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ORIGINAL PAPER

Differentiation of *Aspergillus niger* by random amplification of polymorphic DNA

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Abstract The randomly amplified polymorphic DNA (RAPD) patterns of whole-cell lysates from five *Aspergillus niger* isolates, including one reference strain, two isolated from deep freeze, and two environmental strains from soil and plant infections, were investigated. PCR-RAPD analysis of genomic DNA was performed using eight primers (Tube-A1, Tube-A6, Tube-A17, Tube-B8, Tube-B11, Tube-B15, Tube-C5, Tube-C6). The RAPD assay discriminated between all strains. Comparison of deep freeze isolates showed identical RAPD patterns in some of the reference and environmental isolates. The data indicates that the RAPD technique is useful for fingerprinting *A. niger*.

Keywords *Aspergillus niger* · DNA (RAPD) · Fingerprinting · Saudi Arabia

Introduction

Aspergillus spp. mold can cause various diseases [24]; invasive aspergillosis, a complication often encountered in patients with cancer and immune deficiencies has been associated with various species including *Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger,* and *Aspergillus terreus* [4, 11]. *A. niger* is a common food contaminant which is also responsible for causing aspergillosis (a lung disease) and otomycoses (fungal ear infections).

Several methods have been utilized in the typing and characterization of pathogenic fungi. These include both phenotypic techniques and genotypic techniques. Phenotypic techniques are based on identification of phenotypic characters, and encompass antigenic profiling, immunoelectrophoresis, radioimmunoelectrophoresis, immunoblotting, enzyme electrophoresis, and yeast killer toxin typing systems [4]. Genotypic techniques involve genomic DNA characterization, and encompass a range of techniques like restriction length fragment polymorphism (RFLP), electrophoretic karyotyping, multilocus enzyme electrophoresis, and PCR-based fingerprinting methods including microsatellite PCR, Southern hybridization probe, as well as the rapid amplification of polymorphic DNA (RAPD) [4, 17, 20].

Of these genotype-based methods, the RAPD has grown to become the most popular for the typing of pathogenic fungi, especially the *Aspergillus* species. Originally described in 1990 by two teams of researchers [23, 25], RAPD genomic typing involves the targeting and amplification of amplicons within the genome via the polymerase chain reaction. Single, short, random oligonucleotide primers (around 10-bases long) are used to bind to homologous sequences on the template DNA using suitable annealing temperatures. *Taq* polymerase is used to amplify the DNA region between two suitably positioned hybridization sites [4, 11, 20].

The RAPD method has several unique advantages: it is extremely fast (yielding results within 48 h), it shows a high degree of discriminating power between closely related strains (provided multiple primers are used), it works well with crude DNA lysates hence eliminating the cost of extensive purification, and it has been used with great success for several types of fungal species including *Candida* spp. [20], *Cryptococcus* spp. [20], and *Aspergillus* spp. [1, 14, 20].

Several RAPD primers and protocols have been published for use in different species of the genus *Aspergillus*. We selected the most promising ones and tested their performance, accuracy and reproducibility of discrimination

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Table 1 Strains and sources of *Aspergillus niger*

Source	Strain code
ATCC	TA
Isolation from deep freeze	T1
Isolation from deep freeze(after 1 year)	T2
Isolation from plant infection	CO
Isolation from soil	SO

[14]. This study is aimed to identify genetic changes in the isolates *A. niger* resulting from changing environmental conditions. The characterization was achieved by detecting and analyzing the electrophoresis gel of RAPD-PCR genomic DNA extracted from the selected fungi.

Materials and methods

Isolation and identification of fungi

Five samples from each refrigerator and freezer doors of every household in Riyadh, Saudi Arabia were done. Twenty households were randomly selected for a total of 100 samples. Samples were grown in Saboraud's agar medium for fungal isolation. Four replicate sets of plates were made for each sample. Plates were incubated at 25–27 °C for 5 days. All media used were prepared according to Atlas [2]. Fungi were identified on the basis of macro and microscopical features according to Raper and Thom [16], Gilman [8], Raper and Fennell [15] and Moubasher [12]. The identification was checked to the species level by CABI Bioscience Identification Services, UK.

