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Molecular Characterization of Fungal Isolates of Locally Processed Rice from Five Agro-Ecological Zones of Nigeria

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Abstract

The purpose of this study was to characterize fungal isolates contaminating locally processed rice in Nigeria and to assess the genetic similarity in the fungi isolates. Fungal species were characterized by amplification of a segment of the β-tubulin gene using Bt2aF (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2bR (5'ACCCTCAGTGT GT GACCCTTGGC-3') primers and phylogenetic relationship established using the Maximum Likelihood method. Amplification of between 450 and 550bp of the β-tubulin region of the genomic DNA of the fungal isolates was obtained from thirty-eight samples (89%). Members of Aspergillus section flavi and section nigri groups were differentiated as *A. flavus*, *A. parasiticus, A. tubigensis, A. sydowii* and *A. niger* respectively. Molecular method identified *Paecilomycesformosus* with 99% similarity index while all the morphologically identified Penicillium species were identified to genus level as *P. formosus, P. steckii* and *P. citrinum* with a percentage similarity ranging from 95%-100%. *Collectotrichum fruticola* and *Talaromyces flavus* from SGS and NGS zone respectively were documented for the first time in the locally processed rice with 100% and 89% similarity index respectively. All the Aspergillus species were closely related to one another and narrowly diverged from the *Penicillium* species. *Aspergilla* and *Penicillia* were the predominant genera identified and the isolates were genetically similar irrespective of their agroecological zones.

Keywords: Genetic Similarity; β-tubulin; *Aspergillus* Section Flavi; Phylogeny

Introduction

Identification of microbial species based on morphological and biochemical tests are not always precise [1]. The traditional methods for the identification of fungal species such as direct plating and pour plates methods followed by phenotypic characterization of the fungal species producing the toxins are mainly based on parameters,

including colony diameter, colour, size and texture of conidia and conidiophore structures [2]. However, species classification using these methods may be difficult due to extensive divergence of morphological characters produced by a high level of genetic variability both inter- and intra-specific [3]. These traditional methods are less reliable and less sensitive compared to modern molecular techniques which are rapid, specific and sensitive [4] and appear to be more reliable and robust alternatives to differentiate fungal species. Sequencing data are objective and fast, and leads to reliable identification of uncommon species [5]. Recent advances in molecular based methods have led to many useful techniques taking into account the house-keeping genes as well as targeting other gene catalogues such as 26S, 28S, β-tubulin, calmodin and actin [6].

Beta-tubulin gene has enormous significance in fungi including establishing evolutionary relationship among fungal species and phylogenetic marker for fungal classification. Iqbal and Bakeer [7] reported that β-*tubulin* is among the most conserved genes in fungi while according to Ayliffe, et al. [8], the intron positions within *β- tubulin* genes may provide some clues of evolutionary relationships among species. Literatures also abound of various authors who have characterized fungi using this gene [9-11].

Although the contamination of locally processed rice by fungal colonies and their toxins have been reported Egbuta, et al. [12] but this has been restricted to few selected states of the federation therefore necessitating a nationwide investigation. Abdus-Salaam, et al. [13] reported fungal contamination of locally processed rice in Nigeria with a wider coverage of five agro-ecological zones of Nigeria but there still exists paucity of information on the influence of ecological factors on the incidence of fungal infection of locally processed rice, hence the need to investigate the genetic relatedness of the different fungal ;species colonizing rice in the different agro-ecological zones (AEZ) as Nigeria rises up into self-sufficiency in rice production and export projection. The objective of this study therefore was to characterize potential mycotoxigenic fungal species isolated from locally processed rice samples collected from five AEZs of Nigeria, by the amplification of a segment of β-tubulin gene and establish their phylogenetic relationship.

Methodology

Sample Collection

Samples collection was as described by Abdus-Salaam, et al. [13]. Briefly, different processing centers in five AEZs (Sudan savannah, Northern guinea savannah, Southern guinea savannah, Derived savannah and humid forest) of Nigeria, where rice is traditionally soaked, parboiled, dried and milled, were selected for the study. The milled (dehusked) rice samples were collected from available commercial processors in these centers between November 2011 and February 2012. At each AEZ, samples were collected from four different processing centers in each selected state. At each centre, locally processed rice was obtained from four different processors which were then pooled together to represent one composite sample per Center.

