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Molecular Typing of Iranian Cladosporium Isolates Using RAPD-PCR

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Abstract: Fourteen isolates of Cladosporium were collected from different areas and clinical samples. These isolates were genotypically compared using RAPD (Random Amplified Polymorphic DNA) technique. The data showed high degree of polymorphism between clinical and non clinical isolates and quite low polymorphism within isolates of the same group (clinical or non clinical group). The RAPD's results were subjected to a numerical taxonomy analysis, using UPGMA method. A phenogram was constructed for isolates Based on its structure; we concluded that genotypic data provide enough information to use the unweighted pair-group method to cluster the isolates in accordance to their respective group. The phenogram grouped in a single branch the isolates of C, G, H, K, E, J, I, M, N and F, indicating a great similarity between these fungi that are both clinical and non clinical ones and suggesting that the classification as distinct isolates may not be appropriate for these isolates of the Cladosporium.

Key words: Dematiaceous fungi, RAPD-PCR, phylogeny, genetic similarity, Cladosporium

INTRODUCTION

Although Cladosporium is one of the largest and most heterogeneous genera of hyphomycetes, currently comprising more than 772 names (Dugan *et al*., 2004), only a mere fraction of these species are known from culture and thus the real number of taxa that exist remains unknown. Species of Cladosporium are commonly encountered on plant and other kinds of debris, frequently colonizing lesions of plant pathogenic fungi and are also isolated from air, soil, food, paint, textiles and other organic matters (Schubert and Braun, 2005), they are also common endophytes (Brown *et al*., 1998; El-Morsy, 2000) as well as phylloplane fungi (Islam and Hasin, 2000; Jager *et al*., 2001; Inacio *et al*., 2002; Stohr and Dighton, 2004; Levetin and Dorsey, 2006). In spite of its obvious importance, species of Cladosporium are still poorly understood. Cladosporium species are causative agents of skin and pulmonary infections. The identification of this fungus is based on the ontogenic aspects of the conidia, biochemical and physiological tests (Caligiorne *et al*., 1999; Huerta-Ochoa *et al*., 2005; Pianzzola *et al*., 2004; De Hoog *et al*., 1994, 1995a, b). However, the application of molecular biology techniques such as PCR-ribotyping (Attily *et al*., 1998), DNA sequencing (Masclaux *et al*., 1995), mtDNA sequencing (Ishizaki *et al*., 1995) and RAPD (Random amplified polymorphic DNA) assays (Haase *et al*., 1995) are further tools for resolving the identification and taxonomy of this fungus. Moreover, in taxonomic and molecular systematic, species-specific RAPD markers could be invaluable tools for species variation and establishing the status of organism and its evolution (Rao *et al*., 1996).

MATERIALS AND METHODS

In this research (Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran, 2006), we have investigated genetic polymorphisms of Cladosporium isolates, which obtained from mycology

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Table 1: Characteristics of the Cladosporium isolates examined

Isolates	Location of sampling	Origin
A	Chitgar park, Tehran, Iran	Air
B, C, D, F, I	Mycology laboratory, faculty of hygiene, Tehran university of medical sciences, Tehran-Iran	Clinical (respiratory-secretions)
G, H, J	Barn, veterinary medicine hospital of Tehran university, Karaj-Iran	Air
K	Barn, Kashan-Iran	
E, M, N	Farm, veterinary medicine hospital of Tehran university, Karaj-Iran	Air
L	Bath, dormitory, University of Tehran, Tehran-Iran	Air

Table 2: Temperature cycles in PCR reaction

Stage	First denaturation	Later denaturation	Annealing	Extension	Final extension
Temperature	95°C	95°C	46°C :P4 52°C :P1	72°C	72°C
Time	3 min	60 sec	60 sec	60 sec	5 min
Cycles	1	35	35	35	1

P = Primer

Table 3: Sequences of eight primers (10 m Oligonucleotide) which were used in this study

Primers	Sequence 5′to 3′
P1	GACAGACACG
P2	CAGCGCCCTT
P3	GGTACGTAGG
P4	ATGGATCGGC
P5	GTGCTAGCAG
P6	AGCCAGCGTT
P7	AAAACCTTGC
P8	AATCGCCGTG

collection and air throughout a year (Table 1). We used RAPD to confirm the usefulness of this technique on molecular typing of Cladosporium isolates (Welsn and McClelland, 1990; Williams *et al*., 1990). The isolates were maintained in distilled water and then subcultured on SCC (Sabouraud agar, Chloramphenicol and Cyclohexamid) at 28°C for two weeks. The transfers were done at 1-month intervals. The colonies mass were harvested and washed with distilled water to remove any debris.

