Genetic diversity of aflatoxin-producing Aspergillus flavus isolated from groundnuts in selected agro-ecological zones of Uganda

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Research article

Keywords: Mycotoxins, secondary metabolites, Aspergillus species and agro-ecological zone.

Posted Date: August 21st, 2019

DOI: https://doi.org/10.21203/rs.2.13266/v1

Abstract

Background Aspergillus is the main fungal genus causing pre- and post-harvest contamination of groundnuts. Aspergillus flavus belongs to section Flavi, a group consisting of both the aflatoxigenic species (A. flavus, A. parasiticus and A. nomius) and non-aflatoxigenic species (A. oryzae, A. sojae and A. tamarii). Aflatoxins are food-borne toxic secondary metabolites produced by Aspergillus species, causing hepatic carcinoma and stunting in children and are the most toxic carcinogenic mycotoxins ever identified. Despite the well-known public health problems associated with aflatoxicosis in Uganda, information about the genetic diversity of the main aflatoxin causing fungus, Aspergillus flavus in this country is still limited. Results A cross-sectional survey was therefore carried out in three main groundnut-growing agro-ecological zones (AEZs) of Uganda; West Nile farming system, Lake Kyoga basin mixed farming system and Lake Victoria basin farming system. This was to assess the genetic diversity of A. flavus and to establish the contamination rates of groundnuts with Aspergillus species at pre- and post-harvest stages. Out of the 213 A. flavus isolates identified in this study, 96 representative isolates were fingerprinted using 16 insertion/deletion microsatellite markers. Data from fingerprinting were analyzed through Neighbor Joining while polymorphism was determined using Arlequin v 3.5. The pre- and post-harvest contamination rates were; 2.5% and 50.0% (West Nile farming system), 55.0% and 35.0% (Lake Kyoga basin mixed farming system) and 32.5% and 32.5% (Lake Victoria basin farming system) respectively. The Chi-square test showed no significant differences between pre- and post-harvest contamination rates among AEZs ($p = 0.199$). Only 67 out of 96 isolates produced suitable allele scores for genotypic analysis. Analysis of genetic diversity showed higher variation within populations than among populations. Two major clusters (aflatoxigenic and non-aflatoxigenic isolates) were identified as colonizing groundnuts at pre- and post-harvest stages. Conclusions These findings provide a first insight on the existence of non-aflatoxigenic strains of A. flavus in Uganda. These strains are potential candidates for developing local Aspergillus bio-control agent.

Background

Groundnut (*Arachis hypogaea L.*), is a major legume grown in approximately 25 million hectares of the semi-arid tropical and sub-tropical regions of the world between latitudes 40 $^{\circ}$N and 40 $^{\circ}$S with an estimated yield of 36 million tons every year (1). Groundnut contains proteins, fats, vitamins and fiber as the major components and all are present in their beneficial forms (2). Proteins (25.8 %) with all the 20 amino acids in variable proportions, total lipid/fats (49.2 %), carbohydrates (16.1 %) and dietary fiber (8.5 %) (3). In Uganda, groundnuts are consumed in either of the following forms: roasted seeds, groundnut stew, groundnut paste and sometimes raw seed cake is used as animal feed. Groundnut is the second most important legume in Uganda after common bean (*Phaseolus vulgaris*) (5). However, groundnut faces production and export constraints from mycotoxin accumulation that results from contamination by *Aspergillus* species favoured by the tropical climate experienced in Uganda (4). Mycotoxins are secondary metabolites produced by the fungal metabolism in response to environmental stress which are later channeled by the fungus in defense, virulence or cell signaling (6). Mycotoxins are stable compounds that cannot be degraded by any ordinary cooking temperature or food processing procedures (7, 8). Mycotoxins, when ingested, are a problem to both human and livestock health; causing acute illness, chronic illness, instant death or immunosuppression

among others (9). In most cases the effects of mycotoxins are manifested much later after exposure (10) . Human and livestock exposure to mycotoxins in developing countries results from over reliance on a single staple food crop which is normally grown only once a year (11). Therefore, this food commodity is kept much longer under storage in order to prolong its availability awaiting new harvest in the succeeding year. Since most storage facilities in developing countries are improvised structures, a great proportion of the stored crop produce get contaminated by *Aspergillus* species, resulting into changes in, taste, color, odor and nutritional value of food and feeds (12). The economic losses due to *Aspergillus* contamination may reach 100 % when the presence of aflatoxins beyond acceptable levels results in produce rejection (13). Acute aflatoxicosis is often as a result of subject exposure to high doses of aflatoxins resulting into instant death, whereas chronic aflatoxicosis is due to exposure to sub-lethal doses over a long period of time (7). Chronic aflatoxicosis results into liver cancer, immune suppression and teratogenicity among other complications (7). This problem is a common occurrence in developing countries like Uganda where farmers have inadequate food storage facilities and handling practices (14). In addition, in developing countries, no strict regulatory measures exist against high levels of aflatoxins in food and feed stuffs, leading to frequent episodes of aflatoxicosis and often death in humans (15). In Uganda, the most recent study on aflatoxins in groundnut was done in 2006 by (16). This present study was done to assess the contamination rates of groundnut with major *Aspergillus* species and to examine the genetic diversity of *Aspergillus flavus* isolated from groundnut in six representative districts within the agro-ecological zones (AEZs) of Uganda. These included: West Nile farming system, high altitude districts (districts of Arua and Koboko), Lake Kyoga basin mixed farming system, low altitude districts (districts of Soroti and Ngora) and Lake Victoria basin farming system, mid altitude districts (districts of Tororo and Kamuli).