Fungal Isolates

The dilution plate technique described by Samson, et al. [14] was used for fungal isolation by plating 0.1ml aliquots on ½ strength Potato Dextrose Agar (PDA) plates supplemented with 0.01% chloramphenicol. A set was incubated at 30°C for 3 days for enumeration of *Aspergillus* species while the second set was incubated at 25°C for 7 days for enumeration of *Fusarium* and *Penicillium* species. colonies that bore resemblance to *Aspergillus* and *Penicillium* species were transferred to full strength potato dextrose agar (PDA). The *Aspergillus* and *Penicillium* cultures on PDA were incubated unilluminated at 30°C and 25°C respectively for 7 days. Pure cultures were obtained by repeated sub-culturing and the subcultured isolates were incubated for 5-7 days on PDA. Fungal colonies were harvested and a thin spread was observed in lactophenol in cotton blue solution on microscope slides for identification using the microscope. Pure isolates were identified on the basis of morphological characteristics (macro: colony colour, morphology and size; micro: conidia morphology and size) and comparison with appropriate keys in literature Klich [2], Ehrlich, et al. [15], Pitt, et al. [16]. The identified isolates were further maintained on PDA slants by the single colony transfer technique at 4°C. In all, forty-six representative fungal isolates obtained from thirty-eight composited locally milled rice samples Abdus-Salaam, et al. [13] were used for this study.

Genomic Characterization of Fungi Species

Extraction of genomic DNA: Mycelia of fungal isolates stored under water in vial tubes were subcultured on PDA in a Petri dish for 7 days in the dark at room temperature (28±$^{2°C}$). DNA was extracted using Zymo kit (Inqaba biotech, South Africa) following manufacturer's instructions.

PCR Amplification of β-tubulin gene, Sequencing and Phylogenetic Analysis: The primers Bt2aF (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2bR (5'ACCCTCAGTGTAGTGACCCTTGGC-3') described by Glass, et al. [17] and as reported by Nouripour-Sisakht, et al. [18] was used.The PCR condition included an initial denaturation at 95ºC for 5 min, followed by 40 cycles of denaturation at 95°C for 1min, annealing at 51ºC for 1min and a final

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extension at 72ºC for 10 min. Positive amplicons were sequenced after purification with QIAquick PCR purification kit (Qiagene, Hilden,Germany). Sequencing was done usingBt2aF (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2bR (5'ACCCTCAGTGTAGTGA CCCTTGGC-3') as described by Glass, et al. [17], Hubka, et al. [19]. Gene sequences obtained were compared by aligning with the sequences in the GenBank using (BLASTn) while evolutionary analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) 7 according to Kumar, et al. [20].

The Maximum Parsimony method described by Steel, et al. [21] was used. The consistency index was 0.804697, the retention index was 0.770349 and the composite index was 0.619898 for all sites and parsimony-informative sites. The Maximum Parsimony tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm [22] with search level 0 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 32 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+noncoding. There were a total of 557 positions in the final dataset. Evolutionary analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) 7 according to Kumar, et al. [20].

Results

Molecular Characterization of Fungi Isolated from Locally Processed Rice in Nigeria

Figure 1 shows amplification of between 450 and 550bp of the β-tubulin region of the genomic DNA of each of the fungal isolates. Amplification was obtained from thirty- eight isolates (89%). Table 1 shows blast result of the nucleotide sequences with the percentage similarities. The Table showed that an isolate from the SGS zone which could not be identified morphologically was identified as *Paecilomyces formosus* with 99% similarity index. The *Penicillium* isolates were also identified to species level with a percentage similarity ranging from 95%-100% as *Penicillium citrinum, P. crustosum* and *P. stekii*. However two isolates, each from SGS and NGS that were identified as *Penicillium* spp. were respectively identified as *Colletotrichum fruticola* and *Talaromyces flavus* with 100% and 89% similarity index respectively. One *Aspergillus* species from the DS zone was clearly identified as *Aspergillus sydowii*. There was also specific identification of *A. tubingensis* from *Aspergillus* section *Nigri* and *A .tamari* in *Aspergillus* section *flavi* respectively.