For DNA extraction the Portion of colonies were washed with PBS, 0.5% SDS and 50 mM EDTA. Then, cell walls were disrupted by freeze-thawing method and glass beads and centrifuged at 10000 g for 2 min. Next, that was precipitated by adding 500 μL lysis buffer and 15 μL protein kinase. After that, they were maintained at room temperature for 10 min with 150 μL potassium acetate buffer (pH = 4.8) (60 mL potassium acetate 5 mg, 11.5 mL glacial acetic acid and 28.5 mL distilled water) (by modification in Kresk and Wellington, 1999). The tubes were vortexed briefly and cell debris and precipitated proteins were removed by centrifugation at 12000 g for 2 min. The supernatant was transferred to another eppendorf tube and centrifuged as mentioned earlier. Then, supernatant was transferred to a new 1.5 mL eppendorf tube. An equal volume of isopropyl alcohol was added to the tubes which was mixed briefly by inversion and centrifuged at 12000 g for 5 min. The supernatant was discarded and DNA pellet was washed two times in 300 μL of 70% ethanol. The supernatant was discarded again. The DNA was dried in air and resuspended in 50 μL distilled water. In order to measure the concentration and purification of DNA, its OD (absorption density) was read and run in the 1.2% agarose gel. DNA aliquote were diluted to 20 ng μL^{-1} for the RAPD reaction.

RAPD-PCR reactions were done in a 25 μL volume containing PCR buffer 1x, 10 mM each of dATP, dGTP, dCTP and dTTP, 10 ng of genomic DNA, 2.5 mM of $MgCl_2$, 20 nM of primer (Table 3) and 5 unit μL^{-1} of Taq polymerase. The amplification parameters consisted of 35 cycles: first deneturation at 95°C for 3 min, but later denaturation at 95°C for 60 sec, Primer annealing at 52°C (for P4 primer; 5′-ATGGATCGGC-3′) and 46°C (for P1 primer; 5′-GACAGACACG-3′) for 60 sec and extension at 72°C for 60 sec. However, in the final step 5 min was used in the final extension step (Table 2). Among the eight primers, which used, only two primers (P1 and P4 primers) gave the best amplification of these isolates genomes (Table 3). The fingerprints were produced by electrophoresis of 10 μL aliquots of reaction in 1.2% agarose gels run in TBE (0.45 M tris-borate, 0.001 M EDTA) buffer at 85 V for 80 min. The gels were stained 1 μg mL^{-1} ethidium bromide, photographed under UV light using a Polaroid camera (Model DS-34) with black and white film (type 667, Polaroid corp.).

RESULTS AND DISCUSSION

Using two primers, a total of 29 different reproducible RAPD markers were generated from the isolate genomes. These markers divided into two groups of main and accessory. That is, the main markers exist in most of isolates. However, the accessory ones present in some of them (Table 4, 5). Negative control tubes (without sample) did not indicate any DNA amplification. In addition, P1 and P4 primers gave representative profiles for the isolate clusters (Fig. 1, 2).

Amplified DNA fragments (bands) which were reproducible in two to three reactions, were scored as 0 (fragment absent) and 1 (fragment present) in a data matrix

Table 4: Amplified DNA fragments (bands) that produced by primer P1 in PCR reaction

	Bands (bp)													
Isolates	1750	1500	1200	1100	1031	900	800	700	600	570	500	400	300	200
A	1	1	1	1	1	1	1	1	1	1	0	0	0	0
B	0	1	1	0	1	1	1	1	0	1	1	1	1	0
C	0	0	0	0	1	1	1	1	1	1	0	1	0	0
D	0	0	0	0	1	0	1	0	0	1	1	1	1	1
E	0	0	0	0	1	0	1	1	0	1	0	1	0	0
F	0	0	0	0	0	1	0	0	0	1	0	1	0	0
G	0	0	0	0	1	0	1	1	0	1	0	0	0	0
H	0	0	0	0	1	0	1	1	0	1	0	0	0	0
I	0	0	0	0	0	0	0	1	0	1	0	1	0	0
J	1	0	1	0	1	0	1	1	1	1	0	0	0	0
K	0	0	0	0	1	0	1	1	1	1	0	0	0	0
L	0	1	0	0	1	0	1	1	0	0	0	0	0	0
M	0	0	0	0	0	0	0	1	1	1	0	0	0	0
N	0	0	0	0	0	0	0	0	1	1	0	0	0	0

1 = Fragment present, 0 = Fragment absent

Table 5: Amplified DNA fragments (bands) that produced by primer P4 in PCR reaction

	Bands (bp)														
Isolates	280	310	376	450	585	603	720	800	872	1028	1078	1150	1220	1500	1800
A	1	1	0	0	1	1	1	0	0	1	0	1	0	1	0
B	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0
C	0	0	0	0	0	1	0	1	0	1	0	1	0	1	0
D	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0
E	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0
G	0	0	0	0	0	1	1	0	0	1	0	1	0	1	0
H	0	0	0	0	0	1	0	0	1	1	0	1	0	1	0
I	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
J	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K	0	0	0	0	1	1	0	0	1	1	0	1	0	1	0
L	1	0	0	1	0	0	1	0	1	1	1	0	0	1	0
M	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0
N	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1

