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IDENTIFICATION FINGERPRINT TO ASPERGILLUS NIGER ISOLATES USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

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ABSTRACT

The present study was aimed to identify of *Aspergillus niger* isolates isolated from different sources (soil, peanut, milk and factory waste water) by traditional methods and find the DNA fingerprint of isolates by using RAPD technique. Identification of twenty four *Aspergillus niger* isolate by using traditional methods (macroscopic and microscopically). Genomic DNA of each isolates was extracted at a final concentration of 115-959µg / 2-3 g of wet mycelium, and at a purity of 1.7-1.9. Each DNA sample was amplified with each of 12 primers and the products were resolved electrophoretically on 1.2% agarose gel, stained with ethidium bromide and photographed under UV. Five Primer failed to support amplification, while another seven primers succeeded in that. RAPD analysis fingerprinted nineteen of the twenty four isolates through marker bands with one or more of the 7 primers.

KEYWORDS: *Aspergillus niger*, DNA, RAPD, Fingerprinting.

INTRODUCTION

The genus *Aspergillus*, comprising of more than 180 species, is economically the most important group of filamentous fungi. While some of the members of this group are food spoilage, disease causing.[1, 2, 3] The others, especially *A. niger* are rendered safe for food and drug administration.[4] *A. niger* is of special interest to modern biotechnologists owing to its exploration for secondary metabolites, mycotoxins, plant growth regulators, citric acid, heterologous and homologous proteins and various commercial enzyme production.[5] *Aspergillus niger* is one of the most important microorganisms used in biotechnology. It has already been in use for decades to produce many extracellular enzymes that are considered

GRAS (Generally Regarded As Safe) by the FDA (Food and Drug Administration of the United States of America).[6] Different molecular techniques are frequently being used to evaluate genomic diversity in fungal isolates. RAPD markers are very useful for the determination of genetic diversity, taxonomic identifications and paternity analysis.[7] RAPD markers typically have high overall variability and so can be useful for detecting genetic differences within species as well.[8, 9]

MATERIALS AND METHODS ISOLATION

Isolation of fungi from: Soil and milk

Series dilutions method were used for isolations fungi from soil and milk by taken 5g from each soil and milk samples individually, and put into flask (100ml) contain 50 ml sterile distilled water and shaken for making suspensions and dilutions, after that, was taken 1ml from dilution (of each soil and milk) and spread on autoclaved PDA plates (90mm in diameter were filled with 20ml medium), and incubated at 28±2 °C, after 3 days the growing colonies of fungi isolates were recultured on PDA plates individually and incubated at 28±2°C for 5 days for identification.

Seeds

Fifty seeds (per each sample) were surface-disinfected with 1% sodium hypochlorite solution for 1 min and then rinsed in sterile distilled water three times. Five seeds were then placed onto the surface of agar plates containing potato dextrose agar (PDA. All plates were incubated for 3 days at 28±2°C, after 3 days the growing colonies of fungi isolates were recultured on PDA plates individually and incubated at 28±2°C for 5 days for identification.[10]

Factory waste water

Fungi were isolated from factory waste water by a series of dilution. Where, were taken 5ml from collected samples and put into flask (100ml) contain 50 ml sterile distilled water and shaken for making suspensions, after that, was taken 1ml from dilution (of each soil and milk) and spread on autoclaved PDA plates (90mm in diameter were filled with 20ml medium), and incubated at 28±2 °C, after 3 days the growing colonies of fungi isolates were recultured on PDA plates individually and incubated at 28±2 °C for 5 days for identification.

Fungal DNA

For DNA isolation cultures were inoculated on the PD broth medium and flasks were put on the shaker at 28± 2°C for 3 days. Culture media were filtered and mycelia were used for genomic DNA extraction by using genomic DNA reagent kit (Geneaid Biotech Ltd., Taiwan, Cat # GR100). Genomic DNA was isolated from 2-3 g mycelium. The purified DNA samples were stored at -20˚C for subsequent downstream studies.