Strains and cultivation

A total of five *A. niger* isolates were recovered from different regions; two strains were isolated from deep freeze during September 2006–November 2007, one reference strain was obtained from the American type Culture collection, one isolated from soil and one isolated from plant infection were obtained from the culture collection of the Agrc. Microbiology Dept. (for list of strains see Table 1). All strains were isolated on Sabouraud glucose agar (SGA) plates with chloramphenicol at 28 °C, and identified according to morphological features [5, 9, 18, 19]. Isolates were maintained on SGA slants at 4 °C until tested.

DNA extraction

SGA plates were inoculated by spreading with a suspension of spores and cultivated at 28 °C. DNA extraction was done by the rapid and simple toothpick technique [21] modified for use in filamentous fungi. Briefly, a small amount of hyphae was sampled with a micro-pipette tip from an outgrown mycelium at latest 1 day after inoculation and transferred into 20 µl of lyzing buffer (1 mol/L $MgCl_2$, 2 mmol/L 1,4-dithiothreitol, 48 U of zymolyase; all Sigma–Aldrich, Czechia). The mixture was incubated for 30 min at 37 °C and centrifuged (13,000*g*, 5 min). The supernatant (15 µl) was transferred into a new tube and diluted to 300 µl with TE buffer (10 mmol/L Tris, pH 8; 1 mmol/L EDTA).

RAPD-PCR amplification conditions

The sequence and codes of the utilized primers [Agricultural Genetic Engineering Research Institute (AGERI), Agriculture Research Center ARC, 9 Gamaa St. Giza 12619, Egypt] are listed in Table 2. All of the primers were 10-nucleotide (10nt) in length containing 60–70% of the G/C contents. The reaction was performed in 20-µl mixtures containing 2 µl of extracted DNA, 0.5 µmol/L primer, 10 mmol/L TRIS-HCL (pH 8.8), 50 mmol/L KCL, 0.1% Triton X- 100, 2.5 mmol/L $MgCl_2$, 200 µmol/L of each dNTP and 2 U of *Taq* polymerase Unis (Top-Bio, Czechia). Eight primers sutable for RAPD strain typing of *A. niger* (Table 2) were tested for their discriminatory power. Reaction mixtures were overlaid with mineral oil and subjected to amplification in a Robocycler Gradient 96 Temperature Cycler (stratagene, USA). For amplification with the primers and $(GATA)_4$ initial denaturation was done for 5 min at 94 °C, followed by 45 cycles of denaturation (1 min, 94 °C), annealing (1 min, 36 °C), and extension (1 min, 72 °C). All experiments were done in triplicate.

Electrophoretic analysis

RAPD-PCR patterns were visualized by agarose gel electrophoresis. Aliquots of 10 µl each of the amplification products were loaded onto 1.2% agarose slabs (11 by 14 cm) and run in TAE (40 mM Tris-acetate, 1 mM EDTA) buffer at 2 V/cm during 5 h. Slabs were stained with 0.4 µg of ethidium bromide/ml and documented with a Gel Doc 2000 gel system (Bio-Rad). Molecular weight analysis of patterns was performed with the Quantity One version 4.2.1 software (Bio-Rad), with the 1-kb DNA ladder (Invitrogen) as a molecular weight marker.

Analysis of RAPD-PCR patterns

Polymorphic bands from all the RAPD-PCR patterns were individually identified by their specific migration rates in the electrophoretic analyses. Once bands were properly and distinctively identified, binary (0/1) matrices were constructed to compare the patterns. Dice similarity coefficients [6] were generated by the SIMQUAL subroutine

Table 2 Code and sequences of the eight DNA random primers used for fingerprinting the five *Aspergillus niger* gathered from different locations as well as the number and types of the amplified DNA bands generated by each primer