1 = Fragment present, 0 = F ragment absent

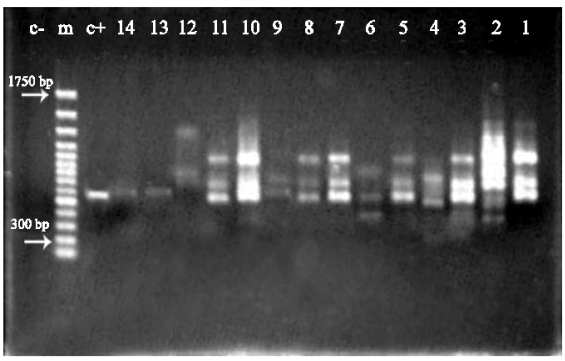

Fig. 1: Example of ethidium bromide-stained 1.2% agarose gel showing the products from RAPD fingerprinting of *Cladosporium* sp. Isolates with primer P4. m = Molecular size standards (100 bp DNA ladder). C+ = Positive control. c- = Negative control. The numbers from 1 to 14, respectively, are corresponded to A, B, C…, M and N isolates

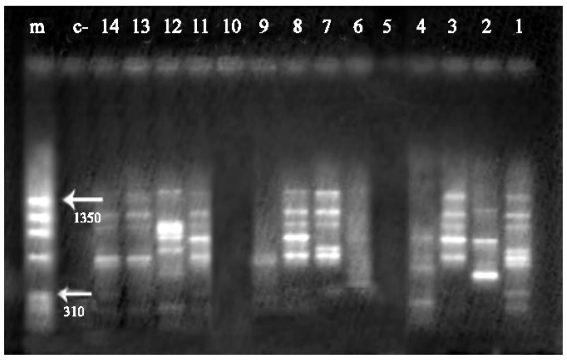

Fig. 2: Example of ethidium bromide-stained 1.2% agarose gel showing the products from RAPD fingerprinting of *Cladosporium* sp. Isolates with primer p4. m = Molecular size standards (1x 174 DNA Hae 111 digested). C+ = Positive control. c- = Negative control. The numbers from 1 to 14, respectively, are corresponded to A, B, C…, M and N isolates

Table 6: Data matrix for association coefficients between each pair of operating taxonomic units of Cladosporium isolates

Isolates	A	B	C	D	E	F	G	H	I	J	K	L	M	N
A	1.00													
B	0.41	1.00												
C	0.66	0.62	1.00											
D	0.38	0.62	0.52	1.00										
E	0.48	0.72	0.76	0.76	1.00									
F	0.35	0.59	0.62	0.62	0.79	1.00								
G	0.69	0.52	0.83	0.55	0.79	0.59	1.00							
H	0.62	0.52	0.83	0.55	0.79	0.66	0.93	1.00						
I	0.48	0.59	0.63	0.69	0.86	0.79	0.72	0.72	1.00					
J	0.59	0.69	0.72	0.65	0.90	0.69	0.69	0.69	0.76	1.00				
K	0.69	0.45	0.83	0.55	0.72	0.59	0.88	0.93	0.72	0.69	1.00			
L	0.55	0.52	0.55	0.55	0.65	0.52	0.72	0.72	0.59	0.55	0.66	1.00		
M	0.59	0.48	0.72	0.59	0.76	0.69	0.76	0.76	0.90	0.72	0.83	0.55	1.00	
N	0.48	0.45	0.62	0.62	0.72	0.72	0.66	0.66	0.86	0.69	0.72	0.45	0.90	1.00