Materials And Methods

Sample collection

The study was conducted in the selected major groundnut-growing AEZs of Uganda and selection was based on the groundnut production statistics in Uganda and the degree of variations in abiotic factors between AEZs. Basing on the groundnut production statistics provided by Uganda Bureau of Statistics, 2014, two districts with the highest groundnut production levels were selected from an AEZ and surveyed.

A total of 240 households and groundnut fields combined, were surveyed. From each household / groundnut field surveyed, at least a sample of groundnut was collected (120 field samples and 120 storage samples). A total of 40 samples were collected from each district (20 field samples and 20 storage samples). From each field, a quadrant measuring 1 m x 1 m was thrown randomly at five different sampling points at least 10 m apart. Then three groundnut stands were pulled and handpicked on the same day of sampling. Extra care was taken to sort out pods that were damaged by soil fauna and later clean pods packed in a paper bag. The groundnut pods were sun-dried for a week, disinfected using a 0.5 % (v/v) sodium hypochlorite solution, hand shelled followed by storage at 4 °C until fungal isolation according to (18). For the case of storage samples, sub-samples of shelled groundnuts from each bag or container were taken randomly from the top, middle and bottom using a sampling probe and later mixed to form a uniform mixture. From this mixture, 250 g were drawn and packed in a sterile paper bag for isolating fungi. Unshelled groundnuts were taken

only once from each storage bag or container and packed for laboratory analysis following the method of (19).

Isolation of *Aspergillus* species from groundnut seeds

Aspergillus species were isolated at National Peanut Research Laboratory, Dawson, Georgia, USA. A selective growth medium, modified dichloran Rose Bengal (MDRB) was used for isolation of *Aspergillus* section *Flavi* (20). The MDRB medium is composed of 10 g/L dextrose, 2.5 g/L peptone, 1.0 g/L di-potassium phosphate (KH_2PO_4), 0.5 g/L magnesium sulphate hepta hydrate ($MgSO_4.7H_2O$), 0.5 g/L yeast extract, 20 g/L agar, 0.5 mL of 0.05 % (w/v) Rose Bengal stock solution in acetone, adjusted to 1 L with distilled water and later modified with 0.8 mg/L dichloran. After sterilization, 30 mg/L streptomycin and 0.15 mg/L tetracycline were added to the medium. Twenty seeds per sample were separately put into a sterile 50 mL falcon tube and 15 mL sterile distilled water added. The seeds were washed by shaking in a pulverizing machine, KLECO (Visalia, California, USA) for 2 minutes (21). Thereafter, 50 µL of each of the suspensions was separately plated onto MDRB medium (22), followed by incubation at 37 °C for 3 days. The *Aspergillus* colonies were counted, and contamination levels (%) were deduced by sample type and AEZ. In a biosafety cabinet, a stereo microscope and a flame sterilized needle were used to isolate conidia from a colony of interest. The conidia were then transferred onto freshly prepared plates of MDRB medium and streaked in a clock-wise pattern so as to effectively disperse the spores into single colonies. After three days of incubation at 37 °C, hyphal tips from single colonies were picked using a flamed scalpel and transferred into Czapek Dox agar (OXOID Ltd, Hampshire, England) slants for identification and storage.

Aspergillus species and strain identification

Morphological characterizations were done on 12-day old pure cultures of *Aspergillus* grown on Czapek Dox agar at 30 ^{o}C. This was to identify the different species and strains in accordance to (23) and comparison to reference cultures in the collection at National Peanut Research Laboratory, Dawson, GA, USA.

Genomic DNA extraction and quantification

Genomic DNA from the *Aspergillus flavus* isolates was extracted at National Peanut Research Laboratory, Georgia, USA, using Qiagen DNeasy Plant kit (QIAGEN, Hilden, Germany). Sterile disposable plastic loops were used to harvest 3 loopfuls of spores from the culture slants and loaded into each sample tube. Following the manufacturer's instructions, a 500 µl clear lysate were pipetted into a 2 ml eppendorf tube and later loaded into a QIAcube robot (QIAGEN, Hilden, Germany). The concentration of the eluted DNA was determined using a Nanodrop ND 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA)

Genotyping of *Aspergillus flavus* isolates

Primers that were previously developed to detect insertions-deletions (InDel) within the aflatoxin-biosynthesis cluster of *Aspergillus* (21) were used in this study for the genetic fingerprinting of the *Aspergillus* isolates (Table 1). Since deletions/insertions in this gene cluster are associated to aflatoxin production (21), clustering of isolates were deduced from the shared deletions/insertions patterns that correspond to aflatoxigenicity or non-aflatoxigenicity.

