ISSN 2664-8075 (Print) & ISSN 2706-5774 (Online)
South Asian Research Journal of Applied Medical Sciences
Abbreviated Key Title: South Asian Res J App Med Sci

| Volume-2 | Issue-3 | May-June 2020 | **DOI: 10.36346/sarjams.2020.v02i03.001**

Original Research Article

Molecular Characterization of Moulds Isolated from Stored Food Products in Imo State, Nigeria

NB Ohabughiro*, W Braide, SI Okorondu, CE Nwanyanwu

Department of Microbiology, Federal University of Technology, P.M.B 1526, Owerri, Imo State, Nigeria

***Corresponding Author**
NB Ohabughiro

Article History
Received: 16.01.2020
Accepted: 23.01.2020
Published: 30.06.2020

Abstract: Cereals and legumes are important food crops and provide cheap sources of energy and protein, and therefore, are good substitutes or supplements to major staples foods and help meet the needs of Africa's teeming population. Five food products (rice, maize wheat, groundnut and beans) stored for 2 - 4 months in different packaging materials were assessed for the presence of mycotoxin producing moulds. These samples were randomly selected from different markets. Standard microbiological and molecular methods were used in the isolation and identification of moulds. The frequently occurring moulds species identified molecularly were *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*, and *Penicillium chrysogenum.* The dominant mould species were *Aspergillus flavus* (46%) followed by *Aspergillus tamari* (23%), *Aspergillus niger* (18%), and *Penicillium chrysogenum* (9%) while the least was (4%) *Aspergillus brunneoviolaceus*. The Phylogenic tree was constructed by using the geneious software version 4.0. (By Neighbor-joining (N-J) method based on the 18s rDNA sequence.) The 18S rDNA sequence analysed showed that strains were most closely affiliated with members of their genus. The occurrence of high contamination level of *Aspergillus* species indicates the possible production of aflatoxin in stored food products. There is a strong need to devise good storage condition for stored food products to avoid mycotoxigenic moulds contamination.

Keywords: Stored food products, *Aspergillus* spp, and Sequencing and Phylogenetic tree.

INTRODUCTION

In Africa, Rice (*Oryza sativa*) Maize *(Zea mays*) Wheat (*Triticum aestivum*) Groundnut (*Arachis hypogaea*) Cowpea (*Vigna sinensis*) are important food crops. Cereals and legumes provide cheap sources of energy and protein [1]. Fungi found in stored food can be classified as "field fungi" and the "storage fungi"). Store fungi include all species of *Aspergillus, Fusarium* and *Penicillium* [2].Common genera of moulds include: *Acremonium, Alternaria, Aspergillus, Cladosporium, Fusarium, Mucor, Penicillium, Rhizopus, Stachybotrys, Trichoderma, and Trichophyton* [3]. Moulds do not only because direct losses but can threaten the health of both man and animals by producing poisonous mycotoxins, which contaminate foods and feeds. Water activity, Hydrogen ion concentration, Temperature, Gas tensions, specifically of oxygen and carbon dioxide, Consistency that is, liquid or solid state, Nutrient status, Specific solute effects and Preservatives are some predisposing factors to fungal growth and proliferation [4]. Damages caused by storage fungus includes: Loss of nutrients, discoloration of grain, reduction in germination ability,caking of grains, increase in the temperature of the stored goods up to spontaneous combustion, mouldy smell and taste, production of mycotoxins [5]. One of the ways of controlling storage fungi is drying of the produce as quickly and evenly as possible after harvesting up to the critical moisture/safe moisture level. The critical water content for safe storagecorresponds to a water activity of about 0.7 [6].

This study reports on the molecular characterization of moulds associated with stored products.

Copyright @ 2020: This is an open-access article distributed under the terms of the Creative Commons Attribution license which permits unrestricted use, distribution, and reproduction in any medium for non commercial use (NonCommercial, or CC-BY-NC) provided the original author and source are credited.