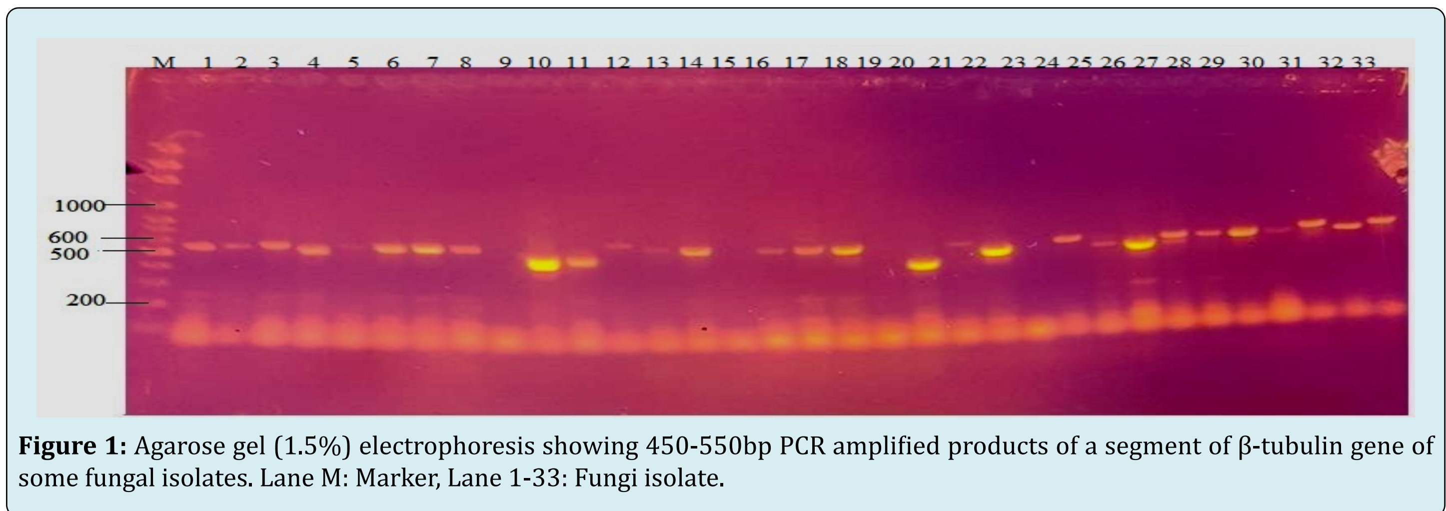

Figure 1: Agarose gel (1.5%) electrophoresis showing 450-550bp PCR amplified products of a segment of β-tubulin gene of some fungal isolates. Lane M: Marker, Lane 1-33: Fungi isolate.

Phylogenetic Relationship of Fungal Isolates

The phylogenetic analyses of the fungal isolates (Figure 2) showed that amongst the isolates, *Aspergillus flavus* was the most recent ancestor with *Aspergillus flavus* (HF570030.1) as the common ancestral species. The *Aspergillus* species were closely related to one another and narrowly diverged from the *Penicillium* species. Next to the ancestral clade were A. *parasiticus* and *A. tamarii* which showed a common evolution from *A. flavus. A. niger and A. tubigensis* shared a common ancestor and individual strains from each specie respectively clustered together except for A. *niger* (KF669403.1) which formed an outgroup.

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S/ No	Location	Item code	Morphological Identification	Molecular identification (Closest known identity)	% Similarity	NCBI Acession no
1	SGS	16781	*A.*section *Flavi*	*Aspergillus flavus*	100%	KR051523.1
2	SGS	16784	*A.*section *Flavi*	*Aspergillus flavus*	99%	KJ482657.1
3	SGS	16785	*A.*section *Nigri*	*Aspergillus tubingensis*	98%	KP329861.1
4	SGS	16786	*UIS	*Paecilomyces formosus*	95%	GU968683.1
5	SGS	16788	*A.*section *Flavi*	*Aspergillus flavus*	100%	KF434077.1
6	SGS	16789	*A.*section *Flavi*	*Aspergillus flavus*	100%	KM491310.1
7	SGS	16790	*A.*section *Flavi*	Aspergillus flavus	100%	KF562211.1
8	SGS	16792	*Penicillium*	*Colletotrichum fructicola*	100%	KP852485.1
9	SGS	16794	*Aspergillus spp*	*Aspergillus niger*	100%	HG325832.1
10	SGS	16797	*A.*section *Flavi*	*Aspergillus tamarii*	99%	EU021673.1
11	SGS	16798	*A.*section *Nigri*	*Aspergillus niger*	98%	KF669403.1
12	SGS	16799	*A.*section *Nigri*	*Aspergillus niger*	98%	KJ136068.1
13	SGS	16801	*A.*section *Flavi*	*Aspergillus flavus*	100%	KF434079.1
14	DS	16802	*A.*section *Nigri*	*Aspergillus niger*	93%	KY416563.1
15	DS	16803	*Aspergillus spp*	*Aspergillus sydowii*	100%	KP329915.1
16	DS	16806	*Penicillium*	*Penicillium citrinum*	99%	KP329977.1
17	HF	16812	*Penicillium*	*Penicillium steckii*	95%	GU944526.1
18	DS	16815	*Penicillium*	Penicillium citrinum	100%	JX141013.1
19	DS	16816	*A.*section *Flavi*	*Aspergillus flavus*	100%	KF434075.1
20	DS	16817	*A.*section *circumdati*	*Aspergillus flavus*	100%	KF225072.1
21	HF	16818	*A.*section *circumdati*	*Aspergillus flavus*	100%	KF434079.1
22	HF	16819	*A.*section *circumdati*	*Aspergillus flavus*	98%	KJ136103.1
23	HF	16822	*Penicillium*	*Penicillium crustosum*	99%	KJ481238.1
24	NGS	16825	*A.*section *Flavi*	*Aspergillus flavus*	99%	HF570030.1
25	NGS	16826	*Penicillium*	*Talaromyces flavus*	89%	AY766252.1
26	SS	16829	*A.*section *Flavi*	*Aspergillus flavus*	100%	KF562206.1
27	SS	16830	*A.*section *Flavi*	*Aspergillus flavus*	100%	KC190483.1
28	SGS	16834	*A.*section *Flavi*	*Aspergillus flavus*	99%	HQ285476.1
29	SGS	16835	*A.*section *Flavi*	*Aspergillus flavus*	100%	KF225050.1
30	HF	16836	*A.*section *Flavi*	*Aspergillus parasiticus*	99%	EF203170.1
31	DS	16837	*Aspergillus* spp.	*Aspergillus tubigensis*	100%	KX961163.1
32	DS	16839	*A.*section *Flavi*	*Aspergillus flavus*	99%	KY288823.1