Table 1: Primer pairs that were used in amplification of aflatoxin biosynthesis gene (21)

Marker	Forward 5'→3'	Reverse 5'→3'	Amplicon size (bp)
AFLC01	CCGACCTCACGACGCATTAT	CCGGCTAGCTTCAACAGACG	140-370
AFLC02	GGTTGGCGGATTGAGAGGTA	GGAGATCAGCCGAGAAGACA	100-296
AFLC03	TCCGCCGAGAGCCATAATAG	GGATGCTGACACCTCGATAG	120-160
AFLC07	GTCAGCAAGAGGAGCCTTCA	GGTCACGGAGATCCTCCATA	159-404
AFLC08	CGCCAGCACGGAGATCGAAT	CGTCTCCTCAGGCGGTCTAT	224-399
AFLC12	CGCAAGGAGCTCGACCAATA	TTCAGCTCAGCGACGAGAGT	241-360
AFLC13	TCGGTTCAATGCTCGAACAC	TCCAACCTTCGGCCTAGTCT	140-410
AFLC15	GCTCTACAGGCTGATTCAAG	TCGACAGTCCGACAATATGC	204-370
AFLC16	ATCGCAGCGGAAGCTTGGAA	AGTCTCGGACTCCGGTGACA	145-410
AFLC17	GCACAACTCGTACAGCTATC	TCTAAGTGCGAGGCAACGAA	125-390
AFLC18	GGCAGCCAGACCAAGGAATA	CCTTCTCGTAGCCGCTCATC	130-400
AFLC19	ACAGGACCGCACGGATCAAT	AGGAGCGGATGTCGAAGTCT	260-491
AFLC20	GCCTAGCGCTCCATTCTCAG	CCATCGTATCCGGCTCTATC	120-370
AFLC21	TACCTTACTCCGCTAAGCAG	GCGGTCACCTACCAATGAAT	150-368
AFLC22	TTCGCAGGAGTGTAGCCAAG	GTTGGAACACGCTCCATAGG	120-371
AFLC24	GAACGAGATAACGGCTGCAT	ATCAATCCACGGACCGTTGT	100-430

The forward primers were tailed with a 5'-CAGTTTTCCCAGTCACGAC-3' sequence and labeled with 6-carboxyfluorescein (6-FAM). The reverse primers were tailed with 5'-GTTT-3' sequence to promote non-template adenylation (24). Amplifications were performed using 10 ng of DNA and Titanium Taq polymerase (Clontech) in 5 µl reactions as described before (25). The labeled PCR amplicons were analyzed using an ABI 3730XL DNA analyzer and data were processed by GeneMapper v 4.0 (Applied Biosystems, Foster city, California, USA).

Data analysis

The GenStat Discovery Edition 4 (2002) for windows (VSN International Ltd, Rothamsted Experimental station, UK) software was used for data analysis. The Chi-square test and One –Way analysis of variance (ANOVA) were used to compare the frequencies of groundnut contamination with *Aspergillus* species and to determine the relative abundance of isolated *Aspergillus* species and strains at pre- and post-harvest stages. Allele sizes observed as relative fluorescence units were converted to binary data, where presence of an amplicon of any size was scored as '1' whereas its absence was scored as '0'. Relationships among the isolates based on InDel data were determined through Neighbor Joining with 1000 bootstrap replications (26), using TREECON for Windows 1.3b version. Analysis of Molecular Variance (AMOVA) in Arlequin version 3.5.2.2 software package using 1000 permutations provided genetic structure based on the AEZs from which

groundnut samples were collected. The principal co-ordinate analysis (PCoA) and Mantel test were done to identify genetic clusters and their associations with geographical locations using GenAlEx version 6.502.

Results

Contamination frequencies of *Aspergillus* in the groundnut samples

Overall, 34.58 % (83/240) of the groundnut samples had *Aspergillus*. The frequencies (%) of *Aspergillus*-contaminated groundnuts are summarized in Table 2. Lake Kyoga basin mixed-farming system had the highest number of groundnut samples contaminated with *Aspergillus* and West Nile farming system had the lowest number of groundnut samples contaminated with *Aspergillus* (Figure 2). However, there were no significant differences in the overall contamination frequencies across AEZs ($p > 0.05$). Pre- and post-harvest contamination frequencies were 30.00% and 39.17 % respectively. Lake Kyoga basin mixed-farming system had the highest pre-harvest contamination frequency whereas West Nile farming system had the lowest pre-harvest contamination frequency (Table 2). West Nile farming system had the highest post-harvest contamination frequency while Lake Victoria basin farming system had the lowest post-harvest contamination frequency (Table 2). Examination of the fungal species contaminating groundnuts revealed presence of *A. flavus*, *A. parasiticus* and *Aspergillus* section *Nigri*. More than one *Aspergillus* species were found co-existing on 70 % (168/240) of the total groundnut samples collected and on 61 % (73/120) of the post-harvest groundnut samples. An example of multiple fungal species co-existing in a groundnut sample is shown below (Figure 2). *Aspergillus flavus* was the most abundant, both as S- and L-strains, whereas *A. parasiticus* was the least abundant species observed (Table 3). The three *Aspergillus* species were distributed throughout the AEZs surveyed (Table 3) and the abundance and distribution of each species never differed significantly across AEZs ($p > 0.05$).

