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Research Article

Isolation and Characterization of Fungal Strains Associated with Mycotoxin Production from Bambara Nuts (*Vigna subterranea* (L.) Verdc) Marketed in Nigeria

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ABSTRACT

Fungal contamination of food commodities is a global food security challenge that impacts negatively on the health of consumers. Mycotoxins are produced as secondary metabolites by some pathogenic fungi and may contaminate agricultural products while on the field or during harvesting and storage. A field survey was conducted in order to isolate and identify the mycotoxin-producing fungal strains in Bambara nut collected from major markets in eight States in Nigeria including Oyo, Ogun, Lagos, Ekiti, Kwara, Kogi, Abuja, and Nasarawa. Samples per site were pooled, processed and screened for the presence of mycotoxin-producing fungi by culture technique and intergenic spacer sequencing (ITS). Mycotoxin production was determined using thin layer chromatography (TLC) with scanning densitometer. Twenty-four mycotoxin-producing fungal species were isolated from Bambara samples, which were identified as *Talaromyces pinophilus*, *Macrophomina phaseolina* (6), *Fusarium oxysporum* (2), *Aspergillus foetidus*, *Aspergillus flavipes*, *Rhizopus oryzae* (2), *Phanerochaete chrysosporium*, *Aspergillus flavus* (2), *Aspergillus terreus*, *Aspergillus awamori*, *Aspergillus welwitschiae*, *Rhizopus microsporus*, *Ceriporia lacerate* and *Fusarium verticillioides* (accession numbers of MN42329-MN4233323) from Bambara nuts. The genera *Macrophomina* were the most dominant. Some of the identified fungi are noted for the production of mycotoxins and mycotoxins exert toxic effects on animals and humans. The fungi associated with Bambara nut diseases were identified and can be taken as targets in varietal improvement of Bambara nut for resistance to fungal diseases in Nigeria.

Keywords: Bambara groundnut, Mycotoxin, Contamination, Fungi, ITS

Introduction

Bambara groundnut (*Vigna subterranea* (L.) Verdc), is a legume with high content of protein and minerals, which originated from Africa [1], although it's now found in every continent of the world [2]. The high nutritional composition promotes its utilization in many food applications. It is presently grown throughout Nigeria, with the exception of the riverine and swampy areas. The shift in cultivation of bambara groundnut from the drier to the wetter parts of the country has subjected the crop to more disease problem due to the prevalent high humidity. It grows near or below the surface of the soil which serves as fungal inoculum [3]. Mold contamination and subsequent mycotoxin occurrence may become inevitable under certain environmental conditions [4]. Countries in Asia and Africa experience high temperature (26–39°C) and high relative humidity (67–98%), conditions which support the growth of mycotoxigenic fungi on agricultural crops and consequently the production of mycotoxins on these crops [5]. However, climate change that results in global warming has led to emergence of a shift in the prevalence of aflatoxigenic fungi and aflatoxin production in temperate regions where fungal contamination and toxin

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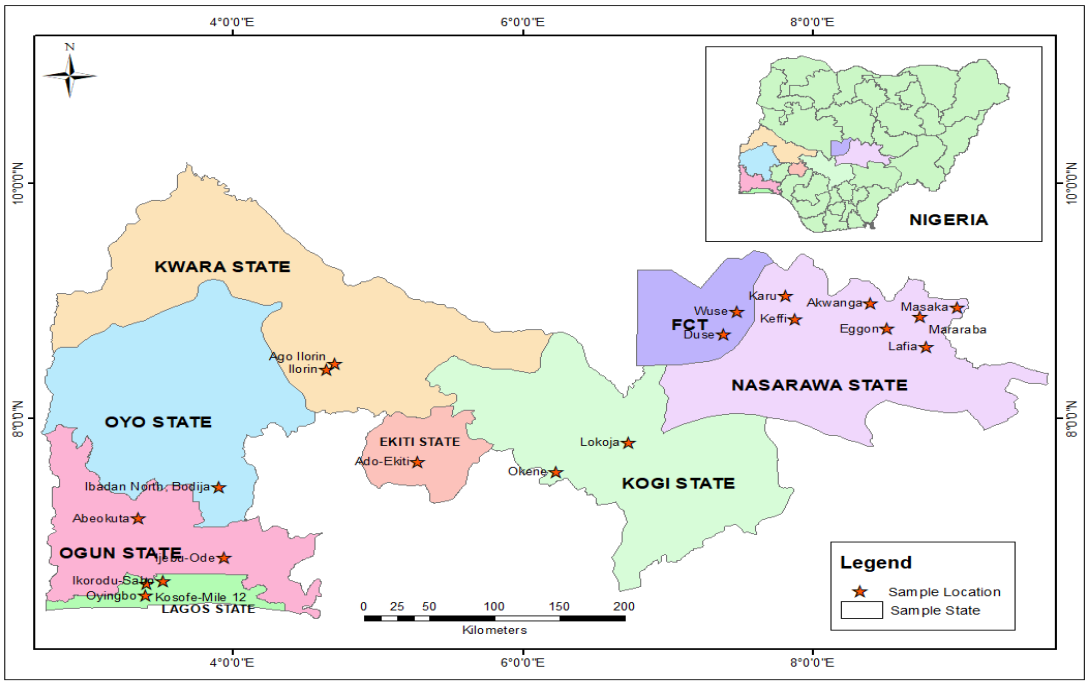