Table 1: Molecular Identification of Fungal Isolates**.**
*UIS= Unidentified isolate

All strains of *Penicillia* formed a good cluster relationship with the exception of *P. crustosum* (16822) which relate closer with *Colletotrichum fructicola*. The phylogenic tree also revealed *Paecilomyces formosus* (16786) and *Talaromyces flavus* (16826) could have evolved from *Penicillium* spp.

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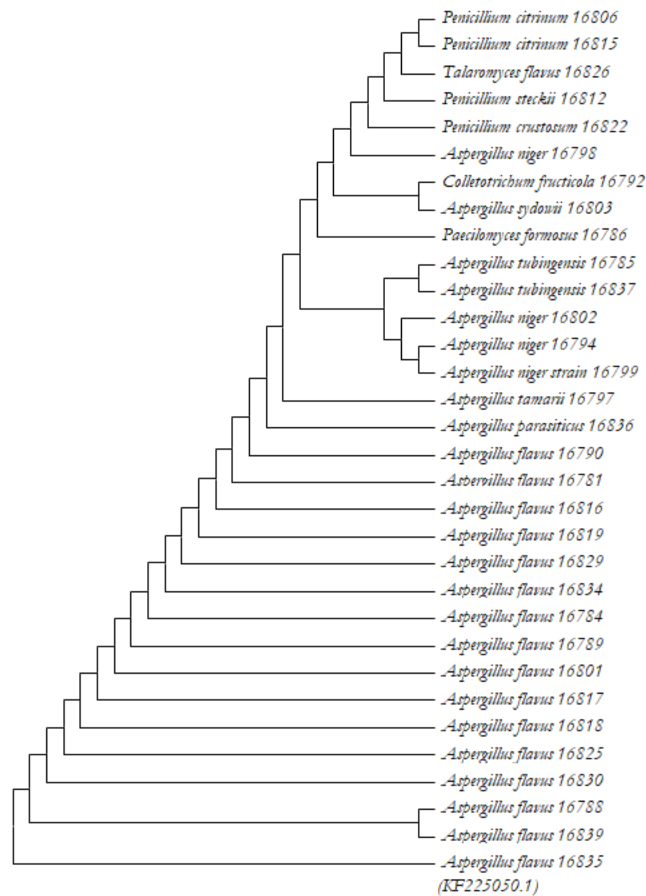

Figure 2: Phylogenetic Relationship of Isolates using the Subtree-Pruning-Regrafting (SPR) algorithm.

Discussion

The percentage similarity of the isolates to the reference strains in the gene bank was high which indicated a good index of identification. Rocha, et al. [23] stated that a similarity of less than 40% indicated that isolates do not belong to the same group while an intermediate degree of similarity occurs when strains share 40% to 60% of fragments and still undergo speciation.