Table 2: Prevalence of *Aspergillus* contaminated groundnut at pre-and post-harvest by AEZs

Agro-ecological zone	Pre-harvest contamination (n = 40)	Post-harvest contamination (n = 40)	Overall contamination (n = 80)
West Nile	1 (2.50 %)	20 (50.00 %)	21 (26.25 %)
L. Kyoga basin	22 (55.00 %)	14 (35.00 %)	36 (45.00 %)
L. Victoria basin	13 (32.50 %)	13 (32.50 %)	26 (32.50 %)
Total (N = 120)	**36 (30.00 %)**	**47 (39.17 %)**	**83 (34.58 %)**

Table 3: *Aspergillus* species and strains identified by AEZs.

Agro-ecological zone	*A. flavus* (S strain) n = 88	*A. flavus* (L strain) n = 46	*A. parasiticus* n = 3	*Aspergillus* section *Nigri* n = 94
West Nile	10 (11.36 %)	2 (4.35 %)	1 (33.33 %)	18 (19.15 %)
L. Victoria basin	36 (40.91 %)	14 (30.43 %)	1 (33.33 %)	46 (48.94 %)
L. Kyoga basin	42 (47.73 %)	30 (65.22 %)	1 (33.33 %)	30 (31.91 %)
Total (N = 231)	**88 (38.10 %)**	**46 (19.91 %)**	**3 (1.30 %)**	**94 (40.69 %)**

Only 67 out of 96 representative isolates of *A. flavus* had adequate allelic regions amplified by all the 16 microsatellite markers for analysis. Analysis of molecular variance (AMOVA) indicated higher degree of variation among individuals within a population compared to that among individuals between populations (Table 4).

Table 4: Analysis of molecular variance statistics for the 67 isolates from four populations

Source of variation	df	SS	MS	Est. var	Percentage
Among populations	3	12.807	4.269	0.180	9.000%
Within populations	63	115.939	1.840	1.840	91.000%
Total	66	128.746		2.021	100.000%

Only 16 out of 25 InDel markers used in genotyping the 96 isolates showed polymorphism. Neighbor Joining of the microsatellite data revealed two major clusters; one with most of the isolates having many loci in the aflatoxin biosynthesis gene cluster amplified and another with most of its isolates possessing few loci in their aflatoxin biosynthesis gene cluster amplified. These two clusters were; aflatoxigenic and non-aflatoxigenic respectively (Figure 3). However, isolates in these two clusters were not distinctively differentiated by the number of gene loci present in each isolate. For example, some few isolates (1608, 1478, 1567, 1631 and 1570) whose aflatoxin biosynthesis gene cluster had minimal deletions were clustered with isolates having major deletions in their aflatoxin biosynthesis gene clusters. Similarly, some isolates (1554, 1462, 1605, 1470, 1482, 1483 and 1546) had very few loci in their aflatoxin biosynthesis gene clusters but were clustered in the same clade with those having almost intact loci in their aflatoxin biosynthesis gene cluster. Principal co-ordinate analysis of the microsatellite data showed four distinct clusters for the entire population. However, isolates were not clustered based on their geographical origins but rather on similar deletion patterns shared among them (Figure 4). The Mantel test revealed insignificant negative correlation

between genetic distances and geographical distances among *A. flavus* isolates (R^2 = 0.0085, p = 0.220) (Figure 5).

Non-aflatoxigenic strains of *A. flavus* were dominant (65.67 %), whereas the aflatoxigenic strains were fewer (34.33 %). The highest proportion of aflatoxigenic *A. flavus* strains was in L. Kyoga basin mixed farming system (68.20%) and none in West Nile. Whereas the highest proportion of non-aflatoxigenic strains was in L. Victoria basin farming system (65.22 %) and West Nile had the lowest proportion (8.70 %).

Discussion

Contamination of groundnut by *Aspergillus* species originates at pre-harvest stage as was previously noted by (27) in their study. This is because *Aspergillus* species are well adapted in soil as conidia, hyphae and sclerotia, which are in direct contact with groundnut pods (28). Other abiotic factors like drought stress, a common experience in the Lake Kyoga basin mixed farming system could be responsible for high susceptibility of groundnut at pre-harvest (29, 30). Cropping system and climate could be responsible for *Aspergillus* species distribution pattern and abundance within these AEZs that are far apart and with different climatic conditions as was noted by (20). No significant statistical difference existed between pre-harvest and post-harvest contamination levels of groundnut with *Aspergillus* species across AEZs. This could be due to common pre- and post-harvest handling methods employed as previously noted by (31).