Primer code	Sequence	Polymorphic bands		Total bands	Polymorphism %
		Unique bands	Non-unique bands		
A17	5′-GACCGCTTGT-3′	1	5	6	100.00
A6	5′-GGTCCCTGAC -3′	1	3	4	100.00
A1	5′-CAGGCCCTTC-3′	2	3	5	100.00
B8	5′-GTCCACACGG-3′	–	2	2	100.00
B15	5′-GGAGGGTGTT-3′	1	5	6	100.00
B11	5′-GTAGACCCGT-3′	1	2	3	100.00
C6	5′-GAACGGACTC-3′	4	4	8	100.00
C5	5′-GATGACCGCC-3′	1	8	9	100.00
Total		11	32	43	100.00

from the NTSYS-pc 2.02j (Applied Biostatistics, Inc.) package. Cluster analyses along with their corresponding dendrograms were generated by the unweighted-pair group method using average linkages (UPGMA), with the SAHN and TREE subroutines from the NTSYS-pc package.

Results and discussion

Eight random primers were successfully used to fingerprint of five *A. niger* isolates gathered from different locations. The primers are A1, A6, A17, B8, B11, B15, C5, and C6. These primers give clear and scorable profiles. The profiles are repeatable with sufficient polymorphism. The sequences of these primers are listed in Table 2. A total of 43 amplified DNA bands were generated across the studied genotypes with all bands being polymorphic. Of these bands ten unique ones were detected. The RAPD profiles of the amplification products are shown in Fig. 1a, b, c, and d. The number of bands and the degree of polymorphism generated by each primer are given in Table 2. The numbers of bands were varied with different primers among the different *A. niger* isolates. A maximum of eight DNA bands were amplified with primer C6 and C5, and a minimum of two with the two primers B8. The size of the amplified bands also varied with the different primers. The largest 2,000-bp band was amplified by the primer A17 in the CO and SO isolate while the smallest one was amplified by the primer B15 and detected at about 100 bp in the CO isolate.

The generated RAPD profile with the primer A17 shows a total of six bands with 100% polymorphism among the studied isolates (Fig. 1a). It comprises one polymorphic unique band and five non-unique ones. The first unique band at apparent molecular weight of 700 bp was recognized in the isolate TA. The second band at about 400 bp was identified in the isolates T1, T2, CO and SO. The third band at about 900 bp was observed in the isolates TA, T1, T2 and CO. The fourth band at about 1,300 bp was recognized in the isolates TA, T1, T2, CO and SO. The fifth band was detected at about 2,000 bp in the isolates T2, CO and SO (Tables 3, 4).

The generated RAPD profile with the primer A6 shows a total of four bands with 100% polymorphism among the studied isolates (Fig. 1a). It comprises one polymorphic unique band and three non-unique ones. The first unique band at apparent molecular weight of 1,500 bp was recognized in the isolate SO. The second band at about 300 bp was identified in the isolates TA, T1, T2, CO and SO. The third and fourth bands at about 800 and 1,300 bp were observed in the isolates TA, T1, CO and SO (Tables 3, 4).

A total of three polymorphic bands were identified in the RAPD profile generated by the primer B11 (Fig. 1b). It comprises one polymorphic unique band and two non-unique ones. The first unique band was scored at about 400 bp in the isolate T1. The second band was observed at about at about 500 bp in the isolates TA, CO and SO. The third band at about 800 bp was identified in the isolates TA, T1, T2, CO and SO (Tables 3, 4).

The generated RAPD profile with the primer A1 shows a total of five bands with 100% polymorphism among the studied isolates (Fig. 1b). It comprises two polymorphic unique bands and three non-unique ones. The first unique band at apparent molecular weight of 500 bp was recognized in the isolate TA. The second unique band at about 800 bp was identified in the isolate SO. The third band at about 400 bp was observed in the isolates TA, T1, T2, CO and SO. The fourth band at about 900 bp was recognized in the isolates T2 and CO. The fifth band was detected at about 1,600 bp in the isolates T1, T2, CO and SO (Tables 3, 4).

A total of two polymorphic bands were identified in the RAPD profile generated by the primer B8 (Fig. 1c). Two non-unique bands were detected in the polymorphic bands. The first band was scored at about 800 bp in the isolates TA, T1, T2, and CO. The second band was observed at about at about 900 bp in the isolates TA, T1, T2, CO and SO (Tables 3, 4).

