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Molecular characterization and strain typing of fungal contaminants of Processed *Manihot esculenta* Crantz ("Garri") in Ogun State, Nigeria

Thomas BT*[1], Ogunkanmi LA[2], Iwalokun BA[3], Agu GC[1]

[1]Department of Microbiology, Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria
[2]Department of Cell Biology and Genetics, University of Lagos, Akoka, Lagos, Nigeria
[3]Division of Molecular Biology and Biotechnology, Nigeria Institute of Medical Research,Yaba, Lagos, Nigeria

*Correspondence: B.T. Thomas, Department of Microbiology,
Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria. Tel: +234-8064011412;
Email: benthoa2013@gmail.com; ORCID: http://orcid.org/0000-0003-0675-5749

Abstract

Background: The lack of standard biochemical tests, coupled with the limitation of the number of micro and macro morphology that can be scored for fungal diagnosis, makes the identification of fungal organisms using microbiological methods less reliable.
Objective: To determine the fungal contaminants of processed *Manihot esculenta* Crantz (Garri) and provide information on their diversity.
Methods: In this study, fungal contaminants of processed *Manihot esculenta* Crantz (Garri) were characterized by sequencing the hyper-variable 18S ribosomal RNA as well as type the isolated organisms using Random Amplified Polymorphic DNA markers.
Results: The PCR amplification of the 18S ribosomal RNA gene of the isolated fungi from processed *Manihot esculenta* Crantz yielded a single fragment of an approximately between 600 and 700 bp. BLAST search using Genbank database showed that the isolate percentage similarity with GenBank accessions ranged between 98% and 100%. The molecular technique successfully identified all the isolated organisms resulting in 100% accurate diagnosis as against 79.3%accurate diagnosis made with the microbiological method. The forty isolated *Aspergillus species* were further resolved into 27 RAPD haplotypes with Simpson Index of Genetic Diversity approaching one for all the isolates. The mean genetic diversity within (G_L) and among (G_S) the isolated *Aspergillus species* were found to be 89% and 11% respectively. The total genetic diversity (H_T) for these organisms was approximately 48%. These results connoted significant strain diversity in the sampled specimens as shown by differences in their electrophoretic patterns.
Conclusion: The study revealed significant strain diversity in the sampled specimens as shown by differences in their electrophoretic patterns.

Key words: Aspergillus species, Electrophoresis, Fungi, Genetic diversity, Processed Manihot esculenta (Garri), Random Amplified Polymorphic DNA (RAPD).

Introduction

Processed *Manihot esculenta* Crantz, popularly known as "Garri" in different West African States, including Nigeria, is the most common form in which cassava is consumed in West Africa, [1] and indeed, in Africa. [2] This food is a roasted granule of cassava that is widely consumed in both rural and urban areas, either with cold water or reconstituted with hot water to form dough which can be eaten with different types of soup. [2] The demand for "Garri" is high due to its affordability, ease of preparation and the fact that it can be consumed in different forms. In Nigeria, the acceptability of "Garri" cuts across the various ethnic and socioeconomic classes, making it the commonest food among the rich and the poor. [3,4]

In recent times, the consumption of "Garri" has increased due to the fortification of this food item with different nutritional supplements such as soybeans and breadfruits, among others. This fortification, no doubts, has led to better nutritional values compared with the unfortified samples. Arisa *et al.* [5] reported higher nutritional values of "Garri" when fortified with different concentrations of groundnut flour. In another study, Osho, [6] also observed improved nutritional protein quality in the "Garri" samples he fortified with soybeans.

Despite the various reported nutritional advantages and the ease of the consumption of this product, majority of consumers prioritize consumption in relation to quality, even among the elites. In Nigeria, for instance, some researchers have characterized the fungal contaminants of this food item using the classical microbiological technique. [3, 4, 7] However, there is paucity of information regarding the molecular characterization of the fungal contaminants of this important cassava food product. To the best of the authors' knowledge, there is no study on strain typing of fungal contaminants of processed *Manihot esculenta* in circulation in Ogun State, Nigeria. Therefore, this study was aimed at identifying fungal contaminants of "Garri" using sequencing of the hyper-variable 18S rRNA gene and providing information on strain diversity using the Random amplified polymorphic DNA markers.

