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TRANSITION BIAS AND NEUTRAL SELECTION DRIVE THE EVOLUTION OF THE POLYKETIDE SYNTHASE GENE IN ASPERGILLUS SECTION NIGRI

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A b s t r a c t

The significance of the polyketide synthase (*pks*) gene in the biosynthesis of ochratoxin A in *Aspergillus* section Nigri has been well reported. However, factors driving the evolution of this gene in black aspergilli are seldom studied. This study, was therefore, aimed at investigating these factors as a means to understanding how to circumvent their wide epidemiological coverage in the nearest future. To achieve this, a total of one thousand cassava powders (*Lafun*) were collected from the four geopolitical zones of Ogun State, Nigeria and processed for black aspergilli using standard mycological procedures. The isolated organisms were subsequently characterised phenotypically, followed by screening for ochratoxin A production and sequencing of the *pks* gene associated with its biosynthesis. The sequenced *pks* genes were used for mutation discovery, estimation of selection, substitution pattern and phylogenetic analyses. The results revealed a total of 279 black aspergilli, distributed as follows: *Aspergillus niger* – 88 (8.8%), *A. welwitschiae* – 82 (8.2%), *A. carbonarius* – 56 (5.6%), and *A. aculeatus* – 47 (4.7%). Among these, 18 strains, comprising 6 *A. niger,* 3 *A. aculeatus,* 7 *A. carbonarius,* and 2 *A. welwitschiae* were identified as ochratoxigenic based on both phenotypic characterization and molecular amplification of the *pks* gene. The quantitative measurements of their ochratoxins ranged from 9.12–11.08 for *Aspergillus aculeatus* through 10.52–12.74 and 19.39–23.61 for *Aspergillus carbonarius* and *Aspergillus niger* to 23.88–27.02 for *Aspergillus welwitschiae.* All the ochratoxigenic *Aspergillus welwitschiae, Aspergillus aculeatus* and *Aspergillus niger* were clustered together on the phylogenetic tree. The maximum likelihood estimate of the transition-transversion bias of the *pks* genes of black aspergilli depicts that the number of proteins in the data set of the *pks* genes,

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the transition-transversion bias ratio and the maximum likelihood estimate were 2215 bp, 1.01 and –13279.996 respectively. The maximum composite likelihood estimates of the pattern of nucleotide substitution revealed frequent transitions than transversions in the polyketide synthase genes of *Aspergillus* section Nigri. The results showed that A/G and T/C transition were more frequent than C/T and G/A while the codon-based Fisher's exact test analysis of selection, the Codon-based *Z*-test of neutral evolution and the results from Tajima's Neutrality Test connotes significant bias for neutral evolution ($p < 0.05$). The above results suggest that transition and neutral selection drive the evolution of the *pks* gene of *Aspergillus* section Nigri.

Introduction

The importance of *Aspergillus* Section Nigri in the contamination of several agricultural products has been well documented (THOMAS et al. 2014, CABANES and BRAGULAT 2018, GIL-SERNA et al. 2019). This is because an estimated 30 to 50% of food commodities are lost due to contamination by filamentous fungi during pre-and post-harvest food processes which in turns threaten global food security in addition to wasting 1.47–1.96 Billion hectares of arable land, 0.75–1.25 trillion cubic meters of water and 1 to 1.5% of global energy (FOX and FIMECHE 2013). What is even more deleterious about the consumption of these organisms and their secondary metabolites is their documented harmful consequences on human and animal health (TANAKA, THOMAS et al. 2021). Consequently, the Food and Agriculture Organization (FAO) has estimated that these secondary metabolites contaminate about 25% of global food and feed production, entailing losses of about 1 billion tons of food and food products (ALTOMARE et al. 2021).

The growing awareness of the reverberations of mycotoxins on livestocks has affected the marketability of food commodities and raise global food safety concerns (MATEUS) because of the recorded literature of the vulnerability of more than five billion people through various unknown pathways (KHODAEI). Of these mycotoxigenic moulds, the black aspergilli are important as ochratoxin-producing organisms which contaminate several agricultural products including grape-derived products (CABAÑES et al. 2002), coffee and cocoa (SAMSON et al. 2004) and even dried cassava powder (*garri*) (THOMAS et al. 2014). Taking in ochratoxin through food can lead to intoxication, known as ochratoxicosis (BATTACONE et al. 2010). This ochratoxicosis, mainly from ochratoxin A, cause diverse toxic effects including hepatic, neural, teratogenic, mutagenic, and carcinogenic outcomes. At cellular levels, they inhibit DNA and RNA synthesis by interacting with nucleic acids (THOMAS et al. 2014, SAMUEL et al. 2021).