Figure 1. Map of Nigeria showing the location of the study area and sampling points

production rarely occur [6, 7]. Generally, fungal growth and mycotoxin accumulation in agricultural crops are affected by several factors which include fungal species potential, stress factors such as temperature and relative humidity, type of substrate, water activity, aeration and duration of fungal growth [8, 9]. There are few reports on isolation and characterization of the natural mycobiota of Bambara groundnut and possible occurrence of mycotoxins in Bambara groundnut flour. The limited knowledge of fungi infecting Bambara groundnut in Nigeria hinders the control of fungal diseases and the creation of resistant varieties.

This study is aimed at isolating and characterizing the filamentous fungi of Bambara groundnut using molecular methods of DNA extraction, polymerase chain reaction (PCR) and sequencing.

Material and Methods

Sample collection for mycotoxin detection and isolation of fungi

For the fungal isolation, one hundred and twenty (120) samples of Bambara groundnut were randomly collected from selected major markets in each of the sampling sites from two geopolitical zones (North Central and South West) in Nigeria. The following States were sampled: Oyo, Ogun, Lagos, Ekiti, Kwara, Kogi, Abuja, and Nasarawa. Figure 1 shows the map of Nigeria showing the location of the study area and sampling points.

Aflatoxin extraction and detection in the Bambara samples

The Bambara samples were collected from major markets in each of sampling sites. The Bambara nut samples were carefully grounded with commercial waring blender and thoroughly mixed. Twenty grams (20 g) of grounded sample was weighed out (2 replicates) for extraction purpose. Each weighed sample was blended with 100 ml of 70% methanol for three minutes using waring blender. The blended mixture was poured into a 250 ml Pyrex conical flask and seal flask with Parafilm. Then sample was shaken using orbit shaker at 4 × 100 rpm for 30 minutes. The filtrate was obtained using No 1 quantitative Whatman filter paper, 185 mm. To the filtrate, 20 ml of distilled water was added and 25 ml of dichloromethane was further added. The bottom phase of the extract was drained through a bed of 20 g anhydrous sodium sulphate into a 150 ml white plastic beaker.

The dried extract in tripour beaker was washed with dichloromethane (1-2 ml) into a cleaned eppendorf tube. The extract was dried overnight in the fume hood. The extracts were reconstituted by vortexing with 1.0 ml dichloromethane each. The developed plates were viewed under the ultraviolet light-box (wavelength = 365 nm) to see whether each extract fluoresces or not.

Table 1. Results of sequence comparisons of isolates with those available in the NCBI database

Isolates	Size of contig sequences (bp)	Accession numbers	Corresponding species in the NCBI database	Identity	Query
Talaromyces pinophilus	563	MN423293	KF031353	90	97
Macrophomina phaseolina	576	MN423294	MK454909	99	98
	575	MN423301MN423302	MK454909	98	87
	574	MN423303	MN423322	98	95
	628		MN423303	99	93
Fusarium oxysporum	549	MN423296	MN298740	90	85
	544	MN423297	MK692865	95	94
	541	MN423332	KJ439169	95	98
Aspergillus foetidus	588	MN423298	MK910068	96	96
Aspergillus flavipes	578	MN423299	MK775952	91	94
Rhizopus oryzae	628	MN423300	LC514328	99	97
	628	MN423321	MH864361	99	97
Phanerochaete chrysosporium	639	MN423305	MN736179	96	97
Aspergillus flavus	591	MN423309	MN565937	96	98
	589	MN423325			
Aspergillus terreus	594	MN423310	MK027221	90	96
	606	MN423326		93	96
Aspergillus awamori	584	MN423313	MH986194	90	88
Aspergillus welwitschiae	601	MN423320	KT826638	96	95
Rhizopus microsporus	694	MN423324	KF709997	99	96
Ceriporia lacerate	634	MN423327	KP326576	71	78
Fusarium verticillioides	556	MN423330	MN882831	83	96