Primer selection and RAPD assay

Twelve of primers random sequence were used (Bioneer) in a lyophilized form and were dissolved in sterile deionizer distilled water to give a final concentration of (10pmol/µl) as recommended by provider the primers were tested in this study show table 1. The 50 µL RAPD mixture contained PCR buffer (10x), MgCl2 (1.5 mM), dNTPs (0.2 mM), primer (25 pmol/µL), taq polymerase (2.5 U), genomic DNA (1.0 µg). PCR tubes with 50 µL reaction mixture were placed in the PCR thermocycler (Gene Amp Biosysytem-9700, Applied Bio-system) and following temperature cycling conditions were programmed; initial denaturation at 94˚C for 5 min, followed by 40 cycle denaturation at 94oC for 1 min, annealing at (35oC) for 1 min, primer extension at 72˚C for 1 min and final extension temperature 72˚C for 5 min. Twelve micro liter of PCR products were analyzed by electrophoresis in a 1.2% agarose gels at 5 Volt/cm for 2 hour in 0.1xTBE buffer, agarose gels were stained with ethidium bromide 1.2 µg/ml for 2h. The 100bp DNA ladder (100 2,000) bp (Bioneer) was used as a molecular size marker. After electrophoresis, images of gels were captured using Gel Documentation System (Consort - Belgium).

Table 1: Numbers and sequences of the RAPD primers used.

Primer name	Sequence
OPA-09	3¯ - GGGTAACGCC - 5¯
OPA-12	3¯ - TCGGCGATAG - 5¯
OPA-13	3¯ - CAGCACCCAC - 5¯
OPA-18	3¯ - AGGTGACCGT - 5¯
OPB-05	3¯ - TGCGCCCTTC - 5¯
OPB-10	3¯ - CTGCTGGGAC - 5¯
OPB-17	3¯ - AGGGAACGAG - 5¯
OPG-04	3¯ - AGCGTGTCTG - 5¯
OPG-11	3¯ - TGCCCGTCGT - 5¯
OPG-17	3¯ - ACGACCGACA - 5¯
OPH-01	3¯ - GGTCGGAGAA - 5¯
OPH-03	3¯ - AGACGTCCAC - 5¯

Data analysis

Molecular Weight Estimation

In this step we used ladder from Bioneer company 2 kbp (which consist of 13 bands from 100 to 2,000 bp Bioneer). It own molecular weight were known, then curve drawing. The molecular weight of the band of ladder on the Y axis and the distance in the gel on the X axis (from the well to the beginning of each band), measured the distance of the sample on the Agarose gel. Then volume will be strait on the curve from the sample and from the intersection cross volume on the Y axis as.[11]

Analysis of RAPD specific primer product

The band that produced from the Amplification has been calculated from the images of the electrophoresis on the Agarose gel since the signal (1) mean found of the band and (0) mean the abscens of the band with calculate molecular weight. Estimation the polymorphism percentage of for RAPD primers Data generated from the detection of polymorphic fragments were analyzed. The amplification profile of all the used varieties for any given primer was scored as "1" with the presence of each band and was scored as "0" with the absence of the same band of the same size in other isolates.[12] Only major bands consistently amplified were scored. Polymorphism of each primer was calculated based on the following formula:

Polymorphism % = (Np/Nt)×100

(Np)= the number of polymorphic bands of random primer (Nt)= the total number of bands of the same primer.

Estimation the efficiency of primers and the discriminatory power

Efficiency discriminatory power of each primer calculated according to the formula below:

efficiency of primer = total number of primer bands / total number of all primer bands according to.[13]

Primer efficiency ranged between (0-1).

