Transition-transversion mutations in the polyketide synthase gene of *Aspergillus* section *Nigri*

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\textbf{A B S T R A C T}

This study determined the transition-transversion mutation in the \textit{pks} gene of *Aspergillus* section *Nigri* in order to gain insight into the patterns of nucleotide base substitution and the process of molecular evolution using standard recommended techniques. Results obtained depict frequent occurrence of transition (23 ± 0.96) than transversion (11.37 ± 1.38) \((p < 0.05\) with C/T being the most frequently observed transitional base substitution and C/A the most frequently occurring transversional base change. The number of single base insertions (56 ± 1.00) were significantly higher than the observed single base deletions (38 ± 2.00) \((p < 0.05\) while varying degrees of two or more base deletions and insertions were also observed both inside and outside the open reading frame. The maximum likelihood value estimated for the \textit{pks} gene was calculated to be -9458.80 in 423 positions of the final dataset while the transition-transversion ratio was estimated to be 0.50. The Tajima's neutrality test approaches seven \((7)\) with the nucleotide diversity estimated to be approximately 65%. Evolutionary test depicts positive selection as ratio of non synonymous to synonymous divergence was found to be greater than ratio of the number of non synonymous to synonymous polymorphisms. The proportion of substitution driven by positive selection was calculated to be approximately 96.2%. This research therefore provides an insight into the understanding of \textit{pks} gene mutation patterns as some of the observed indels resulted in frame shift mutations.

\section{1. Introduction}

Ochratoxigenic moulds are ubiquitous contaminants of pre and post harvest food commodities (Sanchis and Magan, 2004), including the ready to eat foods (Takahashi-Ando et al., 2004; Cavaliere et al., 2006; Trucksess et al., 2006; Thomas et al., 2014). These organisms attract particular attention through the damage they do to plants, humans and animals as a result of their toxic secondary metabolites known as ochratoxins. In Italy, ochratoxigenic moulds were reported to be involved in the contamination of over 57% of marketed foods with ochratoxin A (OTA) (MAFF, 1997; Wolff et al., 2000) and 22% of the sampled cocoa products (Tafuri et al., 2004). In other studies, higher number of black Aspergilli were correlated with higher levels of ochratoxin A (Belli et al., 2005; Kapetanakou et al., 2009; Thomas et al., 2014) while this secondary metabolite (OTA) has been reported to be encoded by the polyketide synthase (\textit{pks}) gene. This \textit{pks} gene was the first gene to be involved in ochratoxin A biosynthesis (O’Callaghan et al., 2003) and has been reported to have five identified \textit{pks} fragments in Aspergillus carbonarius (Atoui et al., 2006). The polyketide synthase gene is a multifunctional enzyme consisting of different domains, including a \(β\)-ketoacylsynthase (KS), an acyltransferase (AT) and an acyl carrier protein (ACP) domain which repeatedly catalyze the condensation of a two carbon molecule to the growing chain (Kroken et al., 2003). One major constraint to controlling these ochratoxigenic organisms and their metabolites despite their ubiquitous presence in several foods, is in their diversity in terms of random amplified polymorphic DNA (RAPD) haplotypes, expressed transcriptional factors and outer membrane proteins (Kolawole et al., 2015a; Thomas et al., 2015, 2017a) among others. Sometimes, inter specific diversity occurs in the internal transcribed spacer regions with limited intra specific variability in *Aspergillus* spp (Kolawole et al., 2015b). Another factor that enhances diversity in microorganisms is continuous accumulation of mutation in their coding region (Luo et al., 2016) and the possibility of some organisms reshuffling their genome (Thomas et al., 2014).

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2. Materials and methods

2.1. Sources of Aspergillus section Nigri

The Aspergillus section Nigri used in this study was isolated from processed Manihot esculenta (garri) collected from the four geopolitical zones of Ogun State, Nigeria in our previous study (Thomas et al., 2017a, b). The four geopolitical zones sampled were Yewa, Egba, Remo and Ijebu. The isolates laboratory code, the species of the Aspergillus section Nigri and the origin of the isolates were properly delineated in Table 1.