Methods

Sources of Processed Manihot esculenta Crantz (Garri)

A total of 1000 different specimens of processed Manihot esculenta Crantz (made up of two hundred and fifty samples each purchased from the local markets in the four geopolitical zones in Ogun State, Nigeria) between March 2013 and December 2014. These four zones include Yewa, Egba, Remo and Ijebu; the purchases were made during the dry and wet seasons. The sample size of 1000 was derived according to a statistical sampling scheme recommended for microbiological testing of foods. [8]

The samples were collected in pre-sterilized aluminium pans. The samples collected in the pre sterilized aluminium pan, the lids of which were opened before getting to the laboratory were excluded from analysis.. The appearance of the "Garri" samples, the sources of each collection site and the geopolitical zones were noted. "Garri" samples autoclaved at 121°C for 15 minutes were used as control samples while the remaining one thousand (1000) processed samples were used as the test samples. Ten gramme of each sample of the processed *Manihot esculenta* Crantz was aseptically transferred into a sterile 500 ml glass beaker. The content was stirred several times with a sterile spatula in order to obtain homogeneous laboratory sample. The beaker was immediately covered with a sterile glass petri dish to prevent the sample from absorbing atmospheric moisture and environmental contamination.

Fungal Isolation and Identification

Microbiological identification

One gram each of the processed *Manihot esculenta* Crantz sample was aseptically seeded in the middle of a sterile potato dextrose agar (PDA) plate in duplicate. This was incubated for one week at 25°C. Following incubation, fungal isolates of public health significance were identified using the rate of growth, colonial and microscopic morphology according to Larone. [9]

Molecular Identification of Fungi

DNA Isolation, Amplification and Sequencing

Each specimen (fungal isolate) was stirred directly into 200 ml sterile saline and was extracted using a QIAamp DNA mini kit (Qiagen), according to a protocol adapted for the extraction of DNA from fungal cells, as earlier described. [10] In brief, each sample was pre-incubated at 99°C for 20 minutes and then processed as suggested by the manufacturer. After the addition of the cellular lysis buffer, the sample was incubated again at 99°C for 10 minutes. The extracted DNA was amplified by PCR using a pair of universal fungal primers (V9D: 59-TTAAGTCCCTGCCCTTTG TA- 39; LS266: 59-GCATTCCCAAACAACTCGACTC-39), encompassing highly conserved regions which encoded fungal rDNA. [11] PCRs were performed in 0.2 ml reaction tubes in a final volume of 50 ml containing 2 to 10 ng of DNA, 1.5 U Platinum Taq DNA polymerase (In vitrogen), 200 mM each of dATP, dGTP and dCTP, 400 mM dUTP (instead of dTTP), 20 mM Tris/HCl (pH 8.4), 50 mM $MgCl_2$, 0.4 mM each primer and 1 U uracil-N-glycosylase.

The amplification reaction included a hold at 50°C for 5 minutes to allow uracil-N-glycosylase activity and an additional hold at 95°C for 5 minutes for Taq activation. This process was followed by 35 cycles at 95°C for 30 seconds, 62°C for 1 minute and 72°C for 2 minutes, with a final extension step at 72°C for 5 minutes. The amplified product was visualized on

agarose gels, purified and sequenced using a 310 auto Genetic Analyzer (PerkinElmer, Applied Biosystems Div., Waltham, USA) with the same primers. For each sample, a pair of primers amplifying the human b-globin gene was included as an extraction/amplification internal control. DNA sequences were analysed using the BLAST database and assigned to the reference isolate sequences with the highest bit score

Random amplified polymorphic DNA analysis [Figure 1]

DNA Isolation, Primer Screening and PCR Amplification

DNA was isolated and purified based on the manufacturer's instruction of DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). A total of 26 RAPD primers were screened and optimized for polymorphisms and annealing temperature (Tm) using the isolated ochratoxigenic moulds.

Optimal PCR amplification across the isolated organisms was achieved with annealing temperature between 40°C and 36°C. Seven primers that showed good and clear polymorphism with the PCR products were, therefore, used for the study.

