Aspergillus niger

Biodiversity along these environmental gradients stems from research on plants and animals. Biodiversity varies along environmental gradients is of longstanding interest to ecologists and 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 distribution of hypovirulent factors such as dsRNA

INTRODUCTION

The diversity of filamentous fungi in every sphere 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 have 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 distribution of hypovirulent factors such as dsRNA.
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 (gari) collected from the four geopolitical zones of Ogun State, Nigeria in our previous study. The four geopolitical zones sampled were Yewa, Egba, Remo and Ijesa. 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 media to the center of the petri dishes containing minimal medium with 1.5% potassium chloride (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 (NaNO3). 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 mycelial 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 25ºC 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 25ºC. 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 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 36ºC. Seven primers that shows good and clear polymorphism with the PCR products were therefore used for the study. These primers include OPX 07(GAGCAGGCGCT), OPR 16 (CTCTGCCCCCTT), OPR 19 (CCTCCCTCATC), OPR 11(CTAGCCCCCT), OPV 06 (GAACGGACTC), OPA 01(CAGGGCCTTC) and OPA 04(AATCGGGCTG). Each 25 µl PCR reaction contained 12.5 µl master mix (2x) (0.05 45 units/µl Taq DNA polymerase in reaction buffer; 4 mM MgCl2, 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 95ºC followed by 40 cycles of 15sec denaturation at 95ºC, the annealing temperature was progressively decreased by 0.5ºC every cycles from 40ºC to 35ºC for 1 min and 2 min elongation at 72 ºC 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).
| LC | Species                  | Origin          | VA | RH |
|----|-------------------------|-----------------|----|----|
| Y1 | Aspergillus niger       | Ilaro           | Z1 |    |
| Y2 | Aspergillus niger       | Owode-yeova     | Z2 |    |
| Y3 | Aspergillus niger       | Oke Odan        | Z3 |    |
| Y4 | Aspergillus niger       | Ihiroko         | Z4 |    |
| Y5 | Aspergillus niger       | Joga Orile      | Z5 |    |
| Y6 | Aspergillus niger       | Igbogida        | Z6 |    |
| Y7 | Aspergillus niger       | Ilogbo          | Z7 |    |
| Y8 | Aspergillus niger       | Obantoko        | Z8 |    |
| Y9 | Aspergillus niger       | Itosin          | Z9 |    |
| Y10| Aspergillus niger       | Sagamu/Falawo   | Z10|    |
| Y11| Aspergillus niger       | Sagamu/Awolowo  | Z11|    |
| Y12| Aspergillus niger       | Sagamu/Sabo     | Z12|    |
| Y13| Aspergillus niger       | Ijesi           | Z13|    |
| Y14| Aspergillus niger       | Odeiro          | Z14|    |
| Y15| Aspergillus carbonarius| Ode-Ikele       | Z15|    |
| Y16| Aspergillus carbonarius| Sagamu/Esoko    | Z16|    |
| Y17| Aspergillus carbonarius| Sagamu/Ikere    | Z17|    |
| Y18| Aspergillus carbonarius| Sagamu/Ijebu    | Z18|    |
| Y19| Aspergillus carbonarius| Sagamu/Ijelia   | Z19|    |
| Y20| Aspergillus carbonarius| Sagamu/Ijebu    | Z20|    |
| Y21| Aspergillus carbonarius| Sagamu/Ijebu    | Z21|    |
| Y22| Aspergillus carbonarius| Sagamu/Ijebu    | Z22|    |
| Y23| Aspergillus carbonarius| Sagamu/Ijebu    | Z23|    |
| Y24| Aspergillus carbonarius| Sagamu/Ijebu    | Z24|    |
| Y25| Aspergillus carbonarius| Sagamu/Ijebu    | Z25|    |
| Y26| Aspergillus carbonarius| Sagamu/Ijebu    | Z26|    |
| Y27| Aspergillus carbonarius| Sagamu/Ijebu    | Z27|    |

**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 geopolitical 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%.
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 depict 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 the ability of these isolates to undergo parasexual recombination. In addition, the values obtained for total gene diversity (Ht), 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 organisms. In summary, the high level of diversity observed in this study may be due to the ability of these isolates to undergo parasexuality 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.

