in Table 3.

Table 3

Proportions of fungal genus with respect to maize collection sites (N=5) and the overall prevalence of each fungal genus

Location	Fungi Genus			
	***Aspergillus* n (%)**	***Fusarium* n (%)**	***Penicillium* n (%)**	***Rhizopus* n (%)**
Mlali	2(40)	2(40)	1 (20)	0 (0)
Mzumbe	1 (20)	2 (40)	1 (20)	1 (20)
Kingalu	0 (0)	2 (40)	3 (60)	0 (0)
Dakawa	1 (20)	2 (40)	1 (20)	1 (20)
Mawenzi	1 (20)	2 (40)	2 (40)	0 (0)
Sabasaba	1 (20)	1 (20)	1 (20)	2 (40)
Prevalence	**6(20, N= 30)**	**11 (36.7, N=30)**	**9(30, N=30)**	**4 (13.3, N=30)**

Figure 3

Fungal growth on blotter plate

Molecular detection and characterization of isolated fungi

Overall, the results showed successful amplification of fungal DNA in all the 6 suspected Aspergillus isolates. Clear and distinct bands of approximately 600bp were observed in lanes 1 to 9, indicating successful amplification of fungal

ITS regions in all tested isolates (Fig 4a&b). These PCR products were submitted for sequencing to confirm the fungal species.

After sequencing followed by bioinformatics analysis specifically BLASTING, four isolates were identified as *Aspergillus flavus, Aspergillus niger, Aspergillus wentii* and *Talaromyces flavus* with percent identity of 92.35%, 98.41%, 92.96% and 80.00% respectively. The phylogenetic tree, showed independence clustering of two Tanzanian *Aspergillus niger and Aspergillus wentii* from other species of *Aspergillus* from Zimbabwe, India and China (Fig 5).

Figure 4a

Gel electrophoresis results of fungi samples after PCR amplification, Lane M is the 100 bp DNA size Marker, lane 1-9 are samples and NC is a negative control.