Generally, ochratoxin A (OTA) production has been strongly associated with the prevalence of black aspergilli (BELLÍ et al. 2005, KAPETANAKOU et al. 2009, THOMAS et al. 2014). The capacity to synthesize OTA is encoded by

a polyketide synthase (*pks*) gene, which belongs to the large fungal polyketide synthase family responsible for producing diverse mycotoxins and secondary metabolites (VARGA et al. 2003). The *pks* gene encodes a multifunctional enzyme containing distinct domains, including *β*-ketoacylsynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP). This enzyme is central to OTA biosynthesis (O'CALLAGHAN et al. 2003). In *Aspergillus carbonarius*, five *pks* fragments have been identified (ATOUI et al. 2006). Given the diversity of these genes and their roles in metabolite production across *Aspergillus* species, understanding how they are regulated, whether at the transcriptional, translational, or epigenetic level, is essential. This study therefore aimed to explore the evolutionary dynamics of the *pks* genes and identify potential strategies to limit their spread and the associated contamination of food substrates.

Materials and Methods

Sample Collection

Cassava flour (*Lafun*) samples were obtained from 24 localities belonging to four different political zones in Ogun state, South-Western Nigeria. These locations were chosen because of their known association with the production of cassava flour. The climate of Ogun state is tropical wet and dry with average annual rainfall of 1,340 mm across a total crop land area of 693.21k ha. Briefly, a total of 1000 *lafun* samples (250 each from 4 different markets in each zone) were collected in pre-sterilized aluminum pan from the four geopolitical zones of Ogun state, Nigeria between March 2013 to February 2014. The samples were collected at intervals and spread over the study period. The collection of samples was done as described by International Commission for Microbiological Specification for foods (ICMSF 2002). Briefly, aseptic techniques were used during sampling with sterile tools and containers, ensuring representative portions were taken from different parts of each batch. Samples were sealed immediately to prevent contamination or moisture uptake and stored at ambient temperature under dry conditions until analysis.

Fungal Isolation and Characterisation

Fungal isolation was carried out as described by VINCENTE et al. (2008) but with little modifications. First, 10 g from each sample was inoculated at room temperature for 30 min in 100 mL of sterile saline solution containing

200 µg/L penicillin, 200 µg/L streptomycin, 200 µg/L chloramphenicol and 500 µg/L cycloheximide. These antibiotics (penicillin, streptomycin, and chloramphenicol) were included to suppress bacterial contaminants, while cycloheximide was used to inhibit rapidly growing saprophytic fungi, thereby facilitating selective recovery of the target *Aspergillus* species. After the initial incubation, 20 mL of sterile mineral oil was added to the solution, followed by vigorous shaking for 5 min. The flasks were then left undisturbed for 20 min to allow phase separation. The oil-water interphase, where fungal spores and conidia typically accumulate due to their hydrophobic properties, was carefully collected using a sterile pipette. This fraction was chosen to enhance recovery of *Aspergillus* species while minimizing bacterial carryover. The collected interphase was inoculated onto Potato Dextrose Agar (Oxoid, United Kingdom) and incubated at room temperature for 72 h. The grown dark colonies were then isolated and stored on Mycosel agar. The fungal isolates were characterized both macro- and micro-morphologically prior to molecular identification. The macroscopic characterization was done by observing the colony features (color, shape, size and hyphae), and microscopically by a compound microscope with a digital camera using a lactophenol cotton blue stained slide mounted with a small portion of the mycelium. This procedure, adapted from GADDEYYA et al. (2012), which provides a standardized approach for fungal morphological identification.

Screening of the black aspergilli for ochratoxin A production

The screening of the black aspergilli for ochratoxin A production was carried out biphasically. First, preliminary screening of potential OTA-producing isolates was done following the method of HEENAN et al. (1998). Briefly, the black aspergilli (*Aspergillus niger* and *Aspergillus carbonarius*) were sub-cultured on Coconut Cream Agar medium following two approaches. First, Commercial coconut cream (Kara, 24% fat, Singapore) was used as the primary source to ensure reproducibility of nutrient composition across batches. Additionally, fresh coconut milk was extracted from mature coconuts (*Cocos nucifera*) obtained from local markets in Nigeria, prepared by blending grated endosperm with warm distilled water (1 : 2, w/v) and filtering through sterile muslin cloth. Both preparations were incorporated into the agar formulation (200 mL coconut milk, 20 g glucose, 15 g agar, distilled water to 1 L) and sterilized by autoclaving at 121°C for 15 min. The use of commercial cream provided consistency, while the fresh extract demonstrated the feasibility of employing locally available raw materials. The inoculated organisms were incubated at room temperature 27 ±2°C for 7 days after which the reverse of the inoculated Coconut Cream Agar medium was checked