MATERIALS AND METHODS

Sample collection and Preparation

Whole grains/fine powder of rice, maize, wheat, groundnut and beans obtained from the markets were stored in four different storage materials (sack, polyethene, plastic containers and metal containers) for two to four months at ambient temperature in a dry environment. Thirty grams (30 g) each of the fresh and stored samples were labelled and transported immediately to laboratory and kept in cool place prior to mycological analysis. A total of two hundred and ten (210) samples were randomly collected following the method of [7].

Isolation of fungi

Three mycological media (Potato Dextrose Agar, Sabouraud Dextrose Agar and Malt Extract Agar) were prepared according to standard methods. An antibacterial agent (50mg/l, chloramphenicol) and 0.1ml of lactic acidwas added to inhibit the growth of bacteria and yeasts respectively [8]. Standard dilution and streaking technique method was adopted. The samples were serially diluted up to dilution factor of 10^{-3} and 10^{-5}. One-tenth milliliter (0.1ml) of suspension was inoculated onto the freshly prepared surface dried media and incubated at 28 ± 2°C for 7 days for fungal growth. Fungal colonies grown on media were subculture on various media [9] for further characterization and identification.

Enumeration, Morphological and Microscopic identification

Total cell counts of each isolate was done on a colony counter and expressed as colony forming units per gram (Cfu/g) using this formula [10]. The isolated moulds were identified based on colonial morphology and microscopic examination. The moulds were mounted on a slide, stained with lacto phenol-cotton blue to detect fungal structures. Microscopically, moulds were identified on the basis of spore characteristics, pigmentation and septation [11].

Molecular identification of moulds

Deoxyribonucleic acid Extraction Protocol

Extraction was done using a ZR fungal DNA mini prep extraction kit supplied by Inqaba, South Africa. A heavy growth of the pure culture of the isolates was suspended in 200 microlitre of isotonic buffer into a ZR Bashing Bead lysis tubes and 750 microlitre of lysis solution added into the tube. The tubes were secured in a bead beater fitted with a 2ml tube holder assembly and processed at maximum speed for 5 min. The ZR bashing bead lysis tubes were centrifuged at 10,000xg for 1 min. Four hundred (400) microlitres of supernatant was transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000 xg for 1 min. One thousand two hundred (1200) microlitres of fungal DNA binding buffer was added to the filtrate in the collection tubes bringing the final volume to 1600 microlitre, 800 microlitre was then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000xg for 1 min. The flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred (200) microlitre of the DNA Pre-Wash buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000xg for 1 min followed by the addition of 500 microlitre of fungal DNA Wash Buffer and centrifuged at 10,000xg for 1 min. The Zymo-spin IIC column was transferred to a clean 1.5 microlitre centrifuge tube, 100 microlitre of DNA elution buffer was added to the column matrix and centrifuged at 10,000xg microlitre for 30 s to elude the DNA. The ultra-pure DNA was then stored at -20^{0}C for other downstream reaction [12].

Polymerase chain reaction amplification protocol

Ten microliters (10µl) of One *Taq*Quick-Load 2X Master was mixed with Standard Buffer (New England Biolabs Inc.); 1µl each of forward and reverse primers; Internal transcribe space 1 and 4 (ITS1 AND ITS4). ITS1 FORWARD PRIMER 5' – TCC GTA GGT GAA CCT GCG G-3' and the ITS4 reverse primer 5' – TCC GCT TAT TGA TAT GC -3'.Seven microliters (7µl) of Nuclease free water and 1µl of DNA template was used to prepare 20µl reaction volume of the PCR cocktail. The reaction was gently mixed and transferred to a preheated thermalcycler. Amplification conditions for the PCR were as follows: 5 min at 94^{0}C to denature the DNA, followed by 35 cycles of denaturation at 94^{0}C for 30secs, primer annealing at 50^{0}C for 30 secs and strand extension at 68^{0}C for 10 minutes on an Eppendorf nexus gradient Mastercycler (Germany). PCR products were separated on a 2% agarose gel and DNA bands were visualized with syber gold [12].