2.2. DNA isolation, amplification, sequencing and mutation discovery

Each specimen (fungal isolate) was stirred directly into 200 ml sterile saline and extracted using a QiAamp DNA mini kit (Qiagen) (Hilden, Germany) as described by the manufacturer. In brief, each sample was pre-incubated at 99 °C for 20 min and then processed as suggested by the manufacturer. After the addition of the cellular lysis buffer, the sample was pre-incubated again at 99 °C for 10 min. The extracted DNA was amplified by PCR using degenerate primer pairs KAF1/KAR2 (KAF1: 5’GARKSICAYGGIAEGGAC-3’; KAR2 5’-CCAYTGICCCYCGGICG-TRAA-3’) encompassing highly conserved amino acid motifs EA/CHGTGT (KS domains) and FTGGQGAW (AT domains) present on fungal type I PKSs (Amnuaykanjanasin et al., 2005). PCRs were performed in 0.2 ml reaction tubes in a final volume of 50 ml containing 10ng of DNA, 1.5µl Platinum Taq DNA polymerase (Invitrogen) (Waltman, USA)), 200 mM each of dATP, dGTP and dCTP, 400 mM dUTP (instead of dTTP), 20 mM Tris/HCl (pH 8.4), 50 mM MgCl2, 0.4 mM each primer and 1 U uracil-N-glycosylase. The amplification was performed with hierarchical reaction steps at 95 °C for 5 min to allow uracil-N-glycosylase activity and an additional hold at 99 °C for 5 min for Taq activation, followed by 35 cycles at 95 °C for 1 min, with a final extension step at 72 °C for 10 min.

Table 1

| LC | Species | Origin |
|----|---------|--------|
| Y1 | Aspergillus niger | Ilaro |
| Y2 | Aspergillus niger | Owode-yewa |
| Y3 | Aspergillus niger | Oke Odan |
| Y4 | Aspergillus niger | Idiriko |
| Y5 | Aspergillus niger | Aiyetoro |
| Y6 | Aspergillus carbonarius | Iseki |
| Y7 | Aspergillus carbonarius | Joga Orile |
| Y8 | Aspergillus carbonarius | Ilubu |
| Y9 | Aspergillus carbonarius | Igbogita |
| Y10 | Aspergillus carbonarius | Oja Odan |
| E1 | Aspergillus niger | Owode egba |
| E2 | Aspergillus niger | Owode egba |
| E3 | Aspergillus niger | Obantoko |
| E4 | Aspergillus niger | Itoin |
| E5 | Aspergillus niger | Itoin |
| E6 | Aspergillus carbonarius | Orile Imo |
| E7 | Aspergillus carbonarius | Kuto |
| E8 | Aspergillus carbonarius | Kuto |
| E9 | Aspergillus carbonarius | Owode egba |
| E10 | Aspergillus carbonarius | Owode egba |
| A1 | Aspergillus carbonarius | Sagamu/Falawo |
| A2 | Aspergillus carbonarius | Sagamu/Awelowo |
| A3 | Aspergillus carbonarius | Ikenne |
| A4 | Aspergillus carbonarius | Ikenne |
| A5 | Aspergillus carbonarius | Itoin |
| A6 | Aspergillus carbonarius | Itoin |
| A7 | Aspergillus carbonarius | Itoin |
| A8 | Aspergillus carbonarius | Itoin |
| A9 | Aspergillus carbonarius | Itoin |
| A10 | Aspergillus carbonarius | Itoin |
| I1 | Aspergillus niger | Ago-iwoye/garage |
| I2 | Aspergillus niger | Ago-iwoye/main mkt |
| I3 | Aspergillus niger | Ijebu-Igbp |
| I4 | Aspergillus niger | Ijebu-Igbp |
| I5 | Aspergillus niger | Ijebu-Igbp |
| I6 | Aspergillus carbonarius | Ijebu-Ode-Oja oba |
| I7 | Aspergillus carbonarius | Ijebu-Ode-Oja oba |
| I8 | Aspergillus carbonarius | Ijebu-Ode-Oja oba |
| I9 | Aspergillus carbonarius | Ijebu-Ode-Oja oba |
| I10 | Aspergillus carbonarius | Ijebu-Ode-Oja oba |

LC = Laboratory code, Y1-Y10 = Isolate from Yewa zone, E1-E10 = Isolate from Remo zone, I1-I10 = Isolate from Ijebu zone, E1-E10 = Isolate from Egba zone.