for characteristic green fluorescence on exposure to long wavelength UV light (365 nm) in the dark environment to depict potential OTA-producing strains. These potential OTA-producing strains were fumigated with 26.8% ammonia and examined under long-wave UV light (365 nm) in the dark. Ammonia vapour was used because it increases the local alkalinity, thereby ionizing OTA's phenolic and carboxylic groups, which enhances its natural fluorescence. Colonies showing intensified fluorescence were recorded as positive OTA producers. Secondly, the positive OTA-producing strains were subjected to ochratoxin A quantitation following the method described by THOMAS and OGUNKANMI (2014). To achieve this, three agar plugs (the diameter was 4 mm) were removed from the inner, middle and outer area of each of the positive isolate and extracted with 25 mL of 50% methanol for 1 hour in darkness. The extracts were vortexed and filtered with whatman no 1 filter paper containing pre-sterilised cotton wool. Ochratoxin A (OTA) levels were quantified using a competitive direct ELISA kit (Veratox® OTA, Neogen Corporation, USA) following the manufacturer's protocol. Briefly, fungal extracts and OTA standards were incubated with enzyme conjugate and transferred into anti-OTA antibody-coated wells. After washing, substrate and stop solutions were applied, and absorbance was measured at 650 nm using a microplate reader (Bioline Technologies, India). All assays were performed in triplicate, with both positive and negative controls included. OTA concentrations were determined from a standard curve and expressed in ng/g.

Molecular characterisation of ochratoxigenic black aspergilli

The molecular identification was carried out polyphasically viz, DNA extraction, amplification and sequencing. The DNA extraction from the black aspergilli was conducted on a week-old fungal culture using DNeasy Plant Mini Kit (Qiagen, The Netherlands). Primers AcPKS-F1 (AGCATC-TATGCTGGCCAATC) and AcPKS-R 1 (AATG – TACTCTCGCGGGCTAA) were used to amplify the ketosynthase domain of *pks* gene of the black aspergilli. The PCR reactions included 100–200 ng DNA, 50 mM KCl, 10 mM Tris-HCl, 80 µM each dNTP, 1 µM each primer, 2 mM $MgCl_2$ and 1 U of Taq DNA polymerase (Thermo Scientific DreamTaq, USA). The thermal cycler was programmed as follows: an initial step at 94°C for 4 min, followed by 35 cycles of 94°C for 40 s, 55°C for 40 s, 72°C for 40 s, and a final elongation step of 72°C for 10 minutes. PCR products were purified using the QIA quick PCR purification kit (BAO et al. 2012) and sent for sequencing at the International Institute of Tropical Agriculture, Ibadan Nigeria. Sequence quality was assessed by inspecting chromatograms for

peak resolution and background noise, and low-quality reads were trimmed prior to other downstream analyses. The obtained sequences were compared with related sequences using nucleotide BLAST (BLASTn) against the NCBI GenBank database (nt). Searches were performed in (accessed July 2024), and the closest matches were retrieved for taxonomic identification.

Mutation discovery, estimation of selection, substitution pattern and phylogenetic analysis

Mutations in the pks gene of the isolated black aspergilli were identified by aligning sequences generated in this study using MEGA software (TAMURA et al. 2021). Recurrent mutations occurring at the same position were counted once. Substitution types were analyzed separately, with transitions (purine ↔ purine or pyrimidine ↔ pyrimidine) distinguished from transversions (purine ↔ pyrimidine), and single-base versus multiple-base substitutions examined independently. Selection pressure on the pks gene was assessed through codon-based analyses. We estimated the ratio of non-synonymous to synonymous substitutions ($dN/dS = \omega$), evaluated changes in amino acid properties, and calculated the ratio of non-synonymous to synonymous polymorphisms. Neutrality tests were also performed to detect deviations from neutral evolution. Different nucleotide substitution models were applied at different steps to account for model fit and accuracy. The Kimura 2-parameter model (KIMURA 1981) was used to estimate substitution patterns and distinguish between transitions and transversions. The JUKES-CANTOR model (1969) was applied for rate variation across sites under a gamma distribution, as it assumes equal base frequencies and simplifies correction for multiple substitutions. For phylogenetic inference, the Hasegawa-Kishino--Yano model (HKY 1985) was employed because it accommodates unequal base frequencies and transition/transversion bias. The TAMURA-NEI model (TAMURA et al. 2011) was also tested, as it accounts for both GC content bias and differences in substitution rates, providing robustness in tree reconstruction. Molecular phylogenetic analyses were performed in MEGA X and MEGA 11 using the maximum likelihood method. Initial heuristic searches were conducted with Neighbor-Joining and BioNJ algorithms, and the best-scoring topology was selected based on log-likelihood values. Branch support was estimated with bootstrap analysis. Final trees were drawn to scale, with branch lengths representing substitutions per site. The dataset included 18 nucleotide sequences and 993 aligned positions.