Aspergillus section *Flavi* and *Aspergillus* section *Nigri* were the most abundant *Aspergillus* species encountered in groundnut samples. This finding concurs with that from a study done by (32). Most of the *Aspergillus* species in section *Nigri* are of great importance in industrial manufacture of amylases, lipases, citric acid and gluconic acid (33). However, they cause food deterioration with subsequent production of mycotoxins; ochratoxin A (OTA), ochratoxin B, fumonisin B2, fumonisin B4, and secalonic acids, A, D, F as the major natural products toxic to humans and animals (34). Ochratoxin A has been reported to be a nephrotoxic compound causing renal cancer (35). Groundnut contamination with *A. flavus* and some species from *Aspergillus* section *Nigri* is also known for lowering the germination ability of groundnut seeds under storage (36), and the longer the storage duration, the higher the frequency of contamination by *Aspergillus* species (37). Thus, reduction in seed quality due to *Aspergillus* contamination results in poor seed germination ability, low productivity and hence food insecurity.

Isolates of *A. flavus* were more genetically diverse within populations than among populations. This could be as a result of genetic exchange and recombination events that normally occur between different *Aspergillus* strains and species (38). Low genetic diversity among populations of *A. flavus* could be due to small sample size (39) or an evidence that the bulk of the isolates shared similar deletions in the aflatoxin biosynthesis gene cluster. Because of this, the clustering of isolates were not based on their geographical origins but rather on the shared deletions pattern. This could be an evidence of gene flow among *A. flavus* populations in the different groundnut-growing AEZs of Uganda.

Our study also shows that amplicon sizes produced by each marker varied so greatly and some isolates had huge chunks of unamplified loci in their aflatoxin biosynthesis gene cluster. Existence of unamplified loci in this gene cluster is an indication of large deletions that may result from complete knock out of a protein-

coding gene, rendering the gene inactive (40). These isolates which had very few loci amplified as a result of large deletion in this gene cluster were therefore non-aflatoxigenic strains, as was observed by (41) in his study. These *A. flavus* strains could be of value as bio-control agents against aflatoxin-producing *A. flavus* strains through exploitative competition (42). Clustering using Neighbor Joining did not reveal any clear cut difference between clusters of aflatoxigenic and non-aflatoxigenic isolates. This was observed where some few isolates meant to be in the aflatoxigenic cluster were clustered with the non-aflatoxigenic isolates and vice versa. The principal co-ordinate analysis and the Mantel test also support the clustering pattern of the Neighbor Joining analysis. These could be due to the inability of the microsatellite markers used in this study to sufficiently resolve the genetic differences among the isolates with respect to aflatoxin production as well as their geographic origins. This same observation was noted by (43) in their study on *A. flavus* isolated from sorghum seeds.

Conclusion

Aspergillus flavus and *Aspergillus* section *Nigri* are the most abundant species contaminating groundnuts in Uganda and they co-exist in groundnuts, therefore human and livestock health are at risk of intoxication by their combined mycotoxins in food and feed. Since contamination starts at pre-harvest stage due to drought stress, early planting should be emphasized so that there is enough rain to take the plants through their growth and development. In case drought sets in unexpectedly, simple irrigation technologies should be used to meet the water demands of the plants, especially during pod development stage.

Both aflatoxigenic and non-aflatoxigenic *A. flavus* strains exist in all the AEZs of Uganda. This finding could provide a basis for the selection and application of indigenous non-aflatoxigenic *A. flavus* strains as bio-control strains against aflatoxigenic *Aspergillus* strains. This technology has been proven to be successful and it is already being applied in many countries that produce groundnut intensively, like the US (44). Low genetic diversity in *A. flavus* across AEZs can be of great value during the design of a control measure, like RNAi technology for groundnut breeding programs. The existing *A. flavus* culture collection from this study can be employed during a screening process to identify groundnut genotypes that are resistant to aflatoxigenic *Aspergillus* contamination and also subsequent aflatoxin formation.

List Of Abbreviations

AEZs Agro-ecological zones

AMOVA Analysis of molecular variance

ANOVA Analysis of variance

DNA Deoxyribonucleic acid

InDel Insertion /deletion

MDRB Modified dichloran Rose Bengal

Pop	Population
PCoA	Principal Co-ordinate analysis
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
UBOS	Uganda Bureau of Statistics

Declarations

Acknowledgements

Valerie Orner trained me at National Peanut Research Laboratories, Dawson, Georgia, USA.

Michael Hillary Otim. Reviewed the manuscript. National Crops Resources Research Institute, P.O Box 7084, Uganda.

Francis Onyilo. Reviewed the manuscript. Muni University, P.O Box 725 Arua, Uganda.

