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

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ABSTRACT

This study determined the transition-transversion mutation in the *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 *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 *pks* gene mutation patterns as some of the observed indels resulted in frame shift mutations.

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 (Bellí 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 (*pks*) gene. This *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 *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 continous 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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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 SNPs in a variety of organisms, including viruses (Spatz and Rue, 2008), bacteria (Holt et al., 2008; Kotewicz et al., 2008; Manning et al., 2008), plants (Novaes et al., 2008) and humans (Qi et al., 2009).

These mutations have been reported for their involvement in changing wild type allele of a gene to a different allele in a process of forward mutation and/or in reverse mutation where mutant strains are reversed to wild type strains. This phenomenon is a weak force for changing allele frequency but a strong force for introducing new alleles and an ultimate source of new alleles in fungal pathogen populations, thus creating new genotypes within the clonal lineages and so heritable changes in DNA base sequences may be classified according to how they change DNA conformation through substitution processes that occur when a base at a certain position in one strand of DNA molecules is replaced by another one in the other strands (Hartwell et al., 2011).

Knowledge of the patterns and frequencies of substitution mutation in genomes is important in studying molecular evolution and understanding the molecular basis of substitution mutations (Topal and Fresco, 1976; Kimura, 1981; Gojobori et al., 1982). Generally in evolutionary theory, mutations are considered random with respect to the adaptive value to the organism. However, it has long been recognized that this does not necessarily imply randomness in other respects. Many studies have indicated that some nucleotides are more mutable than the others (Zhang and Gerstein, 2003; Zhao and Boerwinkle, 2002). For instance, mutations were more biased towards GC in the transposons of Chilo suppressalis than any other nucleotides while the most frequently occurring base changes were A-G and T-C (Luo et al., 2016) in this same organism. Biased patterns of nucleotide substitution are sources of non-randomness in the production of heritable variation (Petrov and Hartl, 1999). This study was therefore aimed at determining the transition-transversion mutations in the *pks* gene of *Aspergillus* section *Nigri* in order to gain insight into the molecular basis of evolution and their substitution patterns.

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.

Table 1
Sources of *Aspergillus* section *Nigri*.

LC	Species	Origin
Y_1	*Aspergillus niger*	Ilaro
Y_2	*Aspergillus niger*	Owode-yewa
Y_3	*Aspergillus niger*	Oke Odan
Y_4	*Aspergillus niger*	Idiroko
Y_5	*Aspergillus niger*	Aiyetoro
Y_6	*Aspergillus carbonarius*	Imeko
Y_7	*Aspergillus carbonarius*	Joga Orile
Y_8	*Aspergillus carbonarius*	Ihubo
Y_9	*Aspergillus carbonarius*	Igbogita
Y_{10}	*Aspergillus carbonarius*	Oja Odan
E_1	*Aspergillus niger*	Owode egba
E_2	*Aspergillus niger*	Owode egba
E_3	*Aspergillus niger*	Obantoko
E_4	*Aspergillus niger*	Itosin
E_5	*Aspergillus niger*	Itosin
E_6	*Aspergillus carbonarius*	Orile Imo
E_7	*Aspergillus carbonarius*	Kuto
E_8	*Aspergillus carbonarius*	Kuto
E_9	*Aspergillus carbonarius*	Owode egba
E_{10}	*Aspergillus carbonarius*	Obantoko
R_1	*Aspergillus niger*	Sagamu/Falawo
R_2	*Aspergillus niger*	Sagamu/Awolowo
R_3	*Aspergillus niger*	Sagamu/Sabo
R_4	*Aspergillus niger*	Ilisan
R_5	*Aspergillus niger*	Ode-Remo
R_6	*Aspergillus carbonarius*	Ode-lemo
R_7	*Aspergillus carbonarius*	Ikenne
R_8	*Aspergillus carbonarius*	Ikenne
R_9	*Aspergillus carbonarius*	Irolu
R_{10}	*Aspergillus carbonarius*	Irolu
I_1	*Aspergillus niger*	Ago-iwoye/garage
I_2	*Aspergillus niger*	Ago-iwoye/main mkt
I_3	*Aspergillus niger*	Ijebu –Igbo
I_4	*Aspergillus niger*	Ijebu - Igbo
I_5	*Aspergillus niger*	Oru
I_6	*Aspergillus carbonarius*	Mamu
I_7	*Aspergillus carbonarius*	Oru
I_8	*Aspergillus carbonarius*	Ijebu Ode/Oja oba
I_9	*Aspergillus carbonarius*	Ilese
I_{10}	*Aspergillus carbonarius*	Ilaporu

LC = Laboratory code, Y_1–Y_{10} = Isolate from Yewa zone, R_I-R_{10} = Isolate from Remo zone, I_1–I_{10} = Isolate from Ijebu zone, E_1-E_{10} = Isolate from Egba zone.