Results

Table 1 above represents the presence and distribution rate of black aspergilli in the sampled cassava powder circulating in Ogun state, Nigeria.

As shown in Table 1, the highest organism isolated from the samples was *Aspergillus niger* 88 (8.8%), closely followed by *Aspergillus welwitschiae* 82 (8.2), and then *Aspergillus carbonarius* 56 (5.6) and finally *Aspergillus aculeatus* 47 (4.7). The increasing order of the isolated organisms per geopolitical zones was *Aspergillus niger, Aspergillus welwitschiae, Aspergillus carbonarius* and *Aspergillus niger* for Egba, Ijebu, Remo and Yewa respectively. In general, a total of 279 organisms representing 27.9% of black aspergilli were isolated from the sampled cassava powders. The cassava powders collected from the Remo zone were the most contaminated followed by the samples from the Yewa region. Even though, the Egba samples were the least contaminated, there was no statistical significant difference between the Egba samples and the samples from Ijebu.

Table 1

Occurrence of black aspergilli in the sampled *Lafun* in Ogun State (n = 1000)

GPZO	n	Black aspergilli				
		AN	AA	AC	AW	N [%]
Remo	(250)	18	12	29	19	78 (31.20)
Egba	(250)	22	6	14	21	63 (25.20)
Ijebu	(250)	14	11	13	26	64 (25.60)
Yewa	(250)	34	18	6	16	74 (29.60)
Total	(1000)	88 (8.8)	47 (4.7)	56 (5.6)	82 (8.2)	279 (27.9)

Explanation: AN – *Aspergillus niger*; AA – *Aspergillus aculeatus*; AC – *Aspergillus carbonarius*; AW – *Aspergillus welwitschiae*; N – total number of isolates; GPZO – geopolitical zones; n – sample size; % – percentages

The mean ochratoxin A (OTA) concentration of the eighteen positive strains of the black aspergilli is depicted in Table 2 above. The quantitative measurements of the OTA in parts per billion (ppb) shows the two positive *Aspergillus welwitschiae* as the highest producers of this toxin with an average value of 25.3 ±1.72 ppb. This observation was closely followed by the six positive strains of *Aspergillus niger* which had a mean ochratoxin A content of 21.5 ±2.11 ppb. *Aspergillus carbonarius* and *Aspergillus aculeatus* also produced mean OTA contents of 11.63 ±1.11 and 10.10 ±0.98 respectively.

Table 2
Mean Ochratoxin A (OTA) concentration in the positive strains of the black aspergilli (n = 24)

Isolates	OTA content [ppb]	Range [ppb]
AN (n = 6)	21.5 ±2.11	19.39–23.61
AA (n = 3)	10.10 ±0.98	9.12–11.08
AC (n = 7)	11.63 ±1.11	10.52–12.74
AW (n = 2)	25.3 ±1.72	23.88–27.02

Explanation: n – positive strains of the individual species; ppb – part per billion

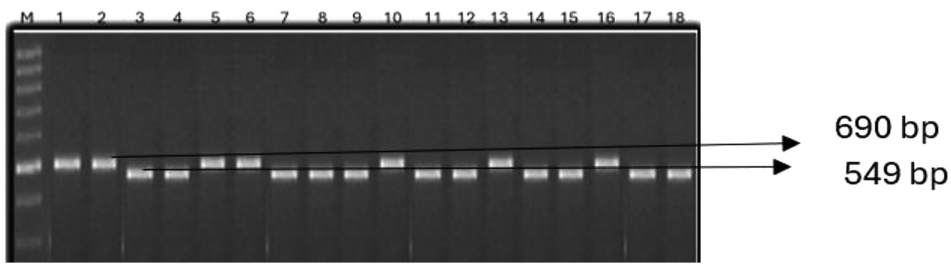

Fig. 1. Characterization of the polyketide synthase gene of black aspergilli. Explanation: *Aspergillus carbonarius* – 1–2, 5–6, 10, 13 and 16; *Aspergillus niger* – 7–9, 11–12, 14; *Aspergillus welwitschiae* – 3–4; *Aspergillus aculeatus* – 12, 17–18