Discrimination power= (total number of primer bands / total number of all primer bands) ×100%.[14]

RESULTS AND DISCUSSION

Twenty two isolates of *Aspergillus niger* were isolated from 78 samples from different sources (10 soil, 45 peanut, 18 milk, 5 factory waste water)

Fungus identification

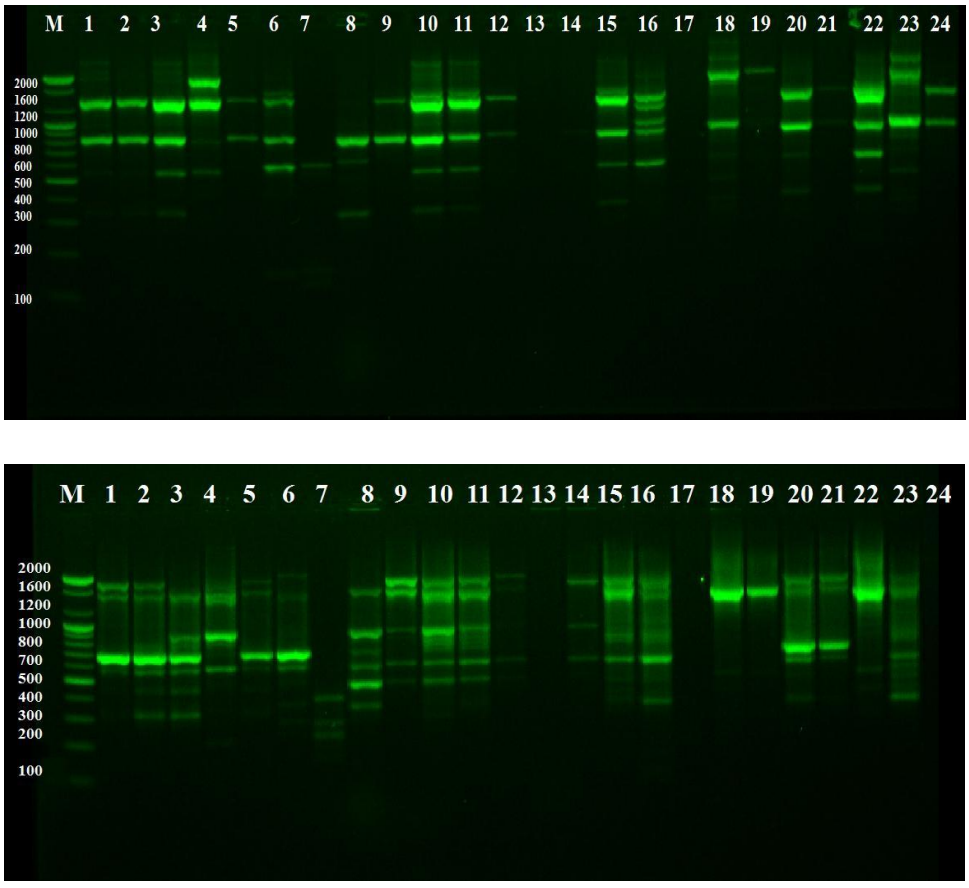
Pure cultures of the isolated fungi were identified on the bases of morphological and microscopically characteristics according to the key of.[15,16]

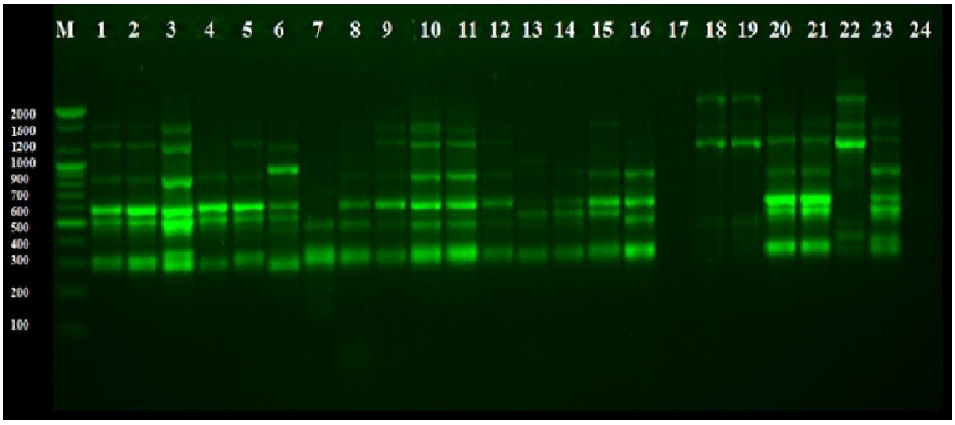
DNA extraction from fresh mycelium of *Aspergillus niger*

The DNA was extracted efficiently by using commercial kit (Geneaid–Taiwan). Purity and concentration of DNA were measured using the nanoodroop. The yield of the DNA extracted from the *A. niger* isolates was in range of (115-959) µg per gram with purity of (1.7-1.9).

RAPD-PCR Analysis

Twelve primers were used in this study, five primers (OPA-09, OPA-13, OPG-04, OPG-11, and OPH-01) did not amplify the DNA, and hence, they were eliminated from the analysis, while another seven primers succeeded in that. Figure 4.




M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
2000
1600
1200
1000
900
700
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500
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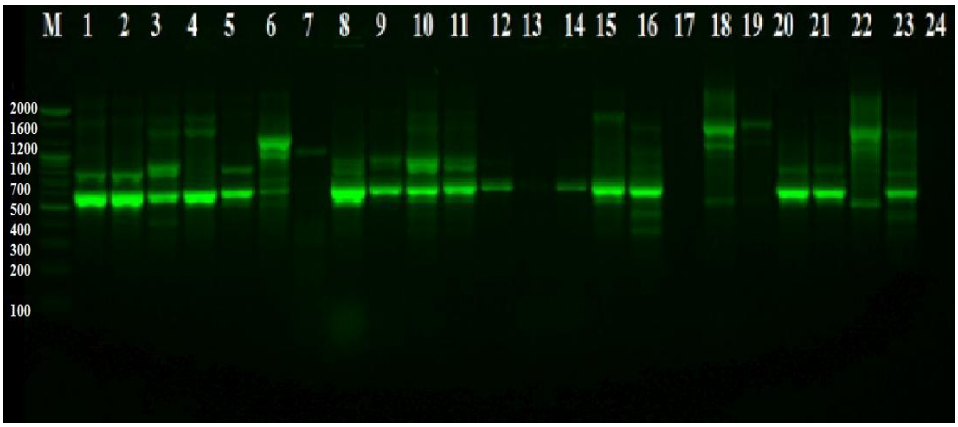
300
200
100


