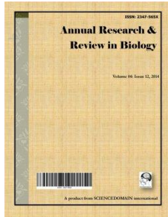

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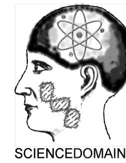

Molecular Characterization of *Penicillium* Isolates Using Rapd Technique

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Molecular analyses were performed on 14 isolates of *Penicillium* species. The *Penicillium* isolates examined were collected from different localities and habitats of Jeddah, Saudi Arabia and maintained on Czapek Dox's and Waksman's media.
Random Amplified Polymorphic DNA (RAPD-PCR) technique was used in this study to distinguish these isolates. A total of 42 DNA bands were generated by these primers in the *Penicillium* isolates grown on Czapek Dox's medium while, *Penicillium* isolates grown on Waksman's medium, 43 DNA bands were generated by the primers. Of the polymorphic bands that were identified in the *Penicillium* isolates grown on Czapek Dox's medium, two were unique, while no such bands could be detected in the *Penicillium* isolates grown on Waksman's medium. *Penicillium* isolates grown on both Czapek Dox's and Waksman's media, 40 monomorphic bands were detected. These monomorphic bands were used to distinguish among the studied *Penicillium* isolates under study.
Data were analysed by a clustering method and similarity coefficients using NTSYSpc version 2.02i. The relationships between the species and isolates are discussed.

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1. INTRODUCTION

The genus *Penicillium* with its species forms a natural monophyletic clade. It is a genus of ascomycetous fungi consisting of 304 species importance in the natural environment as well as in food and drug industry. However, many problems are encountered in either the infrageneric classification of this large genus or even the identification of its species due to the presence of a high degree of homoplasy and overlap in the morphological characters that are used [1] A need of extra criteria that may serve as good taxonomic markers is urgently needed.

The taxonomy of *Penicillium* has always been complex due to its great number of species, which exhibit very few differences. This fact intrigues the ability of researchers to different understand diversity and ecological behavior consequently, explanation of *Penicillium* in industries has become bottleneck. Historically, the classification of organisms has been based on observable morphological characteristics. The growth of isolates in an appropriate culture medium, enabling their most characteristic features to be recognised, is still being practiced in classification of organism. Because many species included in the sub-genus *Penicillium* are morphologically similar, identification of species using traditional morphological techniques proved to be difficult.

The genus *Penicillium* is subdivided into four subgenera (Aspergilloides, Penicillium, Biverticillium and Furcatum) based on the number of branch points between the phialide and the stipe down the main axis of the penicillus and other characteristics, such as the ratio of metula length to phialide length, and the colony diameter on G25N, when the number of branch points is the same [2].

It is repeatedly reported that morphological characteristics of microbes are influenced by environmental factors, and genomic mutations morphological markers are not reliable. The analysis of molecular markers has been taken to overcome the limitations associated with the morphological characterization of some *Penicillium* species [3]. Polymorphisms at the molecular level were studied by the random amplified polymorphic DNA (RAPD) marker technique [4].

In addition, recent trends in taxonomy stress the utmost importance of utilising other criteria that prove to be good phylogenetic or taxonomic markers. One of the methods most widely used for taxonomic identification at the species level has been sequencing and electrophoretic patterns [5-17] have utilized electrophoresis patterns to differentiate between *Penicillium* ssp. isolates.

In the study, molecular assay were performed on 14 isolates of *Penicillium* species grown on Czapek Dox's medium in which $NaNO_3$ is the source of inorganic nitrogen and Waksman's medium in which pepton is the source of organic nitrogen.

Random amplified polymorphic DNA (RAPD-PCR) technique was used to distinguish these isolates. The data were analysed by a clustering method and similarity coefficients using NTSYSpc version 2.02i. The similarities between the isolates are represented as phenograms and the results are discussed.

2. MATERIALS AND METHODS

2.1 Collection of *Penicillium* Isolates

Fourteen isolates of *Penicillium* representing six species *Penicillium corylophilum* Dierckx. (three isolates), *P. rubrum* Stoll. (one isolate), *P. citrinum* Thom. (two isolates), *P. canescens* Sopp. (six isolates), *P. crustosum* Thom. (one isolate) and *Penicillium* sp. (from an oily sewage dump) were included in these studies. These isolates were collected from five different substrates representing different ecosystems in both terrestrial and marine environments of Jeddah, Saudi Arabia (agricultural soil, marine fauna, a sewage dump, an oily sewage dump and wheat grains).

2.2 Culture, Identification and Cultivation of *Penicillium* Isolates

Samples were plated on Czapek (CZ), Potato Dextrose Agar (PDA) and malt extract agar media to isolate the fungal species. The colour of the on the various media and their morphological features under a light microscope were used to identify the different species as suggested by [18,19]. Details of isolates along with original sources are presented in Table 1.

2.3 Rapd-pcr

The isolated fungi were cultivated on here type the name of the media to determine their DNA banding patterns: Czapek Dox's medium, in which $NaNO_3$ is the source of inorganic nitrogen while in Waksman's medium, peptone is the source of organic nitrogen. For each fungus, a triplicate set of 250 ml Erlenmeyer conical flasks, containing 50 ml of medium and, sterilised at 121ºC for 15 minutes under 1.5x atmospheric pressure. The cooled flasks were inoculated with the respective fungi and incubated at 25ºC for seven days. At the ends of incubation period, the cultures were harvested for biomass production.

Approximately 0.5 g of fungal mat was used to perform DNA extraction as described by Dellaporta [20]. A total of fifteen 10-*mer* random oligonucleotide primers (UBC) were independently used in the PCR reactions as described by [21]. The primers were synthesised at the University of British Colombia. Only five primers revealed reproducible polymorphisms in the DNA profiles. Each experiment was repeated two times, and only reproducible products were scored. The codes for and sequences of these primers are précised in Table 2.

The optimal conditions for PCR amplification were as follows: one cycle at 94ºC for 2 min., followed by 40 cycles at 94ºC for 30 sec., 37°C for 60 sec. and 72ºC for 2 min. The reaction was finally incubated at 72ºC for 10 min. The RAPD products were electrophoresed using 1.4% Agarose gel in TAE buffer (0.04 M Tris-Acetate, 1 mM EDTA, pH 8). The run was performed at 100 V for 1 h. The gels were stained with 0.2 µg/ml ethidium bromide and photographed using a gel documentation system (GelDoc, BioRad 2000) and a UV transilluminator. Each PCR reaction was repeated twice to ensure that the RAPD banding patterns were consistent and reproducible, and only reproducible products were scored. A mixture of a 1018 bp fragment and its multimers and pBR328 DNA fragments with lengths of 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 517/506, 396, 344, 298, 220/201 and 154/134 bp was used as a marker.

2.4 Numerical Analysis

The RAPD-PCR data obtained for each isolate were pooled together and coded to create the data matrix used for computation, where the absence of a band was scored as "0" and the presence of a band was scored as "1" for each species (Tables 7 and 8). The relationships between the studied species, expressed by a similarity coefficient, have been represented using a phenogram based on the analysis of the recorded characters using NTSYSpc version 2.02i [22]. The similarity index was estimated using the Dice coefficient of similarity [23]. The average of the similarity matrices was used to generate a tree by the Unweighted Pair-Group Method Arithmetic Average (UPGMA). Three different phenograms were produced for the studied *Penicillium* species based on analysis of their DNA banding patterns.

Table 1. Sample names, sources (Habitats) and species of studied isolates of *Penicillium*

Sample number	Sources (Habitats)	Species of *Penicillium*
1	Agricultural soil	*Penicillium corylophilum* Dierckx.
2	Agricultural soil	*Penicillium corylophilum* Dierckx.
3	Marine fauna	*Penicillium rubrum* Stoll.
4	Sewage dump	*Penicillium citrinum* Thom.
5	Sewage dump	*Penicillium corylophilum* Dierckx.
6	Sewage dump	*Penicillium citrinum* Thom.
7	Oily sewage dump	*Penicillium* sp.
8	Agricultural soil	*Penicillium canescens* Sopp.
9	Agricultural soil	*Penicillium crustosum* Thom.
10	Agricultural soil	*Penicillium canescens* Sopp.
11	Agricultural soil	*Penicillium canescens* Sopp.
12	Agricultural soil	*Penicillium canescens* Sopp.
13	Agricultural soil	*Penicillium canescens* Sopp.
14	Wheat grains	*Penicillium canescens* Sopp.

The first one is representing isolated fungi cultivated on Czapek Dox's medium, the second is representing isolated fungi cultivated on Waksman's medium, and the third one is produced from combining DNA banding pattern data together of both media.

3. RESULTS AND DISCUSSION

3.1 Analysis of DNA Banding Patterns by RAPD-PCR

In this study, randomly amplified polymorphic DNA (RAPD) based polymerase chain reaction (PCR) analysis was conducted to fingerprint and elucidate the similarity indices 14 isolates representing eight *Penicillium* species.

Randomly amplified polymorphic DNA (RAPD) markers are found by the amplification of random locations in the plant genome by polymerase chain reaction (PCR). With this technique, a single oligonucleotide is used to prime the amplification of the genomic DNA. Because these primers are 10-mer long, it is possible that they will anneal at a number of locations in the genome. For amplification products to generate, binding must be to inverted annealing sites, approximately 150-4000 bases apart. The number of amplification products is directly related to the number and orientation of the annealing sites in the genome.

Ten random primers were initially tested, but five-revealed distinct inter and intra specific polymorphisms away the 14 *Penicillium* isolates. Only five primers generated were reproducible polymorphisms in the DNA profiles. The code and sequences of these primers are listed in Table 2. These primers are named OP-A10, OP-A15, OP-A1, OP-O12 and OP-O19. Figs. 1 and 2 illustrate the RAPD profiles generated by these primers respectively. The presence of bands is expressed as (+) and absence as (-) Tables 3 and 5.

3.2 RAPD-PCR Analysis of Fungal Isolates Grown on Czapek Dox's Medium

Out of 42 DNA bands were detected 20 monomorphic and 22 polymorphic bands. Of the polymorphic bands, two were found to be unique and scored (Table 4). The five primers revealed considerable variation among the studied species and within genotypes of the same species from the different sources. The percentage of polymorphisms for these primers varied from 62.5% (primers OP-O12) to 40.0% (primer OP-A15 and OP-A19). All of the primers generated monomorphic bands. The size of the DNA bands ranged from 1900 bp (OP-A15) to 100 bp (OP-A10). The profiles of the DNA bands varied with the primer used (Fig. 1).

Table 2. List of primers (A and O) and their nucleotide sequence. One Kb DNA Ladder 1µg /µl (Invitrogen) was used

No;	Description	Sequence
1	OP-A10	5-GTGATCGCAG-3
2	OP-A15	5-TTCCGAACCC-3
3	OP-A1	5-CAGGCCCTTC-3
4	OP-O12	5-CAGTGCTGTG-3
5	OP-O19	5-GTGAGGCGTC-3

The primer OP-A10 generated a total of ten DNA bands in the isolated genotype (Table 4). No unique polymorphic bands were identified (for the six bands at approximately 1500, 1350, 900, 800, 300 and 100 bp). With respect to the monomorphic bands generated by the primer OP-A10, four bands were detected (at approximately 1000, 700, 600 and 450 bp) (Table 3). These bands allow further discrimination between the studied *Penicillium* isolates. *Penicillium canescens* isolates shown in lanes 13 and 14 were identified by the presence of two polymorphic bands (300 and 100 bp).

The primer OP-A15 generated a total of four polymorphic bands. Six monomorphic DNA bands were observed in the genotypes of the studied isolates (Table 4). Of the polymorphic bands, none was found to be unique. