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GENETIC DIVERSITY OF OCHRATOXIGENIC *ASPERGILLUS* SECTION *NIGRI*, USING RAPD AND VCG TECHNIQUES

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ABSTRACT

This study evaluates the genetic diversity of ochratoxigenic *Aspergillus* section *Nigri* using RAPD and VCG techniques. Results obtained revealed OPX 07 as the most informative of the tested RAPD markers generating 12 polymorphic bands while the least bands were generated by OPR 19. Of the 40 *Aspergillus* section *Nigri* (20 each of *Aspergillus niger* and *Aspergillus carbonarius*), 22 VCGs and 27 RAPD haplotypes were delineated. The two techniques demonstrated similar resolution except in few cases where the RAPD technique further sub divided some VCGs into simpler haplotypes. The average percentage of variable VCG and RAPD reactions were 25 and 50% in that order of sequence while 75 and 50% of the isolates were resolved as same isolates by these techniques respectively. It was also found that the Simpson index of genetic diversity approached one for the isolates from the four geopolitical zones of Ogun State, Nigeria with the mean genetic diversity within isolates (G_L) contributing significantly approximately 89% of the total diversity observed within the isolates ($F=22.23$, $p<0.05$). The remaining 11% of variation could only be allotted to diversity among isolates (G_S). On the whole, the total genetic diversity (H_T) was found to be approximately 48%. In conclusion, RAPD markers provided better resolution than the classical VCG typing technique.

Keywords; Genetic Diversity, Ochratoxigenic *Aspergillus*, RAPD and VCG .

DIVERSITÉ GÉNÉTIQUE DES OCHRATOXIGENIC ASPERGILLUS LA SECTION NIGRI, EN UTILISANT RAPD ET TECHNIQUES VCG

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Résumé

Cette étude évalue la diversité génétique des ochratoxigenic Aspergillus la section Nigri en utilisant RAPD et techniques VCG. 07 résultats obtenus ont révélé que l'OPX plus informatif de l'essai de RAPD générer 12 bandes polymorphes alors que les bandes ont été générés par le RPT 19. Du 40 Aspergillus la section Nigri (20 chacun d'Aspergillus niger et Aspergillus carbonarius), 22 et 27 haplotypes VCGs RAPD ont été délimités. Les deux techniques ont démontré une résolution similaire, sauf dans quelques cas où la technique RAPD plus de sous-divisé certains haplotypes VCGs en éléments plus simples. Le pourcentage moyen de VCG variable et les réactions ont été 25 RAPD et 50 % dans l'ordre de séquence alors que 75 et 50 % des isolats ont été réglées comme mêmes isolats par ces techniques respectivement. Il a également été constaté que l'indice de diversité génétique Simpson approché une pour les isolats provenant des quatre zones géopolitiques de l'Etat d'Ogun, au Nigeria avec la diversité génétique moyenne dans les isolats (GL), contribuant de manière significative à environ 89 % du total de la diversité observée dans les isolats ($F = 22,23$, $p <0,05$). Les 11 autres % de variation ne peut être attribuée à la diversité parmi les isolats (GS). Dans l'ensemble, la diversité génétique totale (HT) est d'environ 48 %. En conclusion, les marqueurs RAPD a fourni la meilleure résolution que la technique classique VCG.

Mots-clés ; la diversité génétique, l'Aspergillus, RAPD et Ochratoxigenic VCG .

INTRODUCTION

The diversity of filamentous fungi in every spheres of life ranging from agriculture through medicine and biotechnology to the environment has long been documented. In medicine, their role in causing various ailments such as asthma, cystic fibrosis and invasive aspergillosis cannot be overemphasized [1-3]. These organisms has since been indicted in agriculture for their ubiquitous contamination of pre and post harvest food commodities including the ready to eat foods [4-7]. Their significance in different environmental hazards such as flooding, Hurricane Katrina among others has also been recorded both in the United State and Denmark [8]. According to Hawksworth [9 and 10], less is known about the variation of fungal diversity and composition along different gradients such as latitude, altitude, productivity and salinity. On the other hand, Fisher [11] and Rosenzweig [12] pointed out that the distribution of biodiversity along these environmental gradients has been of long-standing interest to ecologists and that most of what is known about how biodiversity varies along environmental gradients stems from research on plants and animals.

As early as 1975, the central goal of using sound techniques and statistics in biodiversity research for delineating the abundance, distribution and processes of species coexistence at different spatial and temporal scales has been pointed out [13] in order not to underestimate the biodiversity scores. Before now, genetic diversity in filamentous fungi is majorly carried out using the vegetative compatibility technique which involves characterization of fungi based on heterokaryon formation between different fungal individuals. Heterokaryon formation is an important component of many fungal life cycles and may serve as the first step in the parasexual cycle and the transmission of hypovirulent factors such as dsRNA [14]

This technique however is still being used by many researchers to decipher genetic diversity in phytopathogenic moulds even in the presence of growing advances in the field of mycology and science. Our research however was aimed at comparing the ability of this vegetative compatibility technique in typing ochratoxigenic *Aspergillus* section *Nigri* relative to the random amplified polymorphic DNA (RAPD) method.

MATERIALS AND METHODS

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. The four geopolitical zones sampled were Yewa, Egba, Remo and Ijebu. The isolates laboratory code, the specie of the *Aspergillus* section *Nigri*, the origin of the isolates, the VCG assignments as well as the RAPD haplotypes were properly delineated in table 1

Vegetative Compatibility Grouping (VCGs)

Recovering of nit mutants
Fungal cultures were grown in solid M3 culture medium as explained earlier [15]. A mycelial fragment was then sub cultured from the grown isolates in the solid M3 culture medium to the center of the petri dishes containing minimal medium with 1.5% potassium chlorate (MMC), using the technique described by Brooker *et al*.[16]. The Petri dishes were incubated at room temperature and examined after 14 to 21 days for sector verification. Fragments from these cultures were transferred to petri dishes containing a minimal medium and sodium nitrate ($NaNO_3$). The isolates that presented poor growth colonies in this medium and little mycelial production were considered to be nit mutants, while those presenting dense aerial mycelium growth, or wild-type, were discarded [17]

Phenotypic classification of the nit mutants

For the phenotypic classification of the nit mutants, mycelial fragments from the same petri dishes containing minimal medium were selected and transferred to the center of dishes containing basal medium (BM) supplemented with sodium nitrite (0,5 g/L), sodium nitrate (2,0 g/L), hypoxanthine (0,5 g/L), ammonium tartrate (1,0 g/L) and uric acid (0,2 g/L). Each nit mutant was transferred to three dishes (100 x 15 mm) with each of the aforementioned media; totaling 15 dishes for each isolate. These dishes were maintained in an incubator at 25^{o}C for a period of 21 days. Two evaluations were carried out: the former on the 14th and the latter on the 21st day. The phenotypic classification was done according to the mycelial growth of the mutants in media supplemented with different sources of nitrogen: BM + sodium nitrate (MM), BM + sodium nitrite (NM), BM + hypoxanthine (HM), BM + ammonium tartrate (AM) and BM + uric acid (UAM). Media supplemented with sodium nitrate and ammonium tartrate were used as negative and positive controls respectively [17]

Heterokaryon formation and VCG classification
The heterokaryons were formed when the colonies of different nit mutants were confronted in petri dishes (100 x 15 mm) at a 1 cm distance in nitrate medium (MM). The dishes were stored in a greenhouse, in the dark at 25^{o}C. After 14 to 21 days, they were analyzed on a weekly basis to verify the existence of heterokaryons. In order to carry out the confrontations, combinations were done whereby each dish contained five different isolates and a mycelial fragment was taken from a determined isolate from the center of the dish and in the other four isolates from the margins, i.e., each mutant selected from a determined isolate was paired with all the other mutants from the other isolates so as to determine the number of complementary groups to which the distinct nit mutants belonged [14].