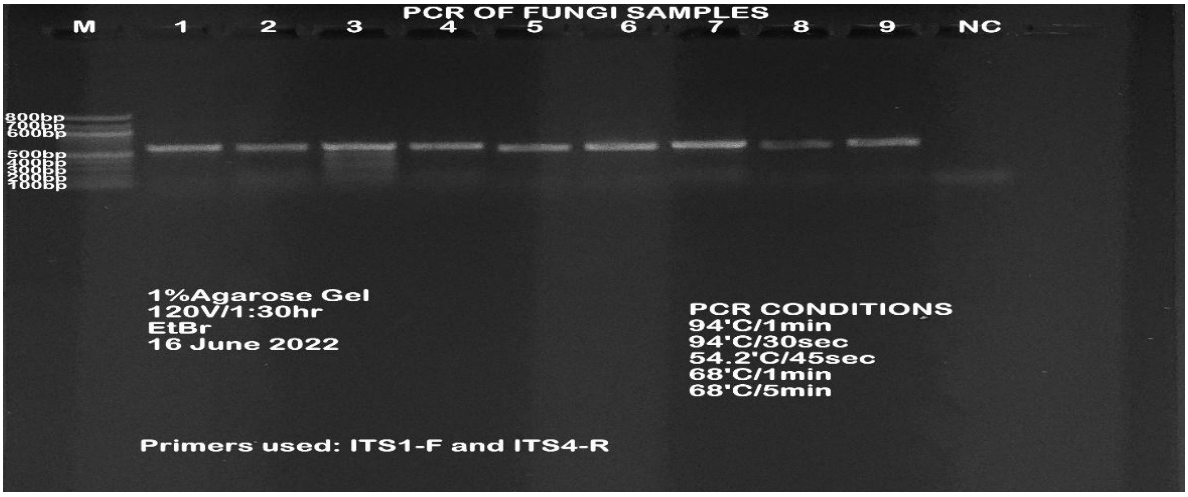


Figure 4b

Gel electrophoresis results of fungi samples prepared for sequencing submission

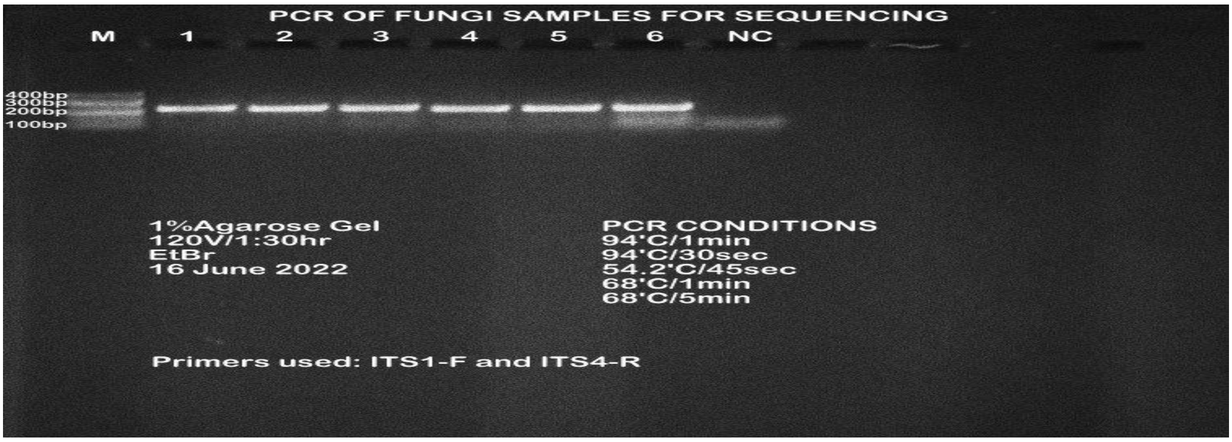
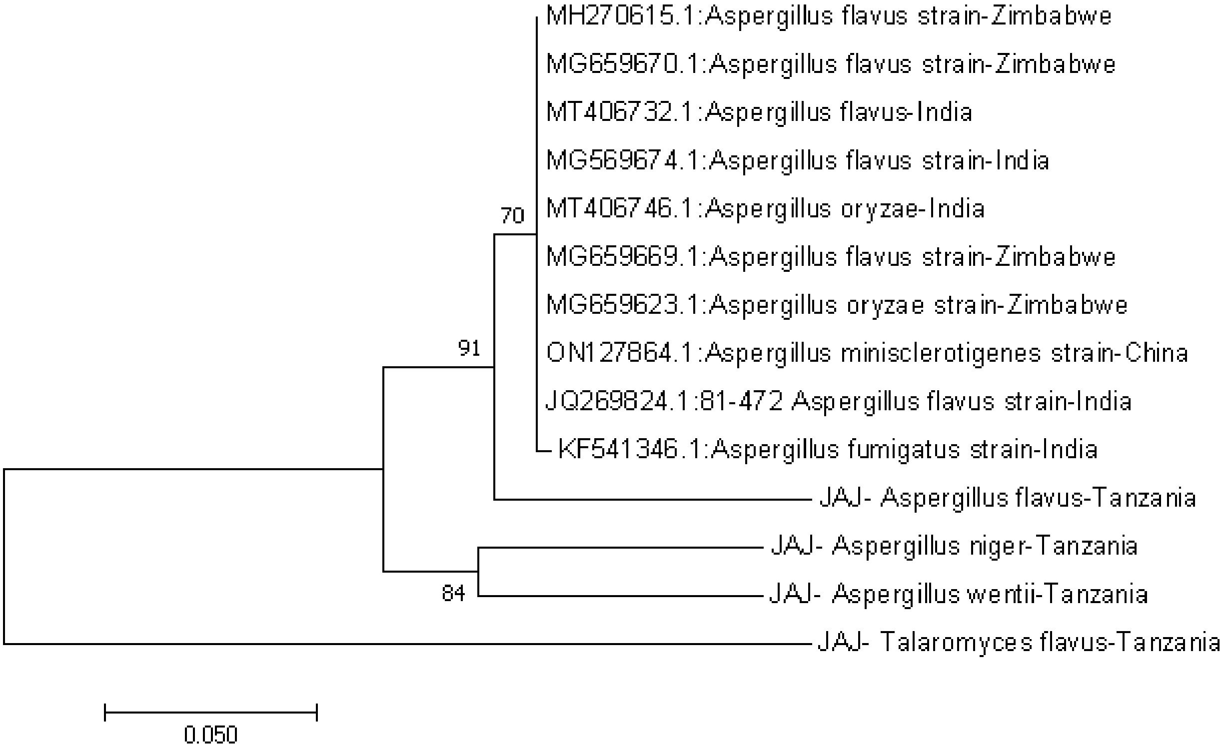