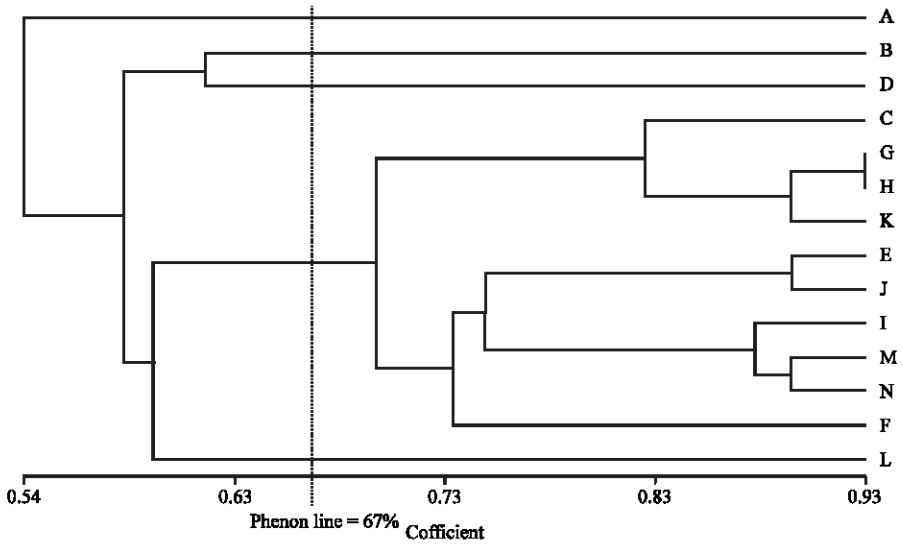

Fig. 3: Phenogram depicting relationships among fourteen operating taxonomic units of Cladosporium fungus using 29 characters and obtained by UPGMA

and Then a phenogram was constructed by the Unweighted Pair Group Method with Arithmetic mean (UPGMA) according to Sneath and Sokal (1973), after determining the association coefficients by the simple matching method (Table 6). The resultant phenogram obtained by UPGMA is illustrated in Fig. 3.

In RAPD-PCR analysis by using P1 and P4 primers different reactions were generated from 14 Cladosporium isolates. The results were obtained in negative control indicate any DNA amplification. The G and H isolates monosporic colonies showed a monomorphic electrophoretical profile, which coincided in all its amplified DNA bands. This result showed the high repetition rate and reliability of RAPD technique and gave more weight to the other results of this research. Although, these colonies were kept for year with one-monthly transfers they showed no differentiation when subjected to tested primers and their variability index remained very low.

G and H isolates indicated coincidence in 93% of its significant amplified DNA bands, even though corresponding to other isolates. On the other hand, clinical isolates (B, C, D, F and I isolates) showed a coincidence that ranged from 52-79%, indicating a not so high intraspecific similarity. The non clinical isolates showed an intraspecific coincidence index that ranged from 45-93%. In addition, the G, H, J and K isolates (from air of barn) showed an intraspecific coincidence index that ranged from 69-93%. Moreover, the E, M and N isolates (from air of farm) showed an intraspecific coincidence index that arranged from 72-90%. The A and L isolates indicated coincidence in 55% of its significant amplified DNA bands. To sum up, the clinical isolates among each other have less similarity than non clinical ones.

In this study, the phenon line, which represents the mean similarities, was at 67%. At this level five groups are characterized: the first one formed by A isolate, the second one formed by B isolate, the third one formed by

D isolate, the forth one formed by C, G, H, K, E, J, I, M, N and F isolates. The similarity index for distinct isolates (A, B, D and L) ranged from 54-62%, staying the below phenon line (Fig. 3). These indexes shows that RAPD profile do not enable the precise study of polymorphism between clinical and non clinical isolates.

In parallel with our study, Rachel *et al.* (1999) by analysis of similarity indexes and phenon line among dematiaceous fungi including *F. pedrosoi*, *F. compacta*, *P. verrucosa*, *C. carrionii*, *E. dermatitidis*, *C. bantiana* strains and *Rhinocladiella* spp. showed that RAPD profiles alone do not enable the study of polymorphism at genus level. However, this technique could detect an interspecific coincidence index that ranged from 73% to 75% between *C. bantiana* and *C. carrionii*, indicating a low similarity among these species. Thus, RAPD technique can be a powerful tool in the study of polymorphism among morphological similar species of the dematiaceous pathogens. In this study that is performed by this technique, there is polymorphism among morphological similar cladosporium isolates as well.

According to our results, the RAPD technique detected a coincidence index between A and D isolates (nonclinical and clinical isolates, respectively) that is 38%, indicating a low similarity among these two isolates. Cladosporium isolates were proposed by De Hoog *et al.* (1995b) to be placed in the genus of *Cladophialophra* by using nutritional physiology and tolerance tests, in accordance with supposed teleomorph connections and along lines of phylogenetic relationships indicated by Masclaux *et al.* (1995) and Kwon-Chung *et al.* (1989). Arnau *et al.* (1994) obtained RAPD markers in *C. fulvum*, in order to establish a genetic map based on mitotic recombination in this imperfect fungus. In addition, a molecular study of 49 RAPDs showed that, with only one exception, all RAPD markers studied represent repetitive DNA. DNA-based methods such as RAPDs and species-specific primers have also been employed to distinguish between *Mycosphaerella* species occurring on *Eucalyptus* (Carnegie *et al.*, 2001; Maxwell *et al.*, 2005). One example of using RAPD is developing molecular markers linked to *C. fulvum* resistant gene Cf-6 in tomato by Wang *et al.* (2007).

The present report shows that based on the polymorphism among the *Cladosporium* isolates, there is an intraspecific genotypical diversity. In addition, better taxonomic classification needs a large number of samples and more RAPD-PCR reactions.

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