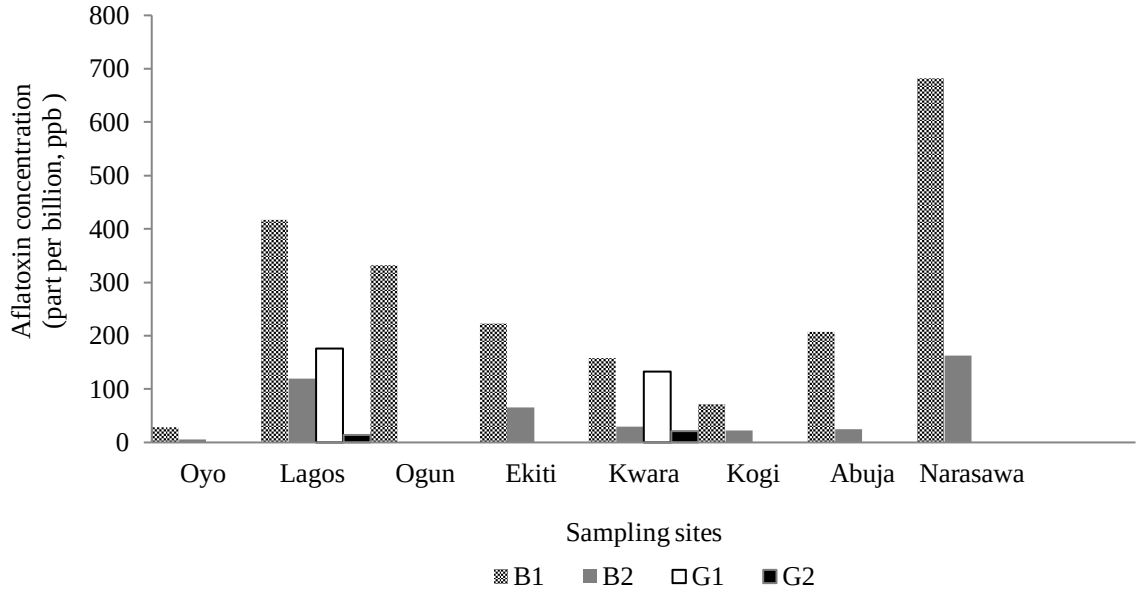

Figure 2. Quantification of Aflatoxin in the Bambara samples

Those with fluorescence and those without are compared with the standards. The extracts were qualified by fluorescence development and during qualitative analysis, the extracts were further subjected to quantitative analysis to ascertain total amount aflatoxins (B1, B2, G1 and G2) in the samples. This was done with the aid of thin layer chromatography (TLC) with scanning densitometer (Camag TLC Scanner 3).

Isolation of mycotoxin producing fungi

To 2 g of sample, 10 ml of distilled water was added and was well mixed. Out of the sample solution, 1ml was plated out on the surface of the solidified Potato Dextrose Agar and Sabouraud Dextrose Agar (Fluka, Sigma-Aldrich, USA) and plates (in triplicates) were incubated at room temperature (28°C) for 72-120 h.

Identification of mycotoxin producing fungi

The isolates were then identified using appropriate microscopic and macroscopic characteristics and ITS gene sequences and stored as agar slants at 4°C until needed.

Cultural and morphological characteristics

The fresh culture samples were used for the microscopy. The samples were subcultured on a freshly prepared Potato Dextrose Agar and Sabouraud Dextrose Agar plates and further processed by Lactophenol staining. They were examined for spore formation and other characteristics by using a compound microscope (Hitachi S–3500N model, ThermoNaran, Hitachi technologies, Schaumburg, Illinois, USA). The pure cultures of the fungal isolates were identified according to the identification criteria of mycology [10, 11].

Purification of cultures through single spore isolation

The test organisms were purified through single spore isolation technique [12]. The single conidial isolates were maintained on low nutrient medium for further studies.