Sequencing protocol

PCR products were cleaned using ExoSAP Protocol described by [12].

Phylogenetic analysis

The obtained nucleotide sequence was analysed using a software, the geneious software version 4.0 (by Neighbor-joining (N-J) method [13] based on the 18s r DNA sequence.

RESULTS AND DISCUSSION

Moulds isolated from the stored products were characterized morphologically and microscopically. They were further identified genetically by sequencing of 18S rDNA gene using ITS1 and ITS4 primers. Their phylogenetic tree was constructed using the neighbor- joining method. Moulds were isolated from all the samples. Table 1 shows the mould species, *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*, and *Penicillium chrysogenum* isolated from the stored and fresh products.

Table-1: Cultural and Microscopic Characteristic of Identified Isolates

Cultural	Microscopic	Probable organism
Observe: Colonies are typically plain green in colour or yellow-green becoming green with age. Reverse- creamish-yellow	Hyphae are septate and hyaline. Conidal head are short columneredbliseriate	*Aspergillus flavus*
Observe: Growing rapidly rusty brown or dark brown Reverse: dull yellow	Conidia head with long chain ofconida, phialides becoming conidiaand rough stipe, has metulae or phialides.	*Aspergillus tamari*
Observe: blackish brown often with yellow mycelium moderately rapid growth rate. Reverse: creamish-yellow to yellow	Septated hyphae, long smooth and colourless conidiophores biseriatephalides, globose conidial head and presence of dark spores from the conidia head.	*Aspergillus niger*
Observe: Brown to dark brown near black commonly abundant Reverse: Uncoloured to Reserve: yellowish gray	Hyaline or pigmented longer stipesuniseriategloboseto ellipsoidal versicle and conidia.	*Aspergillus brunneoviolaceus*
Observe: Blue green with a yellowish pigment, colonies fast growing in shades of green. Reverse: creamish brown	Septate hyphae branched conidiophores with phialides	*Penicillium chrysogenum*

Table-2: Frequency and Percentage Occurrence of Moulds

Moulds	Rice Frequency	Percentage	Maize Frequency	Percentage	Wheat Frequency	Percentage	Groundnut. Frequency	Percentage	Beans Frequency	Percentage	Total Frequency	Total Percentage
A .flavus	11	34%	50	60%	8	27%	30	32%	41	66%	140	46%
A. tamari	7	22%	20	24%	1	3%	25	28%	15	24%	68	23%
A .niger	3	9%	8	10%	18	60%	23	25%	1	2%	53	18%
A.brunneoviolaceus	5	16%	3	3%	1	3%	2	2%	1	2%	12	4%
P.chrysogenum	6	19%	3	3%	2	7%	12	13%	4	6%	27	9%
	32	100%	84	100%	30	100%	92	100%	62	100%	300	100%
		11%		28%		10%		31%		20%		

The study showed that all the stored food products (rice, maize, wheat. Groundnut and beans) analyzed were infested to various degrees of moulds (Table 1). The moulds identified are *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*, and *Penicillium chrysogenum.* These results were considered similar to those results reported by [14]. The most common genera isolated was *Aspergillus* with four different species namely *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*.Result in Table 2 showed that the predominant mould species were in the order *Aspergillus flavus* (46%), *Aspergillus tamari* (23%), *Aspergillus niger* (18%), and *Penicillium chrysogenum.*(9%) while the least was (4%) *Aspergillus brunneoviolaceus*. *Aspergillus flavus* produce Aflatoxins and *Aspergillus* produce Ochratoxin A and their presence in stored and fresh food products could pose a risk to consumer health [15].

Results in Table 2 also showed the frequency and percentage occurrence of mould in different grains, with *A.flavus* (60%) from maize and high *A. niger* from wheat predominating. This finding was similar to those reported by [16]. The occurrence of *Aspergillus flavus* is considered vital because they are known to produce aflatoxins which are considered the most potent carcinogens to human and animals [17]. The moulds with the highest frequency of occurrence were *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*, and *Penicillium chrysogenum.* This has been reported by [18] and [19]. It is likely that post-harvest infections and the storage structures

greatly influence the mycoflora in storage [20]. The two genera *Aspergillus* and *Penicillium* encountered are storage fungi while *Fusarium* is a field fungus [21, 22].