Understanding these have been made possible by the advent of massive parallel sequence technology which has enabled the sequencing of large number of genomes in a short period of time (Mardis, 2008) and this next generation sequencing (NGS) technology has made genome-wide identification of polymorphisms among genetic variants of the same or related species possible. Recently, it was used for comparative genomic analysis in whole-genome sequencing studies to identify insertion-deletion mutations (indels) and SPNs in a variety of organisms, including Chilo suppressalis and humans (Qi et al., 2009). Understanding these have been made possible by the advent of massive parallel sequence technology which has enabled the sequencing of large number of genomes in a short period of time (Mardis, 2008) and this next generation sequencing (NGS) technology has made genome-wide identification of polymorphisms among genetic variants of the same or related species possible. Recently, it was used for comparative genomic analysis in whole-genome sequencing studies to identify insertion-deletion mutations (indels) and SPNs in a variety of organisms, including Chilo suppressalis and humans (Qi et al., 2009).
nonsynonymous/synonymous ratios ($dN/dS = \omega$) and changes in amino acid properties as well as ratio of the number of non synonymous to synonymous polymorphisms. Neutrality test was also carried out on the sequenced isolates to understand the pattern of selection. The substitution pattern and rates were estimated under the Kimura (1981) parameter model. The forty nucleotide sequences belonging to the Aspergillus section Nigri were used for computing the maximum likelihood values. Codon positions considered were the first, second, third and the non coding region. All positions containing gaps and missing data were eliminated while evolutionary analyses were conducted in MEGA X. The maximum likelihood of gamma parameter for sites rate were estimated under the Juke and Cantor (1969) Model. The molecular phylogenetic analysis was inferred using the maximum likelihood method based on the Hasegawa-Kishino-Yano model. The initial trees for the heuristic search were computed automatically by Neighbor-Join and Bio NJ algorithms to a matrix of pair wise distances estimated using the maximum composite likelihood approach and then the topology with superior logarthmic-likelihood value were selected. The tree was drawn to scale with branch length measured in the number of substitutions per site. The McDonald-Kreitman test was used for quantifying the frequency of positive selection as follows:

$$\alpha = 1 - \text{DnPn/DsPs}, \text{where Dn/Ds} = \text{ratio of non synonymous to synonymous divergence}$$
$$\text{Pn/Ps} = \text{ratio of the number of non synonymous to synonymous polymorphisms}.$$ 

The interpretation of the McDonald Kreitman test was as follows:

- $\text{Dn/Ds} < \text{Pn/Ps} = \text{negative selection}$
- $\text{Dn/Ds} > \text{Pn/Ps} = \text{positive selection}$
- $\alpha = \text{proportion of substitution driven by positive selection}$

3. Results

The number of single base transitional substitution (23 ± 0.96) was found to be significantly higher than transversional base change (11.37 ± 1.38) (p < 0.05) (Fig. 1). The most frequently occurring transitional base change was C/T followed by T/C base substitution (p < 0.05) (Fig. 2). When the number of single base substitution in transversion was analyzed, the most frequently substituted base was C/A with a mean substitution of 18.00 ± 1.00, while the least substituted base was T/G (6.00 ± 0.00) (Fvalue = 20.55, p < 0.05) (Fig. 3). There were 15 single base deletions and 23 two or more deletions to give a total of 38 deletions. As for insertions, 20 single insertions and 36 two or more insertions were observed. Some of these indels resulted in frame shift mutation (Fig. 4). Table 2 depicts one or more base deletions and insertions in the pks gene of Aspergillus section Nigri. As shown in this table, three single base deletions each was observed in and outside the open reading frame of Aspergillus niger polyketide synthase gene (pks). Four and two single base deletions were observed in and outside the Aspergillus carbonarius open reading frame. Also, varying degrees of two or more base deletions were observed in their open reading frame. As per the insertions, three different nucleotide base substitutions each was seen inside and outside of the open reading frame except for Aspergillus niger that had a base substitution inside the ORF. Both Aspergillus niger and Aspergillus carbonarius had varying degrees of two or more base insertions. The maximum likelihood estimate of transition-transversion bias is depicted in Table 3. As shown in this table, all the nucleotides have frequency of 25% each with the maximum likelihood value estimated to be -9458.80 in 423 positions of the final dataset. The transition-transversion ratio was estimated to be 0.50. The estimated value of the shape parameter for the discrete distribution was 16.54 with the mean evolutionary rate found to be 0.68, 0.86, 0.98, 1.12 and 1.37 substitution per site while the maximum likelihood estimate of gamma parameter for site rates was -9457.00. The Tajima’s neutrality test was found to be approximately 6.6 (Table 4). The McDonald test for pks gene (Table 5) revealed that the ratio of non synonymous to synonymous divergence is greater than the ratio of the non synonymous to synonymous polymorphisms while a proportion of substitution driven by positive selection was estimated to be approximately 96.2%. The phylogenetic analysis of