Figure 2 represents the phylogenetic relationships of the different black aspergilli. As shown in this Table 3, all the *Aspergillus niger* aggregates (*Aspergillus welwitschiae* and *Aspergillus aculeatus*) clustered together with two other strains of *Aspergillus niger* at 75% percentage level of similarity except for two strains of *Aspergillus aculeatus that clustered with both Aspergillus carbonarius* and *Aspergillus niger* with a percentage similarity of 91%. The strains of *Aspergillus carbonarius* studied were found to share between 84–98% relatedness with themselves and 52% and 56% with *Aspergillus aculeatus* and *Aspergillus niger* respectively (Figure 2).

The maximum likelihood estimate of the transition-transversion bias of the *pks* genes of black aspergilli is depicted in Table 3. The frequency of the DNA building blocks (A, T, C and G) was found to be at equilibrium with a percentage substitution of 25% for each nucleotide of the *pks* genes. The number of protein in the data set of the *pks* genes, the transition-transversion bias ratio and the Maximum likelihood estimate were estimated to be 2215 bp, 1.01 and –13279.996 respectively.

The maximum composite likelihood estimate of the pattern of nucleotide substitution reveals that transitions were more frequent than transversions at the polyketide synthase genes of *Aspergillus* section Nigri. The results

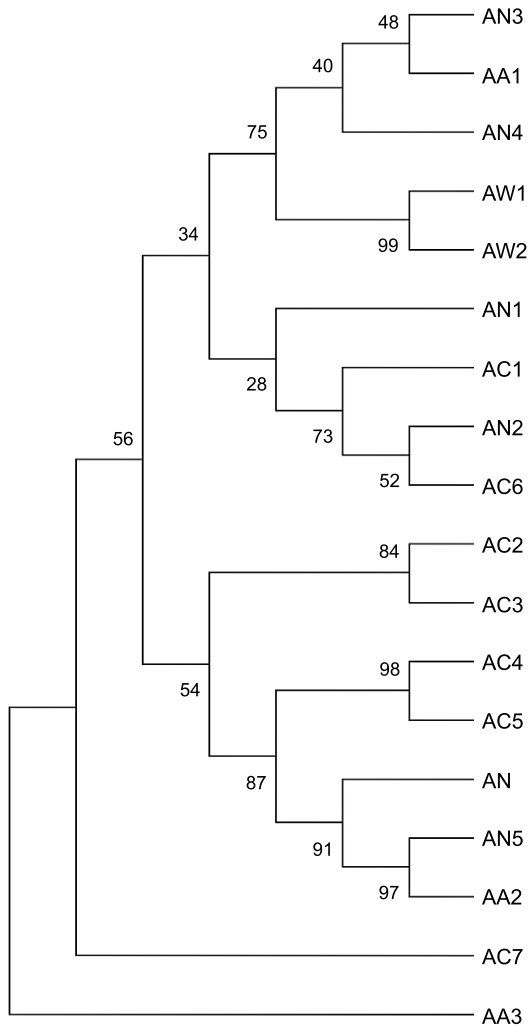

Fig. 2. Phylogenetic relationship of the polyketide synthase genes of isolated black aspergilli AN – *Aspergillus niger*; AA – *Aspergillus aculeatus*; AC – *Aspergillus carbonarius*; AW – *Aspergillus welwitschiae*

also showed that A/G and T/C transition were more frequent than C/T and G/A as evident in Table 4.