Funding

The data collection and subsequent analyses were funded by the NARO under PEARL Project, a project sponsored by Bill and Melinda Gates Foundation. The fungal isolation, genomic DNA isolation, purification and fingerprinting were funded by the US Department of Agriculture - Agricultural Research Services, National Peanut Research Laboratories.

Availability of data and materials: The majority of the data is included herein. The raw data and other materials used in the reported study shall be availed only on request. Please contact Amos Acur (amosacur4@gmail.com) or Stephen Buah (buahs@yahoo.com) to request for additional data.

Authors' contributions

AA - Designed the study, collected the data, analyzed the data, interpreted the results and a major contributor to writing the manuscript

AK - Designed the study, supervised the study and a major contributor to writing the manuscript

RA - Processed the fingerprint results and a major contributor to writing the manuscript

SB - Analyzed the data, interpreted the results and a major contributor to writing the manuscript

JA - A major contributor to writing the manuscript

DM - A major contributor to writing the manuscript

SO - A major contributor to writing the manuscript

ST – Participated in data collection and a major contributor to writing the manuscript

JS – Participated in data collection and a major contributor to writing the manuscript

Declarations

Ethics approval and consent to participate - Not applicable
Consent for publication - Not applicable
Competing interests – There were no competing interests

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Figures

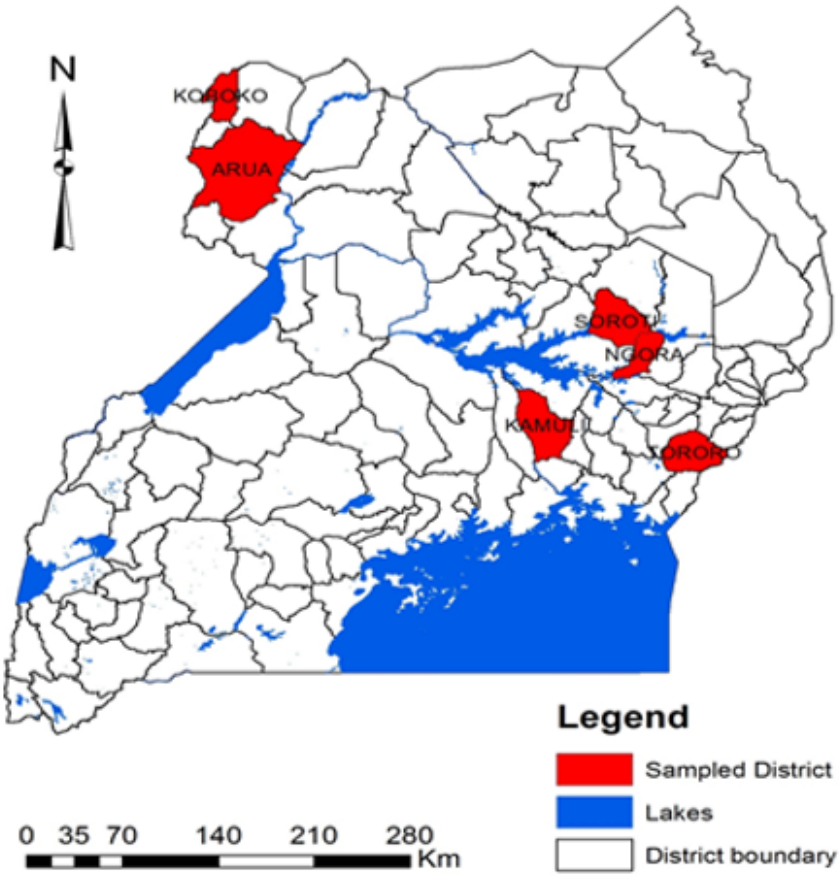

Figure 1

Study sites: Agro-ecological zones from which groundnut samples were collected; West Nile (Arua and Koboko), Lake Kyoga basin (Soroti and Ngora) and Lake Victoria basin (Kamuli and Tororo). Source: UBOS 2014