REFERENCES
1. Denning DW. Invasive aspergillosis. Clin Infect Dis., 1998; 26: 781-803.
2. Taylor R, Dagenais T and Nancy P. Pathogenesis of Aspergillus fumigatus in Invasive

![image]

| Number of Isolates | 10 | 10 | 10 | 10 | 40 |
|--------------------|----|----|----|----|----|
| Number of VCG/RAPD Haplotypes | 6 | 4 | 5 | 7 | 22 |
| Percentage of Variable VCG/RAPD haplotypes | 20 | 20 | 20 | 40 | 25 |
| Percentage of Same VCG/RAPD haplotypes | 80 | 80 | 80 | 60 | 75 |
| Simpson's Index of Diversity | 0.87 | 0.93 | 0.91 | 0.81 | 0.51 |

![image]

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

| RAPD | Frequency in the studied organisms | Heter gene diversity |
|------|-----------------------------------|----------------------|
| YEWA | IGBA | Remo | Ijebu | Total (n=40) | Probability |
| OPX07-0.1Kbp | 0.40 | 0.30 | 0.30 | 0.00 | 0.30 | 0.15 | 0.40 | 0.30 | 0.98 |
| OPX07-0.15Kbp | 0.20 | 0.30 | 0.10 | 0.10 | 0.15 | 0.15 | 0.15 | 0.15 | 0.98 |
| OPX07-0.2Kbp | 0.20 | 0.30 | 0.00 | 0.00 | 0.13 | 0.13 | 0.13 | 0.13 | 0.96 |
| OPX07-0.25Kbp | 0.00 | 0.00 | 0.00 | 0.00 | 0.03 | 0.03 | 0.03 | 0.03 | 0.97 |
| OPX07-0.3Kbp | 0.10 | 0.20 | 0.20 | 0.40 | 0.23 | 0.23 | 0.23 | 0.23 | 0.97 |
| OPX07-0.4Kbp | 0.20 | 0.00 | 0.20 | 0.40 | 0.20 | 0.20 | 0.20 | 0.20 | 0.90 |
| OPX07-0.5Kbp | 0.60 | 0.30 | 0.60 | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 | 0.62 |
| OPX07-0.6Kbp | 0.20 | 0.20 | 0.20 | 0.30 | 0.25 | 0.25 | 0.25 | 0.25 | 0.78 |
| OPX07-0.7Kbp | 0.20 | 0.50 | 0.00 | 0.40 | 0.20 | 0.20 | 0.20 | 0.20 | 0.87 |
| OPX07-0.8Kbp | 0.70 | 0.70 | 0.30 | 0.60 | 0.53 | 0.53 | 0.53 | 0.53 | 0.83 |
| OPX07-0.9Kbp | 0.70 | 0.00 | 0.60 | 0.40 | 0.60 | 0.60 | 0.60 | 0.60 | 0.92 |
| OPX07-1.0Kbp | 0.70 | 0.00 | 0.40 | 0.20 | 0.33 | 0.33 | 0.33 | 0.33 | 0.93 |
prospective multicenter surveillance program. Med. Mycol. 2008;46(S1): S49-S58.

4. Sanchis V, Magan N. Environmental profiles for growth and mycotoxin production. In: Magan NM, Olsen (eds.) Mycotoxins in food: Detection and control. Cambridge: DC/Woodhead Publishing Limited. 2004;174-189.

5. Takahashi-Ando M, Ohsata S, Shibata T, Hamamaoto H, Yamagunich I and Kimura M. Metabolism of zearalenone genetically modified organism expressing the detoxification gene from Clonostachys rosea. Appl. Environ. Microbiol. 2004;70(6):3239-3245.

6. Cavaliere G, Fogia P, Pastorini E, Samperi R and Lagana A. A liquid chromatography/tandem mass spectrometric confirmatory method for determining aflatoxin M1 in cow milk comparison between electrospray and atmospheric pressure photoionization sources. Journal of Chromatography. 2006; 1101: 69-78.

7. Trucksess M, Weaver C, Oles C, Dovido KK and Rader J. Determination of aflatoxins and ochratoxins A in ginseng and other botanical roots by immune affinity column clean up and liquid chromatography with fluorescence detection. JAOAC. 2006; 89(3):624-630.

8. Bennett JW. The moulds of Katrina. Update (NYAcadSci) Jan/Feb., 2006; pp: 6-9.

9. Hawksworth DL. The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycol Res. 2001;105:1422-1432.

10. Hawksworth DL. Fungal diversity and its implications for genetic resource collections. Stud Mycol. 2004; 50: 9-17.

11. Fisher AG. Latitudinal variations in organic diversity. Evolution. 1981;14:64-81.

12. Rosenzweig M. Species Diversity in Space and Time. Cambridge University Press: Cambridge, England. 1995; 20-29.

13. May RM. Patterns of species abundance and diversity. In Ecology and Evolution of Communities. ed. M. L. Cody & J.M. Diamond. 1975; 81-120. Cambridge, MA: Belknap Press of Harvard University Press.

14. Leslie JF. Fungal vegetative compatibility. Annual review of Phytopathology. 1993; 31:127-151.

15. Junqueira NTV, Chaves GM, Zambolim L, Rometo R, Da S and Gasparotto L. Isolamento cultivo e esporulação de Mycrocylus ulei, agente etiológico do mal das folhas da seringueira. Revista Ceres. 1984; 31: 322-331.

16. Brooker NL, Leslie JF and Dickman MB. Nitrate non-utilizing mutants of Colletotrichum and their use in studies of vegetative compatibility and genetic relatedness. Phytopathology 1991; 81: 672-676.

17. Leslie JF, Summerell BA and Bullock S. The Fusarium laboratory manual. Blackwell Publishers, USA.2006.

18. Roote R, Spies JJ and Burger TH. Preliminary DNA fingerprinting of the turfgrass Cynodon dactylon (Poacaeae Chloridoideae). Botulalia. 2002; 82:117-122.

19. Tamura K, Dudley J, Nei M and Kumar S. MEGA 4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution. 2011; 24:1596-1599.

20. Hammer O, Harper DAT and Ryan PD. PAST: Paleontological statistic software package for education and data analysis. Paleontologia Electronica. 2001; 4 (1):1-9.

21. Pekarek E, Jacobson K and Donovan A. 2006. High levels of genetic variation exist in Aspergillus niger populations infecting Welwitschia mirabilis hook. J Hered. 2006; 97(3):270-278.

22. Ogiehor IS and Ikepebomeh MJ. The effect of different packaging materials on the shelf stability of garri. African Journal of Biotechnology. 2005; 523:2414-2416.

23. Gosselin I, Jobidon R and Bernier L. Genetic variability and structure of Canadian populations of Clonostachys rosea, a potential biophyticide. Mol Ecol. 1999; 8:113-122.

24. Johannesson H, Gustafsson M and Stenlid J. Local population structure of the wood decay ascomycete Didinia loculata. Mycologia. 2001;93:440-446.

25. Gryta H, Debaud JC and Marmeise R. Population dynamics of the symbiotic mushroom Helyella cydonioporum: mycelial persistence and inbreeding. Heredity. 2000; 84:294-302.

26. Zeigler RS, Scott RP, Leung H, Bordeos AA, Kumar J and Nelson RJ. Evidence of parasexual exchange of DNA in the rice blast fungus; challenges its exclusive clonality. Phytopathology 1997; 87(5):284-294.

27. Souza-Paccola EA, Fa’varo LCL, Casela CR and Paccola-Meirelles LD. Genetic recombination in Colletotrichum sublineolum. J Phytopathol. 2003;151:329-334.

28. Correll JC and Gordon TR. Population structure of Ascomycetes and Deuteromycetes. In: Structure and dynamics of fungal populations (Worrall JJ, ed). Dordrecht, The Netherlands: Kluwer Academic Publishers. 1999; 227-250.