The generated RAPD profile with the primer B15 shows a total of six bands with 100% polymorphism among the

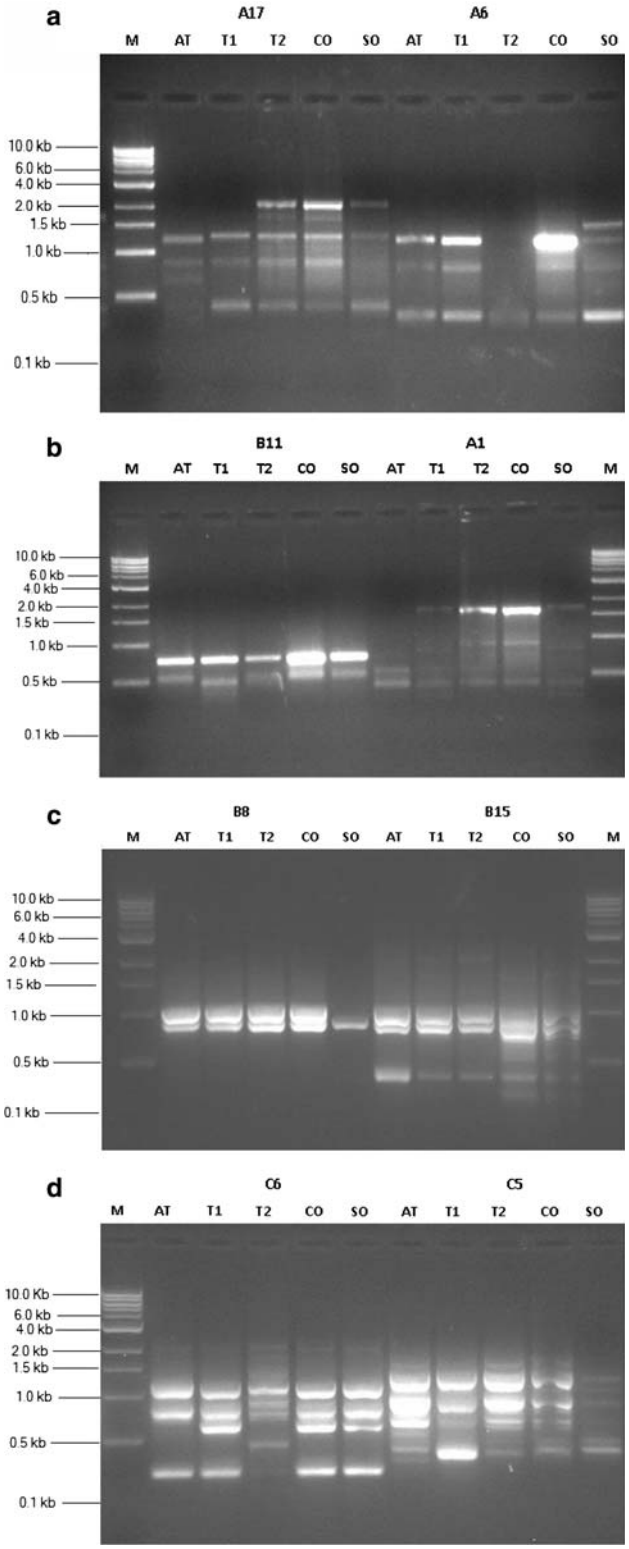

Fig.1 **a** Dendogram derived from RAPD-PCR data of ATCC (*AT* lanes 2,7); isolation from deep freeze (*T1* lanes 3,8); isolation from deep freeze(after 1 year) (*T2* lanes 4, 9); isolation from plant infection (*CO* lanes 5,10); and isolation from soil (*SO* lanes 6,11) using 10-nucleotide primers (*A17*, *A6*); lanes 1, 11: 0.5 mg of 1-Kb DNA ladder visualized by ethidium bromide **b** Dendogram derived from RAPD-PCR data of ATCC (*AT* lanes 2,7); isolation from deep freeze (*T1* lanes 3,8); isolation from deep freeze(after 1 year) (*T2* lanes 4, 9); Isolation from plant infection (*CO* lanes 5,10); and isolation from soil (*SO* lanes 6,11) using 10-nucleotide primers (*B11*, *A1*); lanes 1, 11: 0.5 mg of 1-Kb DNA ladder visualized by ethidium bromide. **c** Dendogram derived from RAPD-PCR data of ATCC (*AT* lanes 2,7); isolation from deep freeze (*T1* lanes 3,8); isolation from deep freeze(after 1 year) (*T2* lanes 4, 9); isolation from plant infection (*CO* lanes 5,10); and isolation from soil (*SO* lanes 6,11) using 10-nucleotide primers (*B8*, *B15*); lanes 1, 11: 0.5 mg of 1-Kb DNA ladder visualized by ethidium bromide. **d** Dendogram derived from RAPD-PCR data of ATCC (*AT* lanes 2,7); isolation from deep freeze (*T1* lanes 3,8); isolation from deep freeze(after 1 year) (*T2* lanes 4, 9); isolation from plant infection (*CO* lanes 5,10); and isolation from soil (*SO* lanes 6,11) using 10-nucleotide primers (*C6*, *C5*); lanes 1, 11: 0.5 mg of 1-Kb DNA ladder visualized by ethidium bromide

Table 3 Molecular size in bp of the amplified unique DNA bands generated by eight random primers used for identifying five *Aspergillus niger* gathered from different locations

Isolates	Primers							
	A17	A6	B11	A1	B8	B15	C6	C5
TA	700	–	–	500	–	–	–	300
T1	–	–	400	–	–	–	–	–
T2	–	–	–	–	–	–	500–750–900–1,100	–
CO	–	–	–	–	–	100	–	–
SO	–	1,500	–	800	–	–	–	–

studied isolates (Fig. 1c). It comprises one polymorphic unique band and five non-unique ones. The first unique band at apparent molecular weight of 100 bp was recognized in the isolate CO. The second and third bands at about 300 and 800 bp were identified in the isolates TA, T1, T2, CO and SO. The fourth band at about 500 bp was observed in the isolates CO and SO. The fifth band at about 700 bp was recognized in the isolates CO and SO. The sixth band was detected at about 900 bp in the isolates TA, T1, T2 and SO (Tables 3, 4).