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
2000
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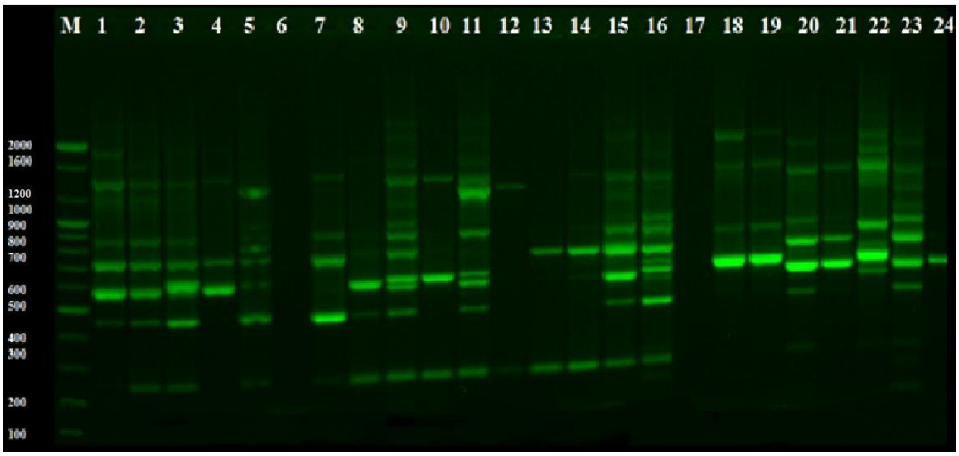
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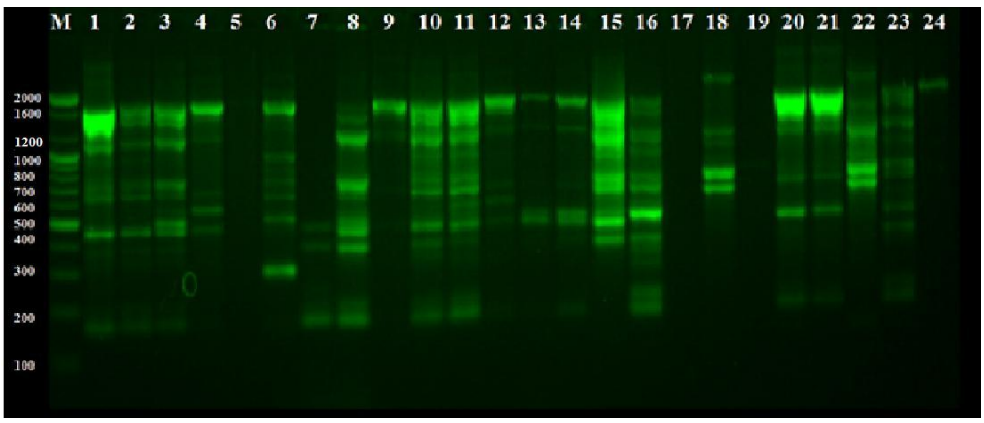
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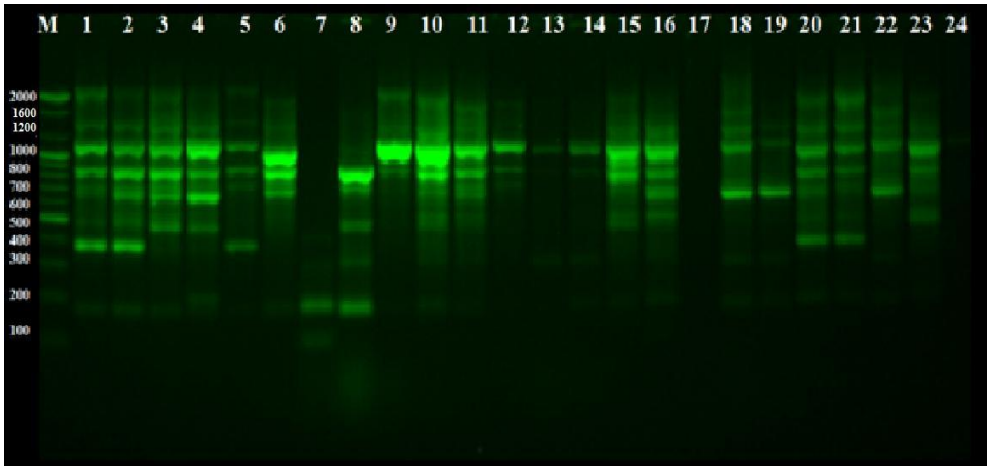

Figure 4: Agarose gel electrophoresis with ethidium bromide of RAPD-PCR reaction all primers (OPA12, OPA18, OPB05, OPB10, OPB17, OPG17, OPH03), respectively. DNA samples of *A.niger* isolates (under optimal conditions). Bands were fractionated by electrophoresis on a 1.2% agarose gel (2hr, 5V/cm, 1XTris-borate buffer) and visualized under U.V. light. M: 100 bp DNA ladder. Lanes: *A.niger* isolates from different source: Lanes 1, 2, 3, 4, 5 (Soil), Lanes: 6, 7, 8, 9 (Ground nut), lanes 10 and 11(Pistachio), Lanes:,12, 13, 14 (Walnuts), lanse 15 and 16 (Cashew), Lanes: ,17, 18, 19, 20 (Almonds), Lanes: 20, 21 (Milk), Lanes: 22, 23, 24 (Factory waste water).

The seven random primers produced total of (149) mine bands for 24 isolates. Of these (149) PCR products were generated 100% (149) polymorphic.

Each primer generates a different number of bands, OPH03 generated maximum number of bands (174) while OPB10 amplified minimum number of bands (82), the differentiation in the number of bands amplified by different primers affected by several factors such as primer structures, template quantity, and less number of priming sites in the genomic DNA.[17]

A total of 149 (100%) polymorphic bands were observed ranging from 12 to 40 bands. The primer OPG17 gave the highest number of polymorphic bands (40), while the minimum number of polymorphic bands (12) by using OPB05 primer.