The high moisture contents could account for the variations in the frequency and percentage occurrence in the stored food products as shown in Table 2 [groundnuts (31%), maize (28%), beans (20%), rice (11%) and wheat (10%)] [23].

Molecular identification of isolated moulds

Five mould isolates were identified on the basis of their molecular characteristics. The Genomic DNA extraction, amplification of 18S rDNA with ITS1 and ITS4 primer. The sequenced data are as follows;

Sequences of various Moulds

Sequence: *Aspergillus flavus* ATCC 1683 ITS region; from type material.

GAGGGATCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTAACCTTAGTTGCTTCGGCGGGCCCGCCTTTA
AGGCCGCCGGGGGGCATCAGCCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGT
GAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGA
AGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACA
TTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTG
TTGGGTCGTCGTCCCCTCTTCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAG
CGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAAACAACCATTCTTTCC
AGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

Sequence*: Aspergillus tamarii* NRRL 20818 ITS region; from type material.

GAGGGATCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTAACCTTAGTTGCTTCGGCGGGCCCGCCTTTA
AGGCCGCCGGGGGGCATCAGCCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGT
GAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGA
AGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACA
TTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTG
TTGGGTCGTCGTCCCCTCTTCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAG
CGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAAACAACCATTCTTTCC
AGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

Sequence: *Aspergillus niger* ATCC 16888 ITS region; from type material

CCTTTCTGCCCGCCTGCCACCCAAGACCAACGCCTAATGTGGGGAATATAGCATATACCGATCGAGAGAAA
TCTACGAAAAACCACTGACCTCCGGACCCCGGACCGCTTGGTTCCCCATCGAACACTGGCGGAAAGAGATG
CGATACCTATGGTTAATTGCAATCATCGAAAATCATCGCATTGGAGAACGCACATTGCGCCCCTCTGATTC
CGGGGGGAATGCCTGTTTCTAATGTGATTTCCTCATGCCCGTGCACAAAGTTGGGGTTGCGGAACATACGA
CCCCAAAGATCGCGGGGGTGTGACTGCCTGAAAAGGATTGAAGCCGGCCGACCAACAGACTTTTTTTTCGG
GACCCTTGGCTGCCGAGAACCAGTGCTGAAAAACAACAAAAACATCGACCTCGAATCTCGAACGAATACC
CGTTGAACTCAAGCTTATCAATAATCGGAGGAACCTGCCGACGTTTTCCAACCATTCTTTCCAGGTTGACCT
CGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAATTGGC

Sequence: *Aspergillus brunneoviolaceus* NRRL 4912 ITS region; from type material.