A maximum of eight polymorphic bands were scored in the RAPD profile of primer C6 (Fig. 1d). Four unique bands were identified. The first, second, third and fourth unique bands were identified at about 500, 750, 900, and 1,100 bp in the isolate T2. The fifth band was detected in the isolates TA, T1, CO and SO at about 200 bp while the sixth band was identified at about 700 bp in the isolates T1, CO and SO. The seventh band was detected at about 800 bp in the isolates TA, T1, T2, CO and SO. The eightth band was scored at about 1,000 bp in the isolates TA, CO and SO (Tables 3, 4).

Figure 1d illustrates the results obtained by primer C5. A total of eight polymorphic bands were scored. One unique band was observed; the first band was identified at about 300 bp in the isolate TA. The second band was recognized at about 400 bp in the isolates TA, T1, T2, CO and SO. The

Table 4 Molecular size in bp of the amplified non-unique DNA bands generated by eight random primers used for identifying five *Aspergillus niger* gathered from different locations

Isolates	Primers							
	A17	A6	B11	A1	B8	B15	C6	C5
TA	1,300–900	1,300–800–300	800–500	400	900–800	900–800–300	1,000–800–200	1,100–800–700–600–400
T1	1,300–900–400	1,300–800–300	800	1,600–400	900–800	900–800–300	800–700–200	1,100–800–700–400
T2	2,000–1,750–1,300–900–400	300	800	1,600–900–400	900–800	900–800–300	800	1,400– 1,200–1,100–800–700–600–400
CO	2,000–1,750–1,300–900–400	1,300–800–300	800–500	1,600–900–400	900–800	800–700–500–300	1,000–800–700–200	1,400–1200–1,100–800–700–500–400
SO	2,000–1,300–400	1,300–800–300	800–500	1,600–400	800	900–800–700–500–300	1,000–800–700–200	1,100–800–500–400

Table 5 Similarity coefficients of the five *Aspergillus niger* isolates obtained from RAPD analysis

T2	T1	AT	CO	SO	Isolates
				1.00	SO
			1.00	0.655	CO
		1.00	0.429	0.56	AT
	1.00	0.723	0.473	0.531	T1
1.00	0.588	0.423	0.667	0.444	T2

third band was detected in the isolates CO and SO at about 500 bp while the fourth band was identified at about 700 bp in the isolates TA, T1, T2 and CO. The fifth and sixth bands were detected at about 800 and 1,100 bp in the isolates TA, T1, T2, CO and SO. The seventh and eighth bands were scored at about 1,200 and 1,400 bp in the isolates T2 and CO (Tables 3, 4).