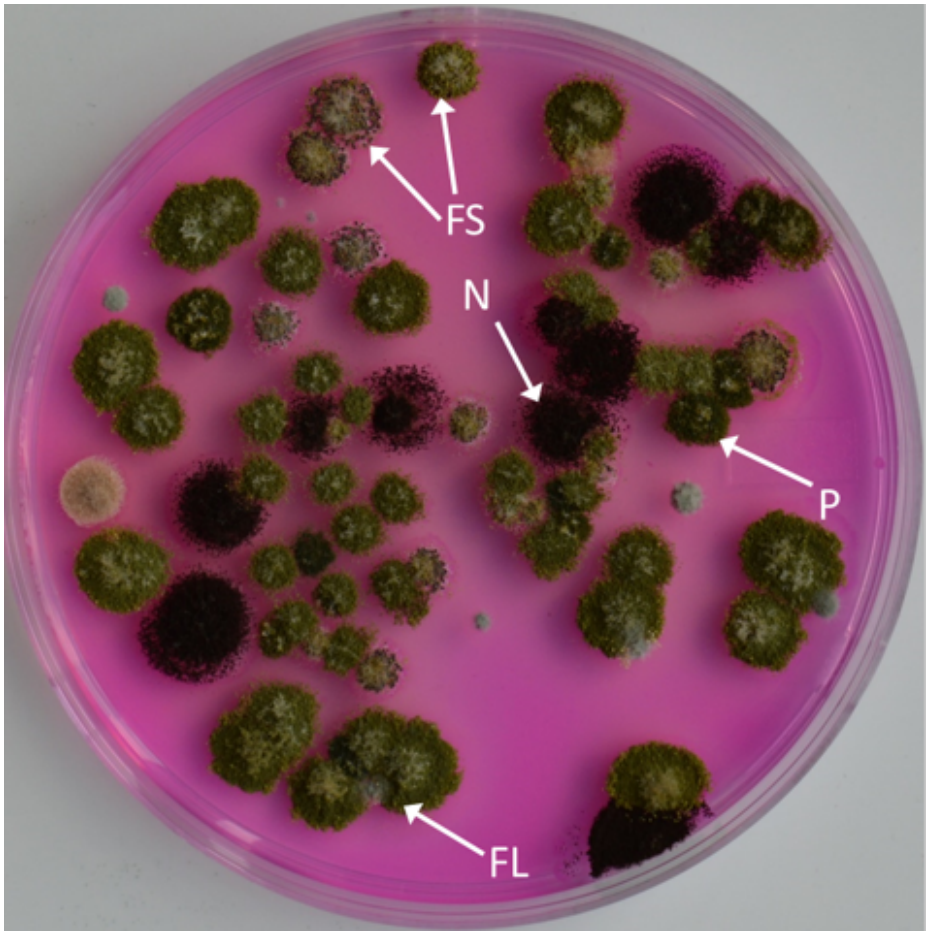

Figure 2

A mixed culture of Aspergillus species isolated from groundnut on MDRB medium. FS: Colony of A. flavus (S strain), FL: Colony of A. flavus (L strain), P: Colony of A. parasiticus and N: Colony of Aspergillus section Nigri