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 incubated again at 99 °C for 10 min. The extracted DNA was amplified by PCR using degenerate primer pairs KAF1/KAR2 (KAF1: 5′GARKSICAYGGIACIGGIAC-3'; KAR2 5′-CCAYTGIGCICCYTGICCIG-TRAA-3′) encompassing highly conserved amino acid motifs EA/CHGTGT (KS domains) and FTGQGAQW (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, 1.5μPlatinum 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 $MgCl_2$, 0.4 mM each primer and 1 U uracil-N-glycosylase. The amplification reaction included a hold at 50 °C for 5 min to allow uracil-N-glycosylase activity and an additional hold at 95 °C for 5 min for Taq activation, followed by 35 cycles at 95 °C for 30s, 62 °C for 1 min and 72 °C for 2 min, with a final extension step at 72 °C for 5 min. 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 analyzed using the BLAST database and assigned to the reference isolate sequences with the highest bit score. Mutation was discovered in the *pks* gene of *Aspergillus* section *Nigri* by aligning the sequenced isolates from this study in MEGA software. Using this software, mutation occurring multiple times at the same position was taken as one. Single base and multiple base substitutions were analyzed separately. The mechanism of mutation was also categorized and identified appropriately.

2.3. Estimation of selection, substitution pattern and phylogenetic analysis

Analysis of selection on *pks gene* was performed with hierarchical strategy. Initially, we evaluated evolutionary patterns using McDonald Kreitman test with sequences from both *Aspergillus niger* and *Aspergillus carbonarius*. In this context, we analyzed the data considering variation in

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 logarithmic-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:

α = 1-DnPn/DsPs, where Dn/Ds = ratio of non synonymous to synonymous divergence
Pn/Ps = ratio of the number of non synonymous to synonymous polymorphisms.

The interpretation of the McDonald Kreitman test was as follows:

Dn/Ds < Pn/Ps = negative selection
Dn/Ds > Pn/Ps = positive selection
α = 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

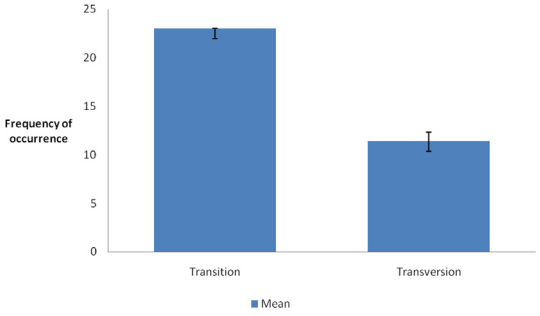

Fig. 1. The number of single base substitution in transition and transversion.

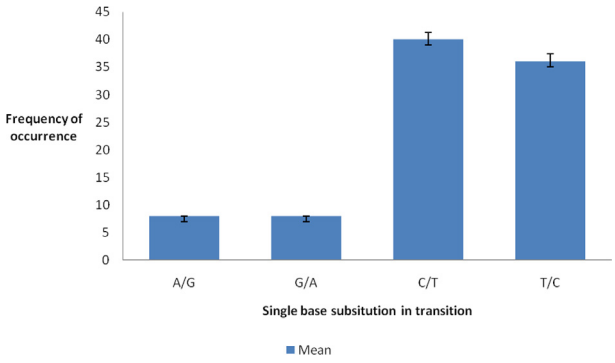

Fig. 2. Single base substitution in transition.