Based on the combined data obtained through the polymorphism of RAPD profiles generated by the eight utilized primers in this study, the similarity coefficient values among the studied *A. niger* isolates were calculated according to the Dice [6] equation (Table 5). The similarity coefficients were used to generate a dendrogram (Fig 2) by UPGMA analysis as implemented in the computer program SPSS version-10. In most cases, the levels of similarity between the studied isolates show low values. The maximum value of similarity was 0.723 and recorded between the two isolates TA and T1. Figure 2 shows the clustering of these isolates in one group. The minimum value of similarity was 0.423 and recorded between the two isolates TA and T2, which indicates that these two isolates were geneti-

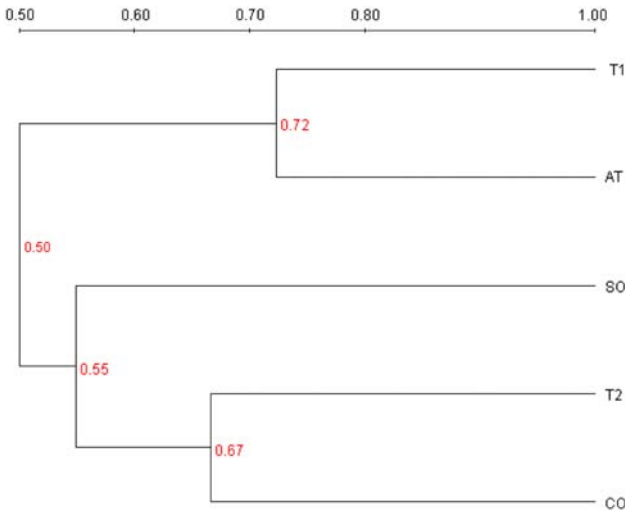

Fig.2 Dendogram derived from RAPD-PCR data of ATCC (*AT*), isolation from deep freeze (*T1*) Isolation from deep freeze(after 1 year) (*T2*) Isolation from plant infection (*CO*) Isolation from soil (*SO*) using (A1, A6, A17, B8, B11, B15, C5, C6) primers. The Dendogram was constructed employing Dice Coefficient Genetic similarity index with a maximum value of 1.0

cally distant from each other (Fig. 2). Generally, the similarity coefficient values recorded between such isolates and the studied isolates were lower than the values recorded between the remaining isolates.

The high level of polymorphism observed between the studied *Aspergillus niger* isolates using eight RAPD primers in this study indicates a remarkable level of intra-species variation among the studied genotypes. Moreover, RAPD analysis showed high discriminatory power at distinguishing the between the strains.

These results accord with the outcome of studies performed by other workers in the molecular typing of various species of *Aspergillus* [3, 7, 10, 13, 17, 22, 24]. The RAPD method is doubtless emerging as a high-utility tool in fingerprinting the genome of pathogenic fungi species.

There are a number of limitations to the RAPD technique, which inevitably apply to the results of this research. Reproducibility of results, even within a laboratory, has been identified as an issue with RAPD; hence, the usefulness of these results in serving as a reference for other researchers remains an unsettled issue. Problems affecting reproducibility range from variation in temperature, primer-to-template ratio, reaction buffer ion concentration, and *Taq* enzyme sourcing [4, 20]. However, we have made diligent efforts to carry out our experiments in triplicate, and the results can be adequately regarded as reproducible under the stated experimental conditions. Also, the use of eight different primers allow for a robust identification of relevant polymorphisms, when the results from each primer's amplification are combined.

This research confirms the suitability of using RAPD to successfully differentiate between genomic strains of *Aspergillus niger*.

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