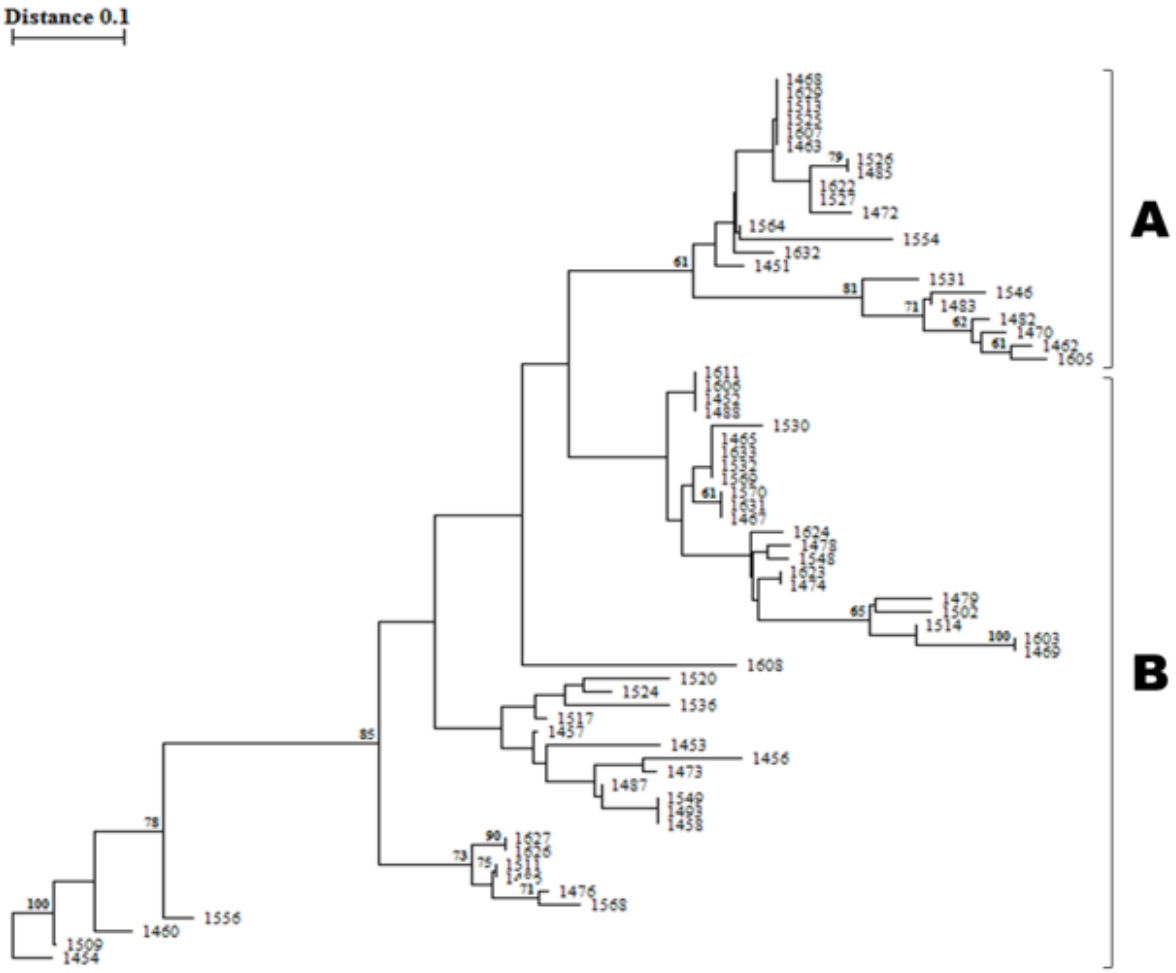

Figure 3

Neighbor Joining Clustal analysis of Aspergillus flavus isolates with 1000 bootstrap replications. Bootstrap values above 60% were considered significant. A: Aflatoxigenic strains B: Non-aflatoxigenic strains.

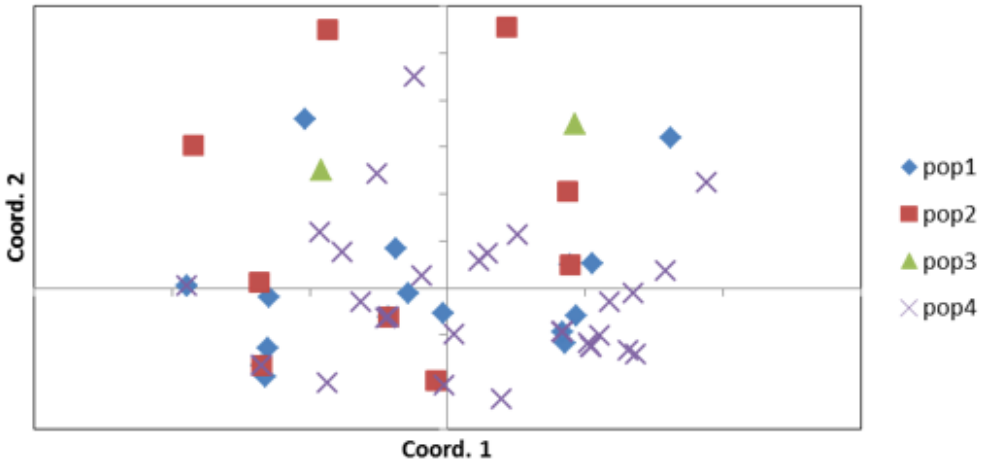

Figure 4

Principal co-ordinate analysis for 16 microsatellite markers used to fingerprint 67 A. flavus isolates.

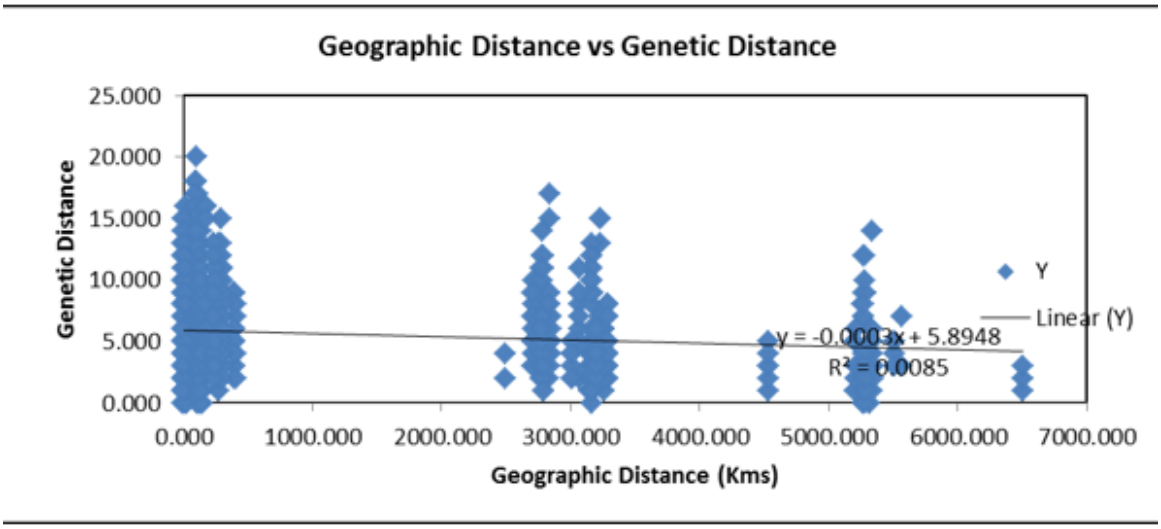

Figure 5

Correlation between the genetic distance and geographic distance of the 67 A. flavus isolates.