The codon-based Fisher's exact test analysis of selection of the *pks* gene of *Aspergillus* section Nigri provides a test of selection based on the comparison of the numbers of synonymous and non-synonymous substitutions between the different sequences of the *pks* gene of *Aspergillus* section Nigri. As shown in Table 5, eighteen *pks* genes of different black aspergilli

Table 3

Maximum likelihood estimates of transition-transversion bias of the *pks* genes of black aspergilli

Nucleotide	NF [%]	ML	NOP	R
A	25	–13279.996	2215	1.01
T	25	–	–	–
C	25	–	–	–
G	25	–	–	–

Explanation: A/T/C/G – adenine/thymine/cytosine/guanine; nt – nucleotide; NF [%] – nucleotide frequency [%]; ML – maximum likelihood estimate; NOP – number of positions in the final dataset; R – transition – transversion bias ratio

Table 4

Maximum composite likelihood estimate of the pattern of nucleotide substitution

Nucleotide	A	T	C	G
A	–	5.61	6.42	**13.73**
T	5.45	–	**14.27**	6.72
C	5.45	**12.46**	–	6.72
G	**11.14**	5.61	6.42	–

were compared in terms of numbers of synonymous and non-synonymous substitutions and none has a probability value of less than 0.05 and so the null hypothesis of neutral evolution is accepted.

The codon-based *Z*-test of neutral evolution connotes significant association between some (18) *pks* gene sequence combinations (AN-5 and AN-1, AA2 and AN-1, AC4 and AN-1, AN-5 and AN-2, AA2 and AN-2, AA2 and AN-5, AA2 and AN-6, AA3 and AA1, AC1 and AN-5, AC1 and AN-6, AC4 and AN-1, AC4 and AN-2, AC5 and AN-5, AC5 and AC4, AC6 and AN-3, AW1 and AA3, AW2 and AN-5, AC7 and AC3, AC7 and AW2) and neutral evolution. Consequently, larger combinations of the compared pks genes (133 compared sequences) have probability value above 0.05 and so the hypothesis upholding neutral evolution was accepted.

As denoted in Table 7, eighteen *pks* gene sequences were used for the Tajima's neutrality test analysis and the number of segregating sites was estimated to be 960 with a nucleotide diversity of 0.136116. The Tajima's neutrality test value was calculated to be 0.343161.

Table 5

Codon-based Fisher's exact test of selection analysis of the *pks* gene of *Aspergillus* section Nigri

	AN-1	AN-2	AN-3	AN-4	AN-5	AN-6	AA1	AA2	AA3	AC1	AC2	AC3	AC4	AC5	AC6	AW1	AW2	AC7
AN-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-2	0.5699	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-3	1.0000	1.0000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-4	1.0000	1.0000	1.0000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-5	1.0000	1.0000	1.0000	1.0000	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-6	1.0000	1.0000	1.0000	1.0000	1.0000	-	-	-	-	-	-	-	-	-	-	-	-	-
AA1	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	-	-	-	-	-	-	-	-	-	-	-	-
AA2	1.0000	1.0000	0.4978	1.0000	1.0000	1.0000	1.0000	-	-	-	-	-	-	-	-	-	-	-
AA3	0.0916	0.0765	0.3934	1.0000	0.3334	0.3585	1.0000	1.0000	-	-	-	-	-	-	-	-	-	-
AC1	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.5427	0.3511	-	-	-	-	-	-	-	-	-
AC2	1.0000	1.0000	0.4835	0.4939	0.3409	0.1806	1.0000	0.3016	1.0000	1.0000	-	-	-	-	-	-	-	-
AC3	0.3082	0.3082	1.0000	0.2990	0.3130	0.4282	1.0000	1.0000	0.0969	1.0000	0.3039	-	-	-	-	-	-	-
AC4	1.0000	1.0000	0.4059	0.4376	1.0000	1.0000	1.0000	0.3017	1.0000	0.0691	1.0000	1.0000	-	-	-	-	-	-
AC5	0.4299	0.7536	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.0765	1.0000	1.0000	0.2769	1.0000	-	-	-	-	-
AC6	1.0000	1.0000	1.0000	1.0000	0.4140	0.5537	0.1263	0.5318	1.0000	0.1675	0.3353	1.0000	0.4341	1.0000	-	-	-	-
AW1	0.5160	0.5160	0.5617	0.5035	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.5160	0.1119	-	-	-
AW2	1.0000	1.0000	1.0000	0.4725	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.5529	1.0000	1.0000	1.0000	1.0000	-	-
AC7	1.0000	1.0000	0.4068	0.3281	0.1786	0.2634	0.4218	1.0000	0.1981	0.3833	1.0000	1.0000	1.0000	1.0000	0.3275	0.5495	1.0000	-