An accurate identification of the species of *Aspergillus* is important because the toxin profile of each species varies and the presence of a particular fungus limits and defines the potential toxicological risks [24]. *Aspergillus flavus* is a known producer of aflatoxins [25]. The relative abundance of *Aspergillus* species and high prevalence of *A. flavus* in this study is supported by previous authors Odhiambo, et al. [26], Egbuta, et al. [12] who reported similar trend in some cereal grains including rice and maize. The high prevalence of *A. flavus* among the section *flavi* in many substrates has led to mis-identifications of other members of the section *flavi* as *A. flavus*. Tam, et al. [11] reported an identification of *A. tamarii* and *A. nomius* (using ITS, β-tubulin and calmodulin genes) which were previously misidentified by phenotypic characteristics as *A. flavus*. In this study, members of *A.* section *flavi* were differentiated and identified as *A. tamarii* and *A. parasiticus*. Although *A. tamari* is non aflatoxigenic [25], it is however infectious to humans while *A. parasiticus* is toxigenic producing both B and G aflatoxins [27].

Identification of *A. tubingensis* which is a member of the species of *Aspergillus* section *nigri* and often misidentified as *A.niger* Palumbo, et al. [28] is a major discovery of this study.

Aspergillus niger is an important member of *Aspergillus* section *nigri* which despite its industrial importance in the production of enzymes and organic acids, is equally of health significance. *Aspergillus niger* has been reported to produce toxic metabolites such as malformin, ochratoxin A and other toxic metabolites while Gniadek, et al. [29] has reported the intermediate cytotoxicity of this species.

DNA sequencing method identified *A. sydowii* which is a thermophillic fungus [30]. The thermotolerant nature

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could enhance this fungus to survive the critical parboiling operation in rice processing. According to Kumar, et al. [31], *A.sydowii* is commonly associated with rice in the field (73%), on threshing floors (65%), or from storage facilities (78%). It is a potential source of fungal secondary metabolites such as sydowinin, deoxymulundocandin and mulundocandin which possess antimycotic properties and are therefore of biomedical interest [32,33]. This study provides the first incidence of the fungus *Colletotrichum fruticola* in Nigerian rice. Reddy, et al. [34] listed *Colletotrichum* spp. as one of the deleterious seed-borne fungi of rice. *Paecilomyces* strains are often heat resistant and may produce mycotoxins in contaminated pasteurized foodstuffs [35]. This genus is similar to *Penicillium* but differs in the absence of greenish coloured colonies and by the short cylindrical phialides that taper into long necks [36]. Some species of *Paecilomyces* have been produce mycotoxins in foods and feedstuffs and can be considered as a potential source of public health problem.

This study also reports the occurrence of *Talaromyces flavus* in Nigerian rice for the first time. Laut, et al. [37] also reported the incidence of *Talaromyces* spp for the first time in rice samples commercialized in Thailand. *Talaromyces flavus* belongs to the genus *Penicillium* and has been reported as a heat resistant fungus that can withstand thermal processes [38]. *Penicillium citrinum, P. stekii* and *P. crustosum* which were among other species of *Penicillium* identified in this study have been reported to be of public health concern. *Penicillium citrinum* is known to produce citrinin; a secondary metabolite which is nephrotoxic while *P. crustosum* produces penitrem A [39].

The phylogenetic tree demonstrated lack of relationship between the strains and their geographical origins because of the high genetic similarity among each group of fungal isolates. This finding is probably due to the easy dispersal of fungi. Al-Wadai, et al. [10] found that using clustering based on RAPD and ISSR dendograms to study genetic diversity in *Aspergillus flavus* population was unrelated to geographic origin. De Oliveira Rocha, et al. [23] reported a similar observation in a study of molecular characterization of *Fusarium verticillioides* isolated from corn grains of different geographical origin in Brazil. Davari, et al. [40] also noted that *A. flavus* isolates can easily adapt themselves to various geographical regions.

Conclusion

Aspergilla and *Penicillia* spp. were the prevalent fungal isolates contaminating milled rice in Nigeria while *Fusarium* species were not detected, despite the occurrence of *Fusarium* metabolites in the zones. While there were significant differences in the distribution of fungal isolates across the zones, the DS zone recorded the highest occurrence of fungal isolates while the SS zone recorded the least. Furthermore, *Colletotrichum fruticola* and *Talaromyces flavus* were documented for the first time in locally processed rice in Nigeria while *A. flavus* and *A. parasiticus* were genetically differentiated from one another. There were genetic similarities among the isolates irrespective of the AEZs thus suggesting the possibility of cross contamination of the organisms on the rice samples.

Conflict of Interest

The authors declared no conflict of interest.

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