These primers included OPX 07(GAGCGAGGCT), OPR 16 (CTCTGCGCGT), OPR 19 (CCTCCTCATC), OPR 11(GTAGCCGTCT), OPV 06 (GAACGGACTC), OPA 01(CAGGCCCTTC), OPA 04(AATCGGGCTG). Each 25 µl PCR reaction contained 12.5 µl master mix (2×) (0.05 45 units/µl Taq DNA polymerase in reaction buffer; 4 mM $Mgcl_2$, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP), 40 pmol oligonucleotide primer and 1 µg of template DNA. The DNA was first denatured for 2 minutes at 95 °C followed by 40 cycles of 15 seconds denaturation at 95°C, the annealing temperature was progressively decreased by 0.5°C every cycles from 40°C to 35°C for 1 minute and 2 minutes elongation at 72°C with a final elongation for 2 minutes. The amplified products were separated on 3% TBE agarose gels stained with ethidium bromide and viewed under a UV Transilluminator. The analyses of the amplification products were done manually with consideration of the number of fragments and repeatability of the reaction following the procedures described by Roodt et al. [12] Each lane of amplified product was checked manually and scored for presence (+) or absence (-) of fragments. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5. [13]

Data obtained from the morphological analysis was subjected to cluster analysis using the PAST (Paleontological Statistics) package. In the process of hierarchical clustering, the un-weighted pair group method of arithmetic average (UPGMA) was employed using the Euclidean similarity distance coefficient within the PAST Package. [14]

Results

The PCR amplification of the 18S rRNA gene of the fungal isolates obtained from the processed Manihot esculenta Crantz yielded a single fragment of an approximately between 600 and 700 bp. BLAST search using Genbank database showed that the isolate percentage similarity of sequence with GenBank ranged between 98% and 100%. The sequence results of all the fungal isolates are summarized in Table I.

The species identifications by the two test systems namely; Microbiological and Molecular methods for the isolated filamentous fungi are depicted in Table II. Except for five (5) *Penicillium chrysogenum* and *Trichoderma atroviride*, two (2) *Fusarium oxysporum* and six (6) *Absidia glauca* which were misidentified as *Penicillium verrucosum*, *Fusarium moniliforme*, *Trichoderma* species and *Absidia corymbifera*, respectively, all the remaining 69 isolates were correctly identified by the microbiological method. Conversely, the comparator molecular technique successfully identified all the isolated organisms resulting in 100% correct diagnosis. The number of discrepant species identified by the microbiological method represented a total of eighteen (18) fungal strains belonging to four different genera connoting approximately 18.3% of the total number of isolated organisms.

The percentage of misdiagnosis for discrepant species was 33.3% for *Absidia glauca*, 27.8% for *Penicillium chrysogenum* and *Trichoderma atroviride* and 11.1% for *Fusarium oxysporum*. Among the isolated *Aspergilli* species, *Aspergillius* niger and *Aspergillus carbonarius* had the highest prevalent rate (21; 24%); this confirmed that these organisms were the major contaminants of "Garri" in Ogun State, Nigeria.

Discussion

The results of the present study showed that the molecular method has enhanced accuracy for fungal identification than the traditional microbiological method. This observation is corroborating the finding of Bhattacharya et al. [15] who reported that microbiological testing is not as sensitive and specific as PCR based technique.

Figure 1: RAPD typing showing different strains of some of the isolated organisms

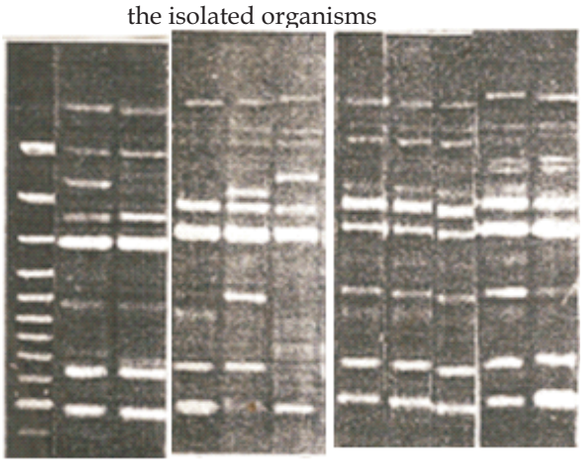

Table I: Molecular identification of fungal isolates from processed Manihot esculenta Crantz

Culture number	Species	Gene Bank accessions for 18S rRNA gene %	Similarity of sequence
FF1	Aspergillus niger	AY214445.1	100
FF2	A. flavus	AY214445.1	99
FF3	A. fumigates	FJ878717.1	99
FF4	A. carbonarius	KR296871.1	98
FF5	A. terreus	KP131622.1	98
FF6	A. wenti	FJ537089.1	99
FF7	Penicillium chrysogenum	L76153.1	98
FF8	Fusarium oxysporum	KJ573079.1	98
FF9	Trichoderma atroviride	KC569355.1	98
FF10	Absidia glauca	AY944877.1	99
FF11	Rhizopus stolonifer	HM051076.1	99

Table II: Identification of fungal agents by the microbiological and the molecular methods