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**Fig. 1.** The number of single base substitution in transition and transversion.

**Fig. 2.** Single base substitution in transition.

**Fig. 3.** The number of single base substitution in transversion.

**Fig. 4.** Total number of insertions and deletions.
The importance of mutation in biological evolution has been documented (Pascarella and Argos, 1992; Benner et al., 1993; Wolf et al., 2007). In this study, transition occurred more frequently than transversion. The predominant presence of singleton indels in this study is in line with what other researchers have documented (Ajawatanawong and Baldauf, 2013). These indels are useful in examining general patterns of indel evolution. One possible explanation of this strong and wide-spread insertion bias is a high background (neutral) bias toward DNA insertion across eukaryotes (Ajawatanawong and Baldauf, 2013). The fact that C/T and C/A were the most frequently substituted nucleotide bases in transition and transversion respectively is contradictory to what was earlier documented in the transposons of Chilo suppressalis (Luo et al., 2016). This may be opining that microbial mutation 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 varying degree of indels observed in the open reading frame of the Aspergillus section Nigri polyketide synthase gene is an indication that this region of the genome is gradually accumulating mutations and so providing insight into how their proteins function (Chan et al., 1998; Moxon and Thaler, 1997). The predominant presence of singleton indels in this study is in line with what other researchers have documented (Ajawatanawong and Baldauf, 2013). These indels are useful in examining general patterns of indel evolution. One possible explanation of this strong and wide-spread insertion bias is a high background (neutral) bias toward DNA insertion across eukaryotes (Ajawatanawong and Baldauf, 2013). The varying degree of indels observed in the open reading frame of the Aspergillus section Nigri polyketide synthase gene is an indication that this region of the genome is gradually accumulating mutations and so providing insight into how their proteins function (Chan et al., 1998; Moxon and Thaler, 1997). The predominant presence of singleton indels in this study is in line with what other researchers have documented (Ajawatanawong and Baldauf, 2013). These indels are useful in examining general patterns of indel evolution. One possible explanation of this strong and wide-spread insertion bias is a high background (neutral) bias toward DNA insertion across eukaryotes (Ajawatanawong and Baldauf, 2013) .

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### Table 2
One base or more deletions and insertions in the pks gene of *Aspergillus* section *Nigri*.

| Pks gene          | Region            | 1 base deletion | 2 or more base | 1 base insertion | 2 or more base insertions |
|-------------------|-------------------|-----------------|----------------|-----------------|--------------------------|
| *Aspergillus niger* | Inside the ORF    | 214,216/T       | 210,216/5nt    | 69,71/T         | 217,225/ATTCTTAG         |
|                   |Outside the ORF    | 216,218/A       | 217,220/2nt    | 91,102/11nt     | 16,18/A                  |
|                   |                   | 220,222/C       |                | 19,20/A         | 69,73/ATT                |
| *Aspergillus carbonarius* | Inside the ORF | 89,91/A        | 89,91/2nt     | 27,25/A         | 30,35/CTATTA            |
|                   |Outside the ORF    | 284,286/T       | 502,600/98nt  | 616,618/T       | 523,536/TTATCTATCTA      |
|                   |                   | 289,291/A       | 287,289/T     | 518,520/C       |                          |
|                   |                   | 600,602/A       |                | 616,618/T       |                          |
|                   |                   | 697,699/G       |                |                |                          |
|                   |                   | 44,46/A         | 38,44/ATTCT    | 17,19/A         | 35,38/G                  |
|                   |                   | 119,121/A       | 27,25/A       | 32,34/G         |                          |

The numbers indicate the position of a nucleotide in sequences. The form of numbers_numbers means that the deletions or insertions occurred between these two position.