Table 6

Codon-based *Z*-test of neutral evolution

	AN-1	AN-2	AN-3	AN-4	AN-5	AN-6	AA1	AA2	AA3	AC1	AC2	AC3	AC4	AC5	AC6	AW1	AW2	AC7
AN-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-2	0.15909	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-3	0.95258	0.95258	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-4	0.81312	0.76647	0.78959	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-5	0.04969	0.03379	0.21231	0.32868	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AN-6	0.08189	0.05723	0.68136	0.27448	0.26507	-	-	-	-	-	-	-	-	-	-	-	-	-
AA1	0.88668	0.88668	0.79289	0.91495	0.13838	0.22067	-	-	-	-	-	-	-	-	-	-	-	-
AA2	0.03680	0.04689	0.74956	0.66034	0.00308	0.01963	0.26376	-	-	-	-	-	-	-	-	-	-	-
AA3	0.15112	0.11714	0.80903	0.13547	0.56306	0.50131	0.00163	0.61756	-	-	-	-	-	-	-	-	-	-
AC1	0.20376	0.20376	0.28737	0.48497	0.00275	0.01515	0.38261	0.97770	0.47602	-	-	-	-	-	-	-	-	-
AC2	0.62513	0.62513	0.66884	0.91360	0.43145	0.25601	0.62846	0.57635	0.39325	0.85779	-	-	-	-	-	-	-	-
AC3	0.58510	0.55329	0.45772	0.51898	0.49520	0.64930	0.16700	0.08101	0.19669	0.55988	0.54052	-	-	-	-	-	-	-
AC4	0.00989	0.01425	0.63329	0.77937	0.30383	0.25739	0.61149	0.50292	0.27942	0.09582	0.24279	0.55616	-	-	-	-	-	-
AC5	0.08494	0.31886	0.95258	0.76647	0.03379	0.05723	0.88668	0.05922	0.11714	0.20376	0.62513	0.55607	0.01425	-	-	-	-	-
AC6	0.47302	0.51015	0.04895	0.99756	0.67162	0.91803	0.14739	0.98629	0.15108	0.30313	0.45709	0.40229	0.76890	0.51015	-	-	-	-
AW1	0.82840	0.82840	0.86637	0.80788	0.19985	0.30666	0.93931	0.50472	0.00238	0.60737	0.52485	0.07296	0.71451	0.82840	0.17288	-	-	-
AW2	0.86702	0.86702	0.50512	0.80952	0.02046	0.06823	0.12559	0.26784	0.62405	0.09398	0.92236	0.96817	0.24293	0.86702	0.19428	0.23000	-	-
AC7	0.84043	0.79427	0.78483	0.69284	0.32365	0.56448	0.81746	0.09029	0.30573	0.50258	0.42202	0.03023	0.46021	0.79427	0.69143	0.95436	0.00160	-

Table 7

Results from Tajima's Neutrality Test

m	*S*	*Ps*	*Θ*	*π*	*D*
18	960	0.433409	0.126007	0.136116	0.343161

Explanation: *m* – number of sequences; *n* – total number of sites; *S* – number of segregating sites; p_s –*S*/*n*; *Θ* – p_s/a_1; *π* – nucleotide diversity; *D* – Tajima test statistic

Discussion and Conclusion

The importance of polyketide synthases (PKSs) in the biosynthesis of polyketides such as OTA and the subsequent association of the polyketide synthase (*pks*) genes in OTA production has been well documented (GEISEN 2004, GALLO et al. 2009, KAROLEWIEZ and GEISEN 2005). In this study, four different black aspergilli namely, *Aspergillus niger, Aspergillus aculeatus, Aspergillus carbonarius* and *Aspergillus welwitschiae* were isolated from different cassava powders sampled in Ogun state, Nigeria. These black aspergilli besides their economic importance, also play important role in ochratoxin A production that contaminates several agricultural products including grape-derived products like coffee and cocoa (CABAÑES et al. 2002, SAMSON et al. 2004), dried cassava powders (THOMAS et al. 2014), among other foods. The *Aspergillus niger* isolated in this study has been regarded to as one of the most important pollutant species of foods in the world, especially in postharvest fruit products (fresh or dried), some vegetables, and several crops and is by far the most common *Aspergillus* species responsible for postharvest decay of fresh foods (PLASCENCIA-JATOMEA et al. 2014). *Aspergillus aculeatus* on the other hand causes post-harvest dry rot of tomatoes (FAJOLA 1979) and, with other members of Section Nigri, is involved in *Aspergillus* bunch rot of grapes (JARVIS and TRAQUAIR 1984, LEONG et al. 2004). *Aspergillus carbonarius* which is one of the three most ochratoxigenic species among the aspergilli, together with *Aspergillus niger* and *Aspergillus ochraceus* (TANIWAKI et al. 2003) was also found in this study. *Aspergillus welwitschiae* have also been found as food-contaminating species (PERRONE and GALLO 2017). The significant contamination of the analyzed cassava powders with different species of black aspergilli may not be unconnected to the practices associated with the production, processing and post processing handling of these cassava products which includes spreading on the floor, mats, displaying in open bowl in the markets as well as the use of various packaging materials to haul finished products from rural to urban areas (OGIEHOR and IKENEBOMEH 2005).