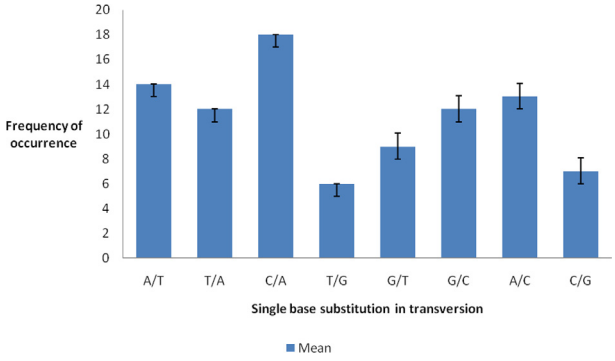

Fig. 3. The number of single base substitution in transversion.

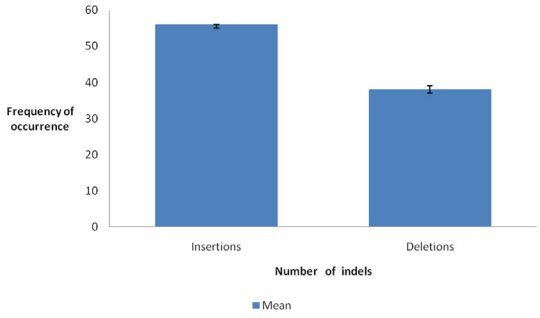

Fig. 4. Total number of insertions and deletions.

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

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

Pks gene	Region	1base deletion	2 or more base	1base insertion	2 or more base insertions
Aspergillus niger	Inside the ORF	214_216/T 216_218/A 220_222/C	210_216/5nt 217_220/2nt	69_71/T	217_225/ATTCTAG
	Outside the ORF	67_69/A 78_80/A 89_91/A	91_102/11nt	16_18/A 19_20/A 27_29/A	69_73/ATT 91_94/AT 80_89/CCTATTGA
Aspergillus carbonariu	Inside the ORF	284_286/T 289_291/A 600-602/A 697-699/G	502_600/98nt	287_289/T 518_520/C 616-618/T	523_536/TTATCCTATCTA
	Outside the ORF	44_46/A 119_121/A	38_44/ATTCT	17_19/A 27_29/A 32_34/G	35_38/GA

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	S	Ps	π	Θ
40	423	1.00	0.65	6.64

m = number of sequences, n = total number of sites, S = number of segregating sites.
Ps = S/n, Θ = Ps/a_1, 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.

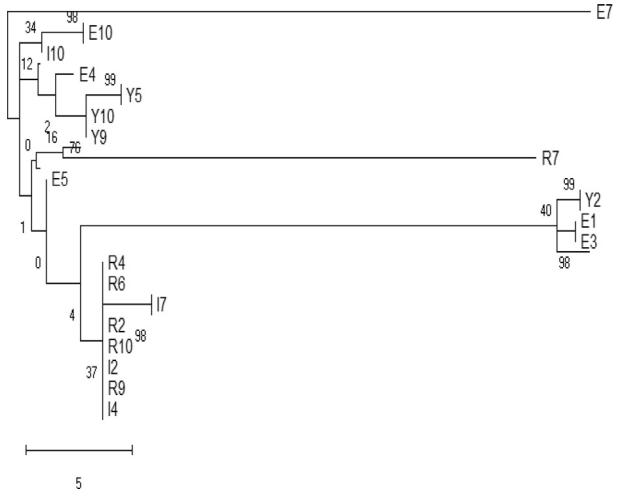

Fig. 5. Phylogenetic analysis of the *pks* genes of *Aspergillus* section *Nigri*.

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 suppresalis* (Luo et al., 2016). This may be opining that microbial mutation rates vary not only among species but also among different genes of the same individual and even within the same gene at different point in time (Drake 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 gene 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).

The fact that the ratio of non synonymous to synonymous polymorphisms emphasizes that pks gene evolve under a positive selection

(Booker et al., 2017) and considering the fact that the *pks* gene is critical to the production of ochratoxin A in *Aspergillus* section *Nigri,* mutation in this region of gene may affect production of this secondary metabolites. The positive value of the Tajima's test of neutrality is compatible with the overall signature of positive selection (Zeng et al., 2007). The affirmation of positive selection in the *pks* gene of the studied *Aspergillus* section *Nigri* was supported by a considerable number of non synonymous substitution involving radical changes in amino acid properties despite the rare presence of non synonymous substitution (Sobrinho and de Brito; 2012).

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.

Liasu Adebayo Ogunkanmi, 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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