Species	Number (n, %) of isolates in each category of diagnostic method					
	Microbiological methods			Molecular methods		
	Correct Diagnosis	Mis-diagnosis	No Diagnosis	Correct Diagnosis	Mis-diagnosis	No Diagnosis
Aspergillus niger	21	0	0	21	0	0
A. fumigatus	8	0	0	8	0	0
A. terreus	5	0	0	5	0	0
A. wenti	4	0	0	4	0	0
Penicillium chrysogenum	5	5	0	10	0	0
Fusarium oxysporum	0	2	0	2	0	0
Trichoderma atroviride	0	5	0	5	0	0
Absidia glauca 0	6	0	6	0	0	
Rhizopus stolonifer	1	0	0	1	0	0
Total	69 (79.3)	18 (8.3)	0 (0.0)	87 (100.0)	0 (0.0)	0 (0.0)

The role of filamentous fungi in food deterioration has been well documented. [16, 17] In the present study, the main filamentous fungi isolated were *Aspergillus niger, Aspergillus flavus, Penicillium chrysogenum, Fusarium oxysporum, Trichoderma atroviride, Absidia glauca and Rhizopus stolonifer*. However, the present findings differed from previous reports in the literature, for the identities of filamentous fungi associated with ready-for-sale "Garri" samples in Edo and Ogun State. [7, 17] Absidia glauca, Trichoderma atroviride and Penicillium chrysogenum were not isolated in the aforementioned previous reports from Edo and Ogun States. However, Thomas et al. [7] recovered Penicillium species, Aspergillus niger and

Aspegillius flavus as reported in a similar study. The variation observed in the two studies may not be unconnected to the fact that a larger sample size and larger number of markets were sampled in the recent previous study. [7]

Irrespective of the types of xerophilic mould isolated, their presence in foods result in remarkable changes in the organoleptic, microbiological as well as the nutritional quality of such foods and subsequently, in food spoilage. [17,18] From the public health perspective, the growth of these xerophilic organisms in processed Manihot esculenta ("Garri") suggest an imminent danger to the consumer, such as the various effects and manifestations of mycotoxicosis, especially when the secondary metabolites of the contaminants have already been produced in the food. [19, 20]

Aspergilli, which were the most predominant in the present study, are among the most abundant organisms that live on the earth without causing diseases. [21-23] Among the isolated Aspergilli in the present study, Aspergillius niger and Aspergillus carbonarius had the highest prevalence rate of 24%, thus affirming their roles as major contaminants of "Garri" in the area studied. This high rate of contamination may be due to the local practices involved in the production, processing and post-processing handling of "Garri". These include spreading of the raw granules on the floor or mats, displaying in open bowls in the open markets as well as use of various packaging materials to haul finished products from the rural to the urban areas. [17]

The RAPD analysis carried out in this study delineated fungal strains diversity as many of the isolates belonging to the same species were further subdivided into different RAPD haplotypes based on the differences in their electrophoretic patterns. [24] The level of genetic diversity observed among the isolates obtained from the four geopolitical zones of Ogun State, Nigeria were examined with parsimony analysis, using PAUP program. The values obtained for total gene diversity (H_T), diversity among and within isolates (GS and GL) were similar to those observed in fungi with known sexual life cycles. [15] According to Grypta et al. [25] regional population structure of this nature is usually the result of more frequent interbreeding events among isolates within a site than between sites, and it is more common among diploid or dikaryotic organisms.

Conclusion

It can be inferred that the molecular method has enhanced the accuracy of fungal species characterization than the classical microbiological techniques. This study should therefore be employed for the identification of the ever dynamic and highly genetically varying strains of filamentous fungi observed in this study. The results of this study also connote significant strain diversity in the sampled specimens as shown by differences in their electrophoretic patterns.

Authors' Contributions: TBT conceptualized the study and drafted the first manuscript. OLA and IBA contributed to the design of the study and revised the final draft of the manuscript. AGC participated in data collections, laboratory and data analysis as well as data interpretation. All the authors approved the final version of the manuscript.
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References

1. Ikediobi CO, Onfia GOC, Eluwah CE. A rapid and inexpensive enzymatic assay for total cyanide in cassava (Manihot esculenta crantz) cassava products. Agri Bio Chem 1980; 44: 2803-09.

2. Oluwole OB, Olatunji OO, Odunfa SA. A process technology for conservation of dried cassava chips with garri. Niger Food J 2004; 22: 65-73.