Molecular identification

Genomic extraction and ITS gene detection

The genomic DNA of the strains was extracted and purified following a standard protocol for fungal genomic DNA preparations using Quick DNA™ fungi/bacterial miniprep kit D6005 (USA). The ITS gene was amplified by Polymerase Chain Reaction (PCR) (94°C for 5 min, 30 cycles consisting of 94°C for 30 s, 50°C for 30 s, 72°C for 45 s followed by a terminal incubation at 72°C for 7 min) using universal ITS-4 (5'TCCTCCGCTTATTGATATGS3') and ITS-5 (5'GGAAGTAAAAGTCGTAACAAGG3') primers. The PCR product purification of isolates was carried by adding 2.5 µL of PCR cocktail mix to 10.0 µL of the PCR product, mixed well and incubated at incubated at room temperature for 30 min. The reaction was stopped by heating the mixture at 94°C for 5 min. The PCR product was purified and sequenced with an automated sequencing apparatus (3130XL, Applied Biosystems). The ITS sequences of the strains were searched for homology with the sequences in public databases using the BLAST search program (http://www.ncbi.nlm.nih.gov/) to find closely related fungal ITS gene sequences. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 [13]. The neighbor-Joining evolutionary model was used to construct the phylogenetic tree.

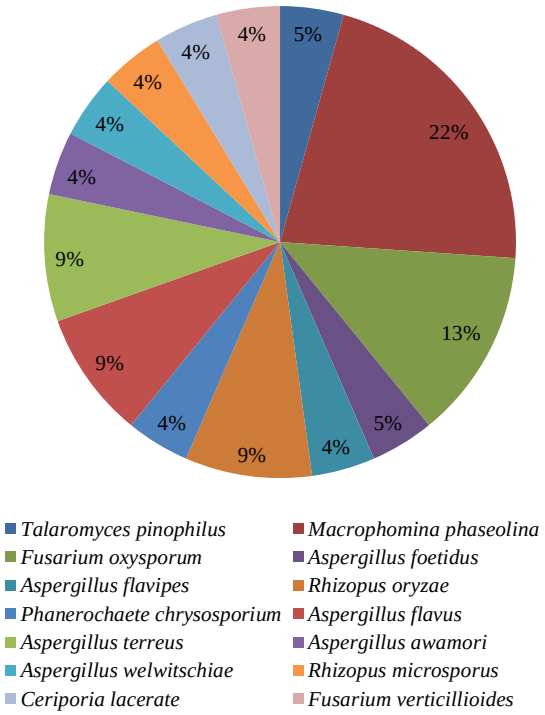

Figure 3. Prevalence of the strains associated with mycotoxin production in Bambara nut

Agarose gel electrophoresis of DNA fragments

The PCR amplified DNA segments were separated by electrophoresis on a 1.5 % agarose gel stained with ethidium bromide, using 100 bp DNA marker (Promega, USA) as DNA standard, Millipore water (blank) was used as negative control. The gel was run for 80 min at 100 V, and the amplified products were observed and imaged

by Kodak fluorescent imaging equipment, model IS 4000R (Kodak image station, care stream molecular imaging health Inc. Rochester, NY, USA.).

Results and Discussions

Aflatoxin extraction and detection in the Bambara samples

In the present study, markets from Nasarawa State recorded highest aflatoxin B1 concentration followed by Lagos State then Ogun State, while Oyo State had the least aflatoxin concentration (Figure 2). Whereas with aflatoxin B2 concentration, Nasarawa State recorded the highest concentration followed by Lagos then Ekiti while Ogun had 0 (zero means the aflatoxin level is below detection limit of the analytical method (1 ppb) (Figure 2). Similarly, Lagos State recorded the highest aflatoxin concentration G1, while same aflatoxin obtained in other States were below the detectable limit (Figure 2). The same trend was observed with aflatoxin G2 concentration where Lagos State had the highest value and others recorded values below the detectable limits (Figure 2). Olagunju *et al.* [14] found the co-occurrence of aflatoxins B1, B2, G1 and G2 and quantified the concentration as (AF) B1 (0.13–6.90), AFB2 (0.14–2.90), AFG1 (1.38–4.60), and AFG2 (0.15–1.00) in the flour samples. Ogiehor *et al.* [15] reports showed total aflatoxin range in some States in Nigeria: Anambra (0.44-3.69 µg/kg), Enugu (0.37- 5.71 µg/kg), Cross River (0.32-4.57 µg/kg), Edo (0.13-4.46 µg/kg), Delta (0.26- 3.64 µg/kg), Imo (0.14-3.16 µg/kg), Rivers (0.17-4.14 µg/kg), Lagos (0.012-2.54 µg/kg), Ondo (0.18-2.41 µg/kg), and Ogun (0.25-1.66 µg/kg). However, some results of this study were above the international permissible level, and pose a source of concern on the safety level of bambara flour on display in our local markets. Limits vary according to the commodity around the world whilst they are 2-4 µg/kg in the European Union (EU) [16]. While USA, the U.S. Department of Agriculture and the U.S. Food and Drug Administration (FDA) have established an "actionable" level of 15-20 ppb or 20 µg/kg for foods except milk and a limit of 0.5 µg/kg for AFM1 in milk [16].