### Table 3
Maximum Likelihood estimate of Transition-Transversion Bias.

| Nucleotide | Nucleotide frequency (%) | ML | NOP | R  |
|------------|--------------------------|----|-----|----|
| A          | 25                       | -9458.0 | 423 | 0.50 |
| T          | 25                       |       |     |    |
| C          | 25                       |       |     |    |
| G          | 25                       |       |     |    |

% = percentage, ML = Maximum likelihood estimate. NOP = Number of positions in the final dataset. R = Transition-Transversion bias ratio.

### Table 4
Results from Tajima’s neutrality test.

| m   | 40 | n   | 423 |
|-----|----|-----|-----|
| S   | 1.00 |
| Ps  | 0.65 |
| π   | 0.64 |

m = number of sequences, n = total number of sites, S = number of segregating sites. Ps = S/n, θ = Ps/a1, π = nucleotide diversity, D = Tajima’s test of statistic.

### Table 5
McDonald test table for pks gene showing numbers of fixed differences and polymorphic sites between and within *Aspergillus niger* and *Aspergillus carbonarius*.

| Mutation    | Differences | Polymorphisms | Dn/Ds | Pn/Ps | α  |
|-------------|-------------|---------------|-------|-------|----|
| Non synonymous | 189         | 62            | 0.37  | 0.1   | 0.962 |
| Synonymous   | 504         | 618           |       |       |    |

Dn/Ds = ratio of non synonymous to synonymous divergence, Pn/Ps = ratio of non synonymous to synonymous polymorphisms, α = proportion of substitution driven by positive selection.

the pks gene is depicted in Fig. 5.

### 4. Discussion

The importance of mutation in biological evolution has been documented (Pascarella and Argos, 1992; Benner et al., 1993; Wolf et al., 2007). In this study, transition occurred more frequently than transversion to further emphasize 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 that 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 fact that C/T and C/A were the most frequently substituted nucleotide bases in transition and transversion respectively is contradictory to what was earlier documented in the transposons of Chilo suppressalis (Luo et al., 2016). This may be opining that microbial mutation 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 varying degree of indels observed in the open reading frame of the Aspergillus section Nigri polyketide synthase gene is an indication that this region of the genome is gradually accumulating mutations and so providing insight into how their proteins function (Chan et al., 1998; Moxon and Thaler, 1997). The predominant presence of singleton indels in this study is in line with what other researchers have documented (Ajawatanawong and Baldauf, 2013). These indels are useful in examining general patterns of indel evolution. One possible explanation of this strong and wide-spread insertion bias is a high background (neutral) bias toward DNA insertion across eukaryotes (Ajawatanawong and Baldauf, 2013).

The varying degree of indels observed in the open reading frame of the Aspergillus section Nigri polyketide synthase gene is an indication that this region of the genome is gradually accumulating mutations and so providing insight into how their proteins function (Chan et al., 2007; Romero et al., 2006; Zhang et al., 2011a, b) and evolve (Wolf et al., 2007). Indel studies generally have also led to the discovery of useful experimental (Podlaha and Zhang, 2003) and drug targets (Cherkasov et al., 2006) as well as powerful taxon diagnostics and phylogenetic markers (Inagaki et al., 2002; Atkinson and Baldauf, 2011).
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Benner, S.A., Cohen, M.A., Gonnet, G.H., 1993. Empirical and structural models for overall signature of positive selection (Zeng et al., 2007). The af (Booker et al. of positive selection in the

5. Conclusion

The results obtained from this study have shown that Aspergillus section Nigri is gradually accumulating mutation in their pks gene and therefore suggest possible evolution of new strain from this isolates in the nearest future. Our study however shows that although pks gene is relatively conserved, mutation in this gene was mostly maintained by positive selection. Therefore, part of the variation found in pks gene may be explained by adaptive changes promoted by positive selection.

Declarations

Author contribution statement

Benjamin Thoha Thomas: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Liusu Adebayo Ogunkami, Bamidele Abiodun Iwalokun: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Omolara Dorcas Popoola: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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