Primer efficiency of each primer ranged between (0-1), primer OPB10 gave the minimum efficiency (0.09), while the primers OPH03 gave the highest efficiency (0.20), primer efficiency represented the ability of primer to appear polymorphic

between isolates and not means gave high number of amplified bands,[18] The value of discrimination power represented the ability to descry and diagnosis of the isolates. The primer OPH03 gave the highest percentage of Discrimination power (20%) while the primer OPB10 gave the lowest percentage (9 %).

DNA Fingerprint for some of these isolates by the presence of a unique band, unique band exist in isolate and does not exist in other.[19]

Fingerprint determined for nineteen isolates the most characteristic banding pattern was for the C1 with OPB17, C2 with OPG17, C3 with OPB10 andOPG17, C4 with OPA12 and OPH03, C5 with OPB05 and OPG17, C6 with OPB05 and OPB10, C7 with OPA18, OPB05, OPG17 OPH03, C8 with OPA12 and OPG17, C9 with OPG17, C10 with OPA12 and OPG17, C12 with OPA12 and OPB05, C13 with OPA18, OPB05 and OPG17, C15 with OPB17 AND OPG17, C16 with OPA12, OPA18 and OPG17, C18 with OPA12, OPA18 and OPG17, C20 with OPG17,C22 with OPB05, OPB10, OPB17 and OPG17, C23 with OPA12, OPA18, OPB17 and OPG17, and C24 with OPB17 and OPH03. The primer OPG17 gave the highest number of unique bands while the primers OPB10 and OPH03 gave the lowest number Fingerprint it through the absence of a common band that is present in other isolates amplified by the primer. Or, through a unique or specific band (s) or marker (s) present in this isolate and not in other isolates when amplified by the same primer. This specific marker or band can be useful in two ways ; first it can be scraped from the gel and reamplified with the same primer but in the presence of a labeled dNTP to produce labeled probes that can be used for hybridization and detection of any Restriction Fragment Length Polymorphism (RFLP) that can be produced by any restriction enzyme within the original specific RAPD fragment (band).[20] The specific RAPD bands or markers can be sequenced and used to prepare primers that are specific for this marker that can be used to specifically identify the isolate producing these specific markers.[21] Table (5).

Table 5: Total bands, polymorphic bands and unique bands of each RAPD primer that observed in 24 isolates; with efficiency and discriminatory power of RAPD primers.

No. of primer	Operon code	No. of bands amplified in all 24 isolates of *Aspergillus niger*				Primer efficiency	Discriminatory power %	Isolates of A.niger	Unique bands molecular weight (base pair)
		Total mine bands	Total bands	Polymorphic bands	Proporation of polymorphic loci (%)				
1	OPA12	20	100	100	100	0.11	11	4, 8, 10, 16, 18, 23	2840; 560; 1330; 1410, 2425, 460, 290; 790, 260
2	OPA18	19	109	109	100	0.12	12	7,12,13,16,23	520; 710; 1450; 1170; 500, 400
3	OPB05	12	127	127	100	0.14	14	5,6,7,12,13,22	320; 960; 700, 1560; 1280; 1580, 1445, 820, 625, 440
4	OPB10	13	82	82	100	0.09	9	3, 6, 22	880; 710; 580
5	OPB17	20	135	135	100	0.15	15	1, 15, 22, 23, 24	800, 570, 350; 1520; 2030; 1710; 1850
6	OPG17	40	138	138	100	0.15	15	2, 3, 5, 7, 8, 9, 10, 12, 13, 15, 16, 20, 22, 23	1120; 595; 800,250; 260; 265; 2390, 2135; 275; 285; 290; 295; 300; 320; 1780, 550; 1550, 345
7	OPH03	25	174	174	100	0.20	20	4, 7, 24	200; 490, 285, 100; 1510
Total		149	865	865					

CONCLUSION

- Fingerprint for ninetten isolates was achieved by RAPD - PCR technique according to their unique band.

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