Isolation and Identification of mycotoxin producing fungi

Also, in the present study, twenty-four mycotoxin-producing fungal species were isolated from Bambara samples, which were identified as *Talaromyces pinophilus*, *Macrophomina phaseolina* (6 strains), *Fusarium oxysporum* (2 strains), *Aspergillus foetidus, Aspergillus flavipes, Rhizopus oryzae* (2 strains), *Phanerochaete chrysosporium, Aspergillus flavus* (2 strains), *Aspergillus terreus, Aspergillus awamori, Aspergillus welwitschiae, Rhizopus microsporus, Ceriporia lacerate* and *Fusarium verticillioides* (accession numbers of MN42329-MN4233323). Ouoba *et al*. (17) reported that few studies that have been investigated on mycotoxin producing from Bambara nut from some African countries such as *Fusarium* sp. (Zambia, Togo, and Zimbabwe), *Cercospora* sp. (Botswana, Togo) and *Fusarium oxysporium* (Burkina Faso, Tanzania). The most predominant strain out of the whole fungi strains was *Macrophomina phaseolina* (Table 1). Ouoba *et al.* [17] isolated and identified nineteen fungal strain associated with mycotoxin production from Bambara nut using morphological and ITS gene sequence analysis. Moreover, the same authors reported *Macrophomina phaseolina* as the most predominant fungi strain followed by *Cercospora* sp. from the Sahel, Sudan-Sahel and Sudan zones in Burkina Faso. In this study the same fungi strains were obtained and *Macrophomina phaseolina* was the predominant fungi strain.

Similarly, in this study twenty-four fungi strains were identified using the same approach. Fungal strains such as *Macrophomina phaseolina*, *Cercospora* sp., *Fusarium* sp. *Aspergillus niger, and Aspergillus flavus* were equally identified in this present study.

Evidences from ITS gene sequence analyses as summarized (Table 2) showed that the ITS genes of the strains had 90-100% similarity to other strains from the GenBank. The phylogenetic tree showed five distinct clusters; strains are closely related by being in the same cluster and having genetic distance (Figure.4). However, the mycotoxin producers recovered in this study were further stratified into eight different subgroups within their common cluster. The sequence analysis of ITS gene and unrooted phylogenetic tree showed that fungal species are likely to have evolved from the same ancestor (Figure 4). The cultural characteristics, as well as the ITS gene sequences, confirmed the association of strains to the genera. The characteristic features of the strains were similar to those reported previously by Ouoba *et al.* [17]. To the best our knowledge, this is the first report on identification of tropical toxigenic fungi isolates obtained without any precedent in Nigeria from Bambara nut through morphological characters and ITS