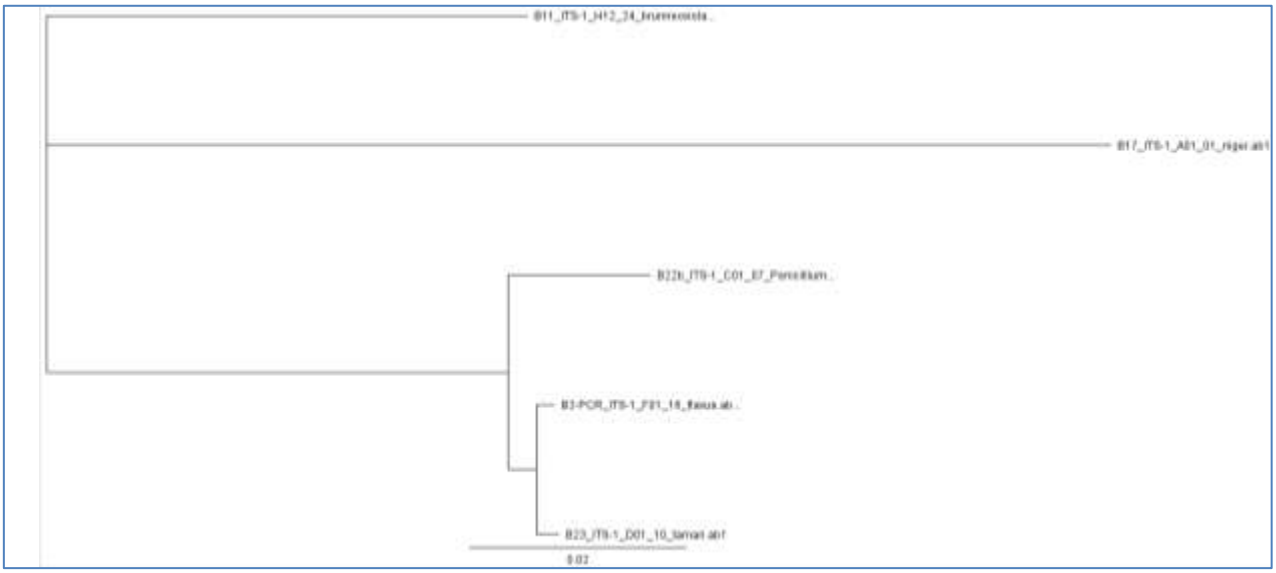
CGCGGCTGGGGTCTTCGGGGCCCAACCTCCCACCCGTGCTTACCGTACCCTGTTGCTTCGGCGGGCCCSCCT
TCGGGCGGCCCGGGGCCTGCCCCCGGGACCGCGCCCGCCGGAGACCCCAATGGAACACTGTCTGAAAGCG
TGCAGTCTGAGTCGATTGATACCAATCAGTCAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATG
AAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCA
CATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTTCTCCCCTCCAGCCCCGCTGGTTG
TTGGGCCGCGCCCCCCCGGGGGCGGGCCTCGAGAGAAACGGCGGCACCGTCCGGTCCTCGAGCGTATGGG
GCTCTGTCACCCGCTCTATGGGCCCGGCCGGGGCTTGCCTCGACCCCCAATCTTCTCAGATTGACCTCGGAT
CAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

Sequence: *Penicillium chrysogenum* CBS 190.68 ITS region; from type material.

GAAGGGTCCTAGCGAGCCCACCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGCCCCGCCATTCA
TGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGA
AGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAG
AACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACATT
GCGCCCCCTGGTATTCCGGGGGGCATGCCTGWCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGT
TGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAG

CGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGYTTGCCGAACGCAAATCAATCTTTTCCAGG
TTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAASCGGAGGA

PHYLOGENETIC TREE



	B2-PCR_ITS-...	B11_ITS-1_...	B17_ITS-1_...	B22b_ITS-1_...	B23_ITS-1_...
B2-PCR_ITS-1_F01_16_fla...		0.097	0.136	0.018	0.004
B11_ITS-1_H12_24_brunn...	0.097		0.142	0.089	0.095
B17_ITS-1_A01_01_niger....	0.136	0.142		0.163	0.143
B22b_ITS-1_C01_07_Penic...	0.018	0.089	0.163		0.017
B23_ITS-1_D01_10_tamari...	0.004	0.095	0.143	0.017	

CONCLUSION

The stored food products were contaminated with various species of moulds. There is a strong need to devise good storage condition for stored food products to avoid mycotoxigenic moulds contamination. This will increase food export and build a healthy nation.