Figure 5

Molecular Phylogenetic analysis by Maximum Likelihood method



Discussion

This study investigated the occurrence and molecular characterization of aflatoxin-producing fungi associated with maize grains collected from selected areas of Morogoro Region, Tanzania. The findings demonstrate a considerable diversity of fungal genera colonizing maize, with important implications for food safety and public health.

The predominance of *Fusarium* spp. observed in this study indicates that maize grains are highly susceptible to colonization by this genus. *Fusarium* species are well known for their ability to produce fumonisins and other mycotoxins, which may have detrimental effect to both human and animal health (Wacoo *et al.*, 2014). The high occurrence of *Fusarium* spp. may be attributed to favourable environmental conditions such as temperature, humidity, and agronomic practices, particularly in areas like Mlali and Dakawa. Similar findings have been reported in other African countries (Okoth *et al.*, 2012; Probst *et al.*, 2014), suggesting that *Fusarium* contamination is widespread in maize production systems.

Penicillium spp. were the second most frequent isolates, indicating their significant role in post-harvest contamination. These fungi are commonly associated with poor storage conditions, including high moisture content and inadequate ventilation. The relatively high prevalence of *Penicillium* spp. observed especially in market sites such as Kingalu and Mawenzi suggests that storage and handling practices may contribute substantially to fungal contamination.
Although *Aspergillus* spp. accounted for 20.0% of the isolates, their presence is of particular concern due to their ability to produce aflatoxins (Boni *et al.*, 2021). Molecular characterization confirmed the presence of *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus wentii*. Among these, *A. flavus* is a well-established aflatoxin producer. The detection of *A. flavus*, even at relatively low frequency, highlights the possibility for aflatoxin contamination in maize. This is consistent with

previous reports by (Mollay *et al.*, 2020; Msemwa *et al.*, 2024) from Tanzania where aflatoxin outbreaks have been linked to contaminated maize.

The occurrence of *Rhizopus* spp. and *Talaromyces flavus*, although not aflatoxin producer, indicates an expansion in fungal diversity associated with maize grains. These fungi are not major producers of aflatoxins however, their presence indicates poor post-harvest handling and storage conditions, which may facilitate the growth of other toxigenic fungi.

Sequencing results further validated species identification and revealed genetic relationships among isolates. The clustering of Tanzanian *Aspergillus flavus* isolate with reference strains from other regions confirms their existence in the study sites.

Despite the relatively small sample size, the study provides important insights into fungal contamination of maize in Morogoro Region. The findings underscore the need for improved pre- and post-harvest management practices, including proper harvesting, drying, storage, and handling of maize grains. Additionally, the integration of molecular techniques with conventional methods enhances the accuracy and reliability of fungal identification.

Conclusion

In conclusion, the presence of diverse fungal genera, including aflatoxin-producing *Aspergillus* species, highlights the need for broader screening by expanding the sample size while considering the spatial and temporal characteristics to plan for fungal contamination mitigation measure in future.

Acknowledgement

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Conflict of interest

The authors declare no conflict of interest.

Study limitations

The sample size was relatively low because of limited funding to accommodate the actual sample size that could give sound representation of the study areas.

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