Of the twenty-four toxigenic strains, six *Aspergillus niger*, three *Aspergillus aculeatus,* seven *Aspergillus carbonarius* and two *Aspergillus*

welwitschiae were delineated. This observation is not completely unexpected because *Aspergillus* species have been labelled as a major OTA-producing species in the tropics (ZHANG et al. 2016), of which this study sites belong to. Ogun State which is one of the thirty-six states in Nigeria has a tropical monsoon climate that supports the distribution characteristics of OTA-producing strains in different climates (HOCKING 2006). The fact that all of the black aspergilli screened were phenotypically positive also exhibits discrete amplification of the polyketide synthase gene further reinforces the involvement of this gene in the biosynthesis of ochratoxin A (GALLO et al. 2014, WANG et al. 2015, ZHANG et al. 2016).

The maximum likelihood estimate of the transition-transversion bias of the *pks* genes which was estimated to be 99% contradicts the general observation that transitions are more common than transversions (STOLTZFUS and NORRIS 2016, LYONS and LAURING 2017). The reason for this observation may be linked to the molecular mechanisms generating this type of mutational substitution (THOMAS et al. 2019, POPOOLA et al. 2024). However, it is believed that if all of the possible pair-wise nucleotide substitutions occur at the same rate, the transition-transversion ratio is expected to be 50%, because there are twice as many possible transversions as transitions (SZCZEPANOWSKA and TRIFUNOVIC 2020). For example, the transition-transversion ratio for *Drosophila* nuclear genome, humans and primate mitochondrial DNA are 2, 4 and 15 respectively (MEYER et al. 1999) and these therefore make transitions highly sensitive to mutational saturation because transitions will saturate more rapidly than transversions (PURVIS and BROMHAM 1997).

The maximum composite likelihood estimate of the pattern of nucleotide substitution reveals that transitions were more frequent than transversions at the polyketide synthase genes of *Aspergillus* section Nigri. The results showed that A/G and T/C transition were more frequent than C/T and G/A. This observation further emphasizes the widely reported significant bias of nucleotide base substitution toward transition than transversion (LUO et al. 2016). The reason for this may be due to differences in the conformation of purines and pyrimidines because purines have a bicyclic structure while pyrimidines have a single ring structure and these therefore make the process of transversion probably more complicated than the process of transition (SMITH and SIMMONDS 1997, ZHANG and GERSTEIN 2003). Our findings are however, contrary to, those observed in grasshopper pseudogenes where no significant difference was observed between transition and transversion rates (KELLER et al. 2007). The explanation for this may be that transition–transversion bias differs according to the region of the genome as well as the type of organism. The codon-based Fisher's exact test analysis of selection of the *pks* gene of *Aspergillus* section Nigri provided

a test of selection based on the comparison of the numbers of synonymous and non-synonymous substitutions between the different sequences of the *pks* gene of *Aspergillus* section Nigri to depict a probability value of less than 0.05 and so the null hypothesis of neutral evolution was accepted. This finding was further supported by the results of Tajima's Neutrality Test ($D = 0.343161$), which is very close to zero. A value near zero indicates that the observed nucleotide variation does not significantly deviate from the expectations under neutral evolution, suggesting the absence of strong selection pressures (either purifying or positive). Since the p-value was greater than 0.05, the null hypothesis of neutral evolution could not be rejected, implying that the mutations in the pks gene are likely evolving neutrally (KORNELIUSSEN et al. 2013). Our results therefore showed population evolving at drift–mutation equilibrium with no evidence of selection (SHRINER et al. 2004). This observation was also strongly supported by the codon-based *Z*-test of neutral evolution which rejects the alternative hypothesis ($dN > dS$) that states that positive selection is the major driving force of evolution of the *pks* genes in favor of the null hypothesis of strict-neutrality ($dN = dS$). These results therefore suggest that transition and neutral selection drives the evolution of the *pks* gene of *Aspergillus* section Nigri.

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