3. Jekayinfa SO, Olajide JO. Analysis of energy usage in the production of three selected cassava based foods in Nigeria. J Food Eng 2007; 82: 217-26.

4. Ogiehor IS, Ikenebomeh MJ, Ekundayo AO. The bioload and aflatoxin content of market garri from some selected states in southern Nigeria: public health significance. Afr Health Sci 2007; 7(4): 223-27.

5. Arisa NU, Omosaiye OB, Adelekan AO, Alabi-Mac FA. Chemical and sensory qualities of gari fortified with groundnut flour. Afr J Food SciTechnol 2011; 2(5): 116-19.

6. Osho SM. The processing and acceptability of a fortified cassava-based product (gari) with soybean. Nutri Food Sci 2003; 33(6): 278-83.

7. Thomas BT, Effedua HI, Agu GC, Musa OS, Adeyemi MT, Odunsi OD, et al. Fungi associated with the deterioration of garri in Ogun State, Nigeria. Reseacher 2012; 4(2): 8-12.

8. International Commission of Microbiological Specification for Food (ICMSF). Microorganisms in foods. Kluwer academic/plenum. 2002Vol7. USA.

9. Larone DH. Medically important fungi: a guide to identification. 4th Edition. Washington D.C, ASM Press. 2002: pp. 150 310.

10. Fredricks D, Smith C, Meier A. Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. J Clin Microbiol 2005; 43(10): 5122-28.

11. Pryce TM, Palladino S, Kay ID, Coombs GW. Rapid identification of fungi by sequencing the ITS1 and ITS2 regions using an automated capillary electrophoresis system. Med Mycol 2003; 41: 369-81.

12. Roodt R, Spies JJ Burger TH. Preliminary DNA fingerprinting of the turfgrass Cynodon dactylon (Poaceae: Chloridoideae). Bothalia 2002; 32: 117-22.

13. Tamura K, Dudley J, Nei M, Kumar S. MEGA 4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biol Evolution 2007; 24: 1596-99. Available at http://www.kumarlab.net/publications)

14. Hammer O, Harper DAT, Ryan PD. PAST: Paleontological statistic software package for education and data analysis. Paleontologia Eletronica 2001; 4(1): 1-9. Available at: http://palaeoelectronica.org/2001 _1/past/issue1_01.htm [Accessed: 04/06/ 2015]

15. Bhattacharya S, Mondal AS. Clinical microbiology in the intensive care unit: strategic and operational characteristics. Ind J Med Microbiol 2010; 28(1): 5-10.

16. Schwan RF, Wheals AE. The microbiology of cocoa fermentation and its role in chocolate quality. Crit Rev Food Sci Nutri 2004; 44: 20521.

17. Ogiehor IS, Ikenebomeh MJ. The effect of different packaging materials on the shelf stability of garri. Afr J Biotechnol 2005; 523: 2412-16.

18. Magnolia C, Hallak C, Astoreeca A, Ponsone L, Chiazchiera S, Dalcero AM. Occurrence of ochratoxin A producing fungi in commercial kernels in Argentina. Mycopathologia 2006; 161: 53-8.

19. Zimmerli B, Dick R. Ochratoxin A in table wine and grape-juice: occurrence and risk wine and its geographical origin. Food Additives and Contaminations 1996; 17: 79398.

20. Otteneder H, Majerus P. Occurrence of ochratoxin A (OTA) in wines: influence of the type of wine and its geographical origin. Food Additives and Contaminations 2000; 17: 79398.

21. Samson RA. Current fungal taxonomy and mycotoxins. Mycotoxins and phycotoxins in perspective at the turn of the millennium. In: Proceedings of the Xth International IUPAC Symposium on Mycotoxins and Phycotoxins, 21-25 May, 2000. Guarujá (Brazil). Ponsen & Looyen, Wageningen, The Netherlands.

22. Bennett JW, Klich J. Mycotoxins. Clin Microbiol Rev 2003; 16(3): 497.

23. Cheesebrough LM. Medical Laboratory Manual for Tropical Countries (Volume II). London, UK.. Butterworth & Co. Publishers. 2005.

24. Pekarek E, Jacobson K,. Donovan A. High levels of genetic variation exist in Aspergillus niger populations infecting Welwitschia mirabilis hook. J Hered 2006; 97(3): 270-78.

Grypta H, Debaud JC, Marmeise R. Population dynamics of the symbiotic mushroom Hebeloma cylindrosporum: mycelial persistence and inbreeding. Heredity 2000; 84: 294302.