Random amplified polymorphic DNA analysis (RAPD)

DNA Isolation, Primer Screening and PCR Amplification
DNA was isolated and purified based on the manufacturer's instruction of DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). A total of 26 RAPD primers were screened and optimized for polymorphisms and annealing temperature (Tm) using the isolated ochratoxigenic moulds. Optimal PCR amplification across the isolated organisms was achieved with annealing temperature between 40 and 36^{o}C. Seven primers that shows good and clear polymorphism with the PCR products were therefore used for the study. These primers include OPX 07(GAGCGAGGCT), OPR 16 (CTCTGCGCGT), OPR 19 (CCTCCTCATC), OPR 11(GTAGCCGTCT), OPV 06 (GAACGGACTC), OPA 01(CAGGCCCTTC) and OPA 04(AATCGGGCTG). Each 25 µl PCR reaction contained 12.5 µl master mix (2×) (0.05 45 units/µl *Taq* DNA polymerase in reaction buffer; 4 mM $Mgcl_2$, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP), 40 pmol oligonucleotide primer and 1 µg of template DNA. The DNA was first denatured for 2 minutes at 95^{o}C followed by 40 cycles of 15sec denaturation at 95^{o}C, the annealing temperature was progressively decreased by 0.5^{o}C every cycles from 40^{o}C to 35^{o}C for 1 min and 2 min elongation at 72 ^{o}C with a final elongation for 2 min. The amplified products were separated on 3% TBE agarose gels stained with ethidium bromide and viewed under a UV Transilluminator. The analyses of the amplification products were done manually with consideration of the number of fragments and repeatability of the reaction following the procedures described by Roodt *et al*.[18]. Each lane of amplified product was checked manually and scored for presence (+) or absence (-) of fragments.

RESULTS

The table 1 below shows the vegetative compatibility assignment and the random amplified polymorphic DNA haplotypes of the studied *Aspergillus* section *Nigri*. Out of the total 40 *Aspergillus* section *Nigri* analyzed (20 each of *Aspergillus niger and Aspergillus carbonarius*), 22 different VCGs and 27 RAPD haplotypes were found. In our data set, the two methods provided similar resolution except in few cases, where RAPD markers divided some VCG into different haplotypes (Fig.1).

TABLE 1: *ASPERGILLUS* SECTION NIGRI; THEIR ASSIGNMENT AND RAPD HAPLOTYPES

LC	Species	Origin	VA	RH
Y_1	*Aspergillus niger*	Ilaro	Z_1	1
Y_2	*Aspergillus niger*	Owode-yewa	Z_1	1
Y_3	*Aspergillus niger*	Oke Odan	Z_2	2
Y_4	*Aspergillus niger*	Idiroko	Z_2	3
Y_5	*Aspergillus niger*	Aiyetoro	Z_{19}	3
Y_6	*Aspergillus carbonarius*	Imeko	Z_5	4
Y_7	*Aspergillus carbonarius*	Joga Orile	Z_8	5
Y_8	*Aspergillus carbonarius*	Ihubo	Z_8	5
Y_9	*Aspergillus carbonarius*	Igbogita	Z_3	6
Y_{10}	*Aspergillus carbonarius*	Oja Odan	Z_3	7
E_1	*Aspergillus niger*	Owode egba	Z_7	8
E_2	*Aspergillus niger*	Owode egba	Z_7	8
E_3	*Aspergillus niger*	Obantoko	Z_7	8
E_4	*Aspergillus niger*	Itosin	Z_7	9
E_5	*Aspergillus niger*	Itosin	Z_7	10
E_6	*Aspergillus carbonarius*	Orile Imo	Z_{22}	11
E_7	*Aspergillus carbonarius*	Kuto	Z_6	12
E_8	*Aspergillus carbonarius*	Kuto	Z_{10}	13
E_9	*Aspergillus carbonarius*	Owode egba	Z_{10}	14
E_{10}	*Aspergillus carbonarius*	Obantoko	Z_{10}	15
R_1	*Aspergillus niger*	Sagamu/Falawo	Z_4	16
R_2	*Aspergillus niger*	Sagamu/Awolowo	Z_4	16
R_3	*Aspergillus niger*	Sagamu/Sabo	Z_4	16
R_4	*Aspergillus niger*	Ilisan	Z_9	17
R_5	*Aspergillus niger*	Ode-Remo	Z_9	17
R_6	*Aspergillus carbonarius*	Ode-lemo	Z_{11}	18
R_7	*Aspergillus carbonarius*	Ikenne	Z_{11}	18
R_8	*Aspergillus carbonarius*	Ikenne	Z_{11}	18
R_9	*Aspergillus carbonarius*	Irolu	Z_{13}	19
R_{10}	*Aspergillus carbonarius*	Irolu	Z_{21}	20
I_1	*Aspergillus niger*	Ago-iwoye/garage	Z_{16}	21
I_2	*Aspergillus niger*	Ago-iwoye/main mkt	Z_{16}	21
I_3	*Aspergillus niger*	Ijebu –Igbo	Z_{15}	22
I_4	*Aspergillus niger*	Ijebu - Igbo	Z_{15}	22
I_5	*Aspergillus niger*	Oru	Z_{17}	23
I_6	*Aspergillus carbonarius*	Mamu	Z_{20}	24
I_7	*Aspergillus carbonarius*	Oru	Z_{18}	25
I_8	*Aspergillus carbonarius*	Ijebu Ode/Oja oba	Z_{18}	25
I_9	*Aspergillus carbonarius*	Ilese	Z_{14}	26
I_{10}	*Aspergillus carbonarius*	Ilaporu	Z_{12}	27