REFERENCES

1. Kassam, A., Friedrich, T., Derpsch, R., & Kienzle, J. (2016). Overview of the Worldwide spread of Conservation Agriculture. *The Journal of Field actions. Science Report*. 8: 241-242.
2. Multon, J.L. (1988). *Preservation and Storage of Grains, Seeds and their By-Products*, Paris, 51.
3. Moore, D., & Robison, G.D. (2011). *21st Century Guidebook to Fungi*. (Ist ed.) Cambridge University Press. 50-55.
4. Pitt, J.I., & Hocking, A.D. (1985). *Fungi and Food Spoilage*. Sydney, Academic Press.Amsterdamp, 48-54.
5. Christensen, C.M., & Meronuck, R.A. (1986). *Quality Maintenance in stored Grains and Seeds*.University of Minnesota press, Minneapolis, 138.
6. Akerstrand, K. (1995). Mould and Yeast Determination in Foods. *Nordic Committee on Food Analysis*, 23:633.
7. Hamed, T. (2016). Sampling methods in research methodology; How to choose a sampling technique for research.Electonic journal, *5*(2): 18-27.
8. Valerie, T., Micheal, E.S., Philip, B. M., Herbert, A. K., & Ruth, B. (2001). *Bacteriological Analytical Manual*. Yeast, Molds and Mycotoxins.Cambridge University Press, 74- 76.
9. Larone, D.H. (2011). *Medically Important Fungi*. A Guide to Identification.ASM Press, Washington, D.C. 185.
10. Bennette, J.W., & Klich, M. (2003). Mycotoxins. *Clinical Microbiology Rev*., 16: 497: 516.

11. Pitt, J.I., Hocking, A.D., Samson, R, A., & King, A.D. (1992). Recommended Methods for the Mycological Examination of Foods. *Modern Methods in Food Mycology*. Elsevier Science Ltd., Amsterdam, 388-389.
12. Inqaba biotech West Africa. (2007). InqabaBiotechonogicalindustries (pty) Ltd. Africa's Genomic Company. Pretoria South Africa. www. Inqaba biotech.co.za.
13. Schmidt, H.A. (2009). Testing Tree Topologies. In Lemey, P., Salemi, M., Vandamme, A.M. *The Phylogenetic Handbook Analysis and Hypothesis Testing*. 2nd edition Cambridge University Press, 381-404.
14. Ranjana, K., & Ananta, K.G. (2016). Molecular characterization of fungi present in stored food grains. Conference in emerging Technologies in Agricultural and food Engineering. *Excel India Publisher ISBN*. 978-93-86256-30-0.
15. Sambrook, J., & Russell, D. W. (2001). *Molecular cloning: A Laboratory Manual*. 3rd Edition, Cold spring harbor laboratory press New York. USA. 2344.
16. Jedidi, L., Cruz, A., Gonzalezjaen, M.T., & Said, S. (2017). Aflatoxin and ochratoxin A and their Aspergillus causal specie in Tunisia.*Cereal food Addit.Contam.part B surveill*. *10*(1): 51-58.
17. Shalini, R. V., & Amutha, K. (2014). Identification and Molecular Characterization of *Aspergillus fumigatus* from soil. *J. Med Pharm. Innov*, 1: 12-15.
18. Omaima, A H., Sorbhy, H. M., Amal, S. H., & Ahmed, S.M F. (2018). Isolation and molecular Identification of *Fusarium*fungi from some Egyptian Grains. *Asian Journal of Plant Sciences*, 17: 182-190.
19. Ryan, K.J., & Ray, C.G. (2004). *Sherries Medical Microbiology*. 4thedn McGraw Hill, New USA.
20. Wagacha, J.M., & Muthomi, J, W. (2008). Review on mycotoxin problem in Africa: Current status, implications to food safety and health and possible management strategies, *int J. Food Microbiology*, 124: 1-12.
21. Bullerman, B.L., & Bianchini, A. (2011). The microbiology of cereals and cereals, 20-23.USA.
22. Andrew, A.M., & Beatrice, A.A. (2017). Detection and Enumeration of moulds on some legumes and a cereal grain from two local markets and two shopping malls in Accra Metropolis. *African Journal of Microbiology Research*, 18: (3) 1-9.
23. Pitt, J.I., & Hocking, A.D. (1997). *Fungi and food spoilage*. 3rdedition. Blackie Academic Professional London.