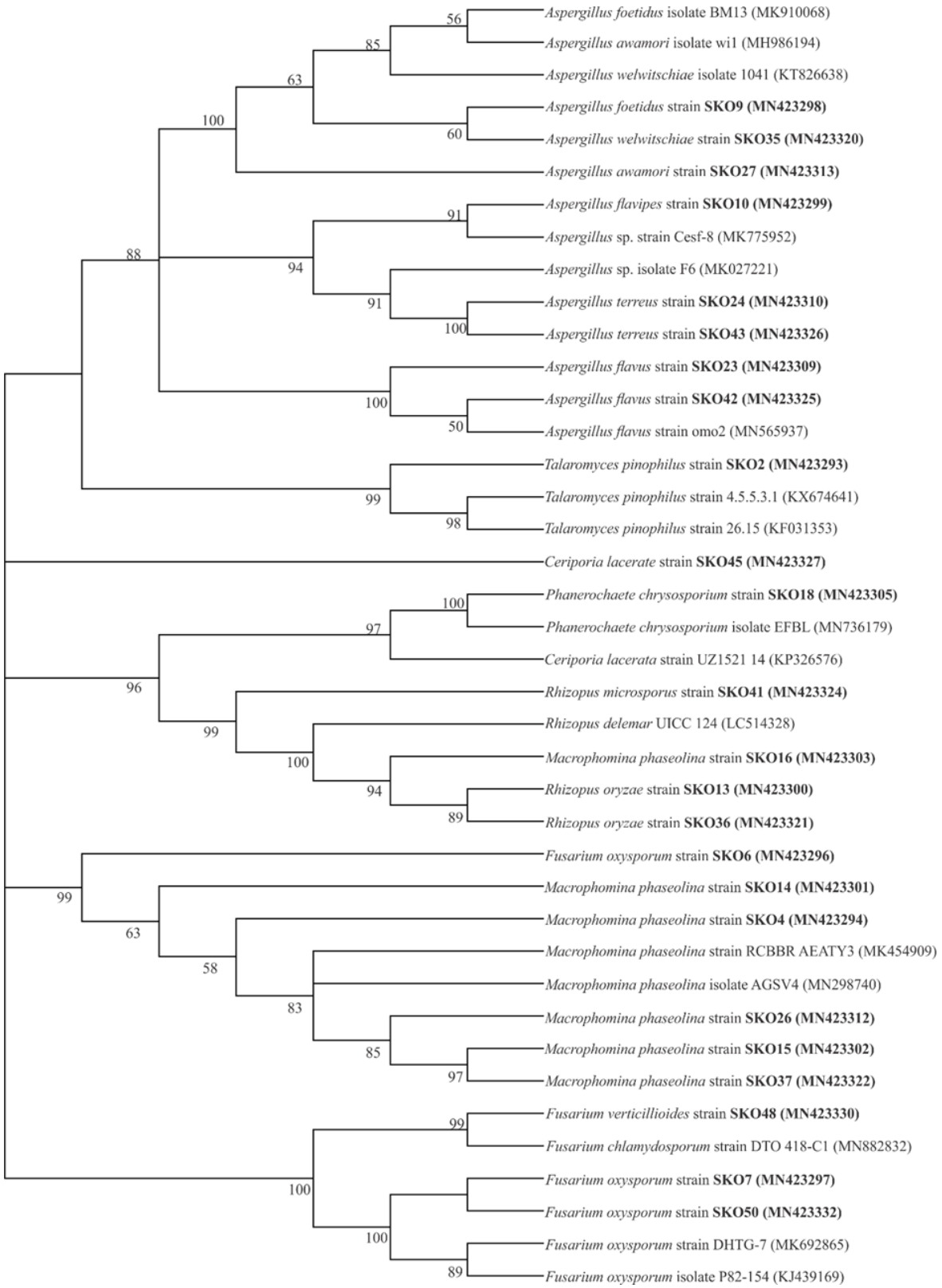

Figure 4. Phylogenetic tree (dendrogram) of mycotoxin-producing fungal species ITS sequences using the neighbor-joining method [17]. Bootstrap test = 1000 replicates. The evolutionary distances were computed using Tamura-Nei model. Analysis involving 40 nucleotide sequences was computed using Mega 5 software.

gene sequence analysis. Among the twenty-four (24) main fungi associated to Bambara groundnut diseases in Nigeria that this study allowed to characterize, only the species *Macrophomina phaseolina* and *Fusarium* sp. were previously reported to be associated to Bambara groundnut fungal diseases in Africa [18]. Heller *et al.* [18] reported only *Macrophomina phaseolina* to be associated to Bambara groundnut fungal diseases. However, Ouoba *et al*. [17] and this present study disagreed with this fact that *Macrophomina phaseolina* was the only fungi strain responsible for Bambara groundnut disease.

Conclusion

The combination of morphological identification and molecular approach based on sequencing of rDNA-ITS region of fungi, has strengthened our knowledge about fungi microflora associated to Bambara groundnut diseases in Nigeria. This study reveals the occurrence of twenty-four fungi associated to Bambara groundnut diseases. These fungi can be taken as targets in varietal improvement of Bambara groundnut for resistance to fungal diseases in Nigeria.

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