KEY; LC = laboratory code, VA = VCG Assignment, RH = RAPD haplotypes

There were also cases where both techniques gave equal resolution to certain isolates. However, there were no cases where a RAPD haplotype was further divided by the VCG typing. In the description of the genetic variation and genetic diversity in *Aspergillus* section *Nigri* using VCG typing and RAPD markers. The ratio of VCGs classification to that of the RAPD technique was 22 to 27. The percentage of variable resolution to same resolution was 25 to 75% in VCG typing and 50 to 50 in the RAPD technique. The Simpson's index of genetic diversity approached one in all the four geo political zones of Ogun State for both techniques. However, the sum total of this diversity index for both technique was 0.51 and indifferent (table 2). Table 3 connotes the RAPD band frequencies and genetic diversity of *Aspergillus* section Nigri. The mean genetic diversity within isolates (G_L) contributes approximately 89% of the total diversity ($F=22.23$, $p<0.05$) while the remaining 11% of variation could only be allotted to diversity among isolates (G_S). On the whole, the total genetic diversity (H_T) was found to be approximately 48%.

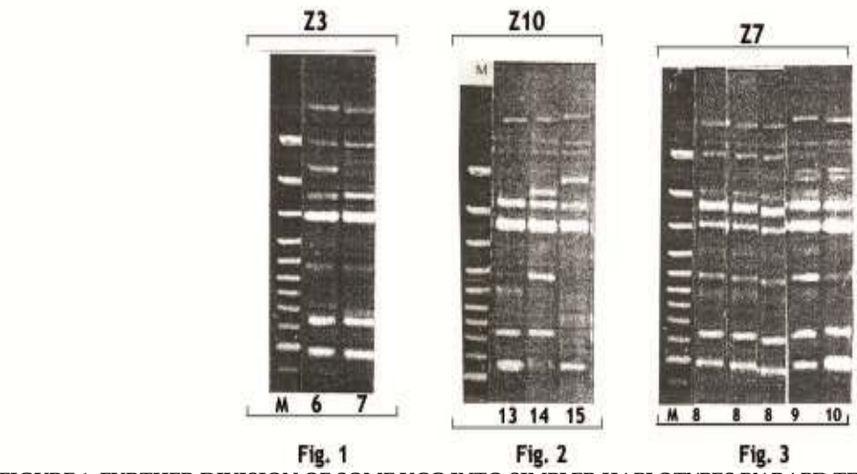

FIGURE 1: FURTHER DIVISION OF SOME VCG INTO SIMPLER HAPLOTYPES BY RAPD TECHNIQUE

TABLE 2: DESCRIPTION OF GENETIC VARIATION AND GENETIC DIVERSITY IN *ASPERGILLUS* SECTION *NIGRI* USING VCG TYPING AND RAPD MARKERS

	VCG TYPING					RAPD TECHNIQUE				
	Y	E	R	I	S_T	Y	E	R	I	S_T
Number of Isolates	10	10	10	10	40	10	10	10	10	40
Number of VCG/RAPD Haplotypes	6	4	5	7	22	7	8	5	7	27
Percentage of Variable VCG/RAPD haplotypes	20	20	20	40	25	40	70	20	40	50
Percentage of Same VCG/RAPD haplotypes	80	80	80	60	75	60	30	80	60	50
Simpson's Index of Diversity	0.87	0.93	0.91	0.81	0.51	0.8	0.7	0.9	0.8	0.51

Table 3: RAPD band frequencies and genetic diversity of *Aspergillus* section Nigri from the four geopolitical zones of Ogun State, Nigeria

RAPD	RAPD band Frequency in the studied organisms						Hier.gene diversity		
	YEWA	EGBA	Remo	Ijebu	Total (n=40)	Probability	HT	GS	GL
OPX07 – 0.1Kbp	0.40	0.20	0.00	0.00	0.15	0.40	0.20	0.02	0.98
OPX07- 0.15kbp	0.20	0.30	0.10	0.10	0.18	0.40	0.15	0.02	0.98
OPX07 – 0.2kbp	0.20	0.30	0.00	0.00	0.13	0.38	0.13	0.04	0.96
OPX 07 – 0.25kbp	0.00	0.00	0.00	0.10	0.03	0.50	0.01	0.03	0.97
OPX07 – 0.3kbp	0.10	0.20	0.20	0.40	0.23	0.40	0.25	0.03	0.97
OPX07- 0.4kbp	0.20	0.00	0.20	0.40	0.20	0.40	0.24	0.10	0.90
OPX07 – 0.5kbp	0.60	0.30	0.60	0.40	0.40	0.40	0.88	0.38	0.62
OPX07- 0.6kbp	0.20	0.20	0.20	0.30	0.25	0.37	0.26	0.22	0.78
OPX04- 0.7kbp	0.20	0.50	0.00	0.40	0.20	0.40	0.24	0.13	0.87
OPX07-0.8kbp	0.70	0.70	0.30	0.60	0.53	0.09	1.19	0.71	0.83
OPX07-0.9kbp	0.70	0.00	0.60	0.40	0.60	0.44	1.50	0.08	0.92
OPX07-1.0kbp	0.70	0.00	0.40	0.20	0.33	0.09	0.69	0.07	0.93

DISCUSSION AND CONCLUSION

The major aim of this research was to use the VCG method to genotype isolates of *Aspergillus* section Nigri relative to the RAPD technique. Our findings however depicts that the VCG typing provided similar resolution as that of the RAPD technique except in few cases where the RAPD method further subdivided some VCGs into RAPD haplotypes. This finding is not unexpected as the vegetative compatibility technique has long been documented as a reliable method for scoring diversity in phytopathogenic fungi [17]. The fact that the RAPD technique subdivided some VCGs into different haplotypes demonstrates the superiority of the latter technique. This observation is not surprising, as the vegetative compatibility techniques scores diversity based on only one marker as against a number of markers that can even be increased to meet specific needs, simply by using more primers [21]. Isolates from most locations were present on different clusters for both VCG and RAPD analyses. This might be the result of gene flow between the geopolitical zones. This is because, garri from which the isolates was obtained are displays in open bowls in markets and various packaging materials use to haul this food from rural to urban areas might exacerbate fungal contamination [22], thereby causing multiple contamination which could be an important prerequisites to evolution of a new fungus as a result of sexual or parasexual recombination. In addition, the values obtained for total gene diversity (H_T), diversity among and within isolates (GS and GL) are similar to those observed in fungi with known sexual life cycles [23, 24]. According to Grypta *et al*.[25], regional population structure of this nature is usually the result of more frequent interbreeding events among isolates within a site than between sites and is more common in diploid or dikaryotic organisim. In summary, the high level of diversity observed in this study may be due to the ability of these isolates to undergo para sexuality under controlled field conditions [26,27] and studies have assessed neither the degree to which parasexuality occurs in natural populations nor the significance of such asexual horizontal gene transfer as an adaptive mechanism relative to migration and genetic drift [28]. Any efforts taken to control fungal contamination should bear in mind the high levels of genetic diversity found from this study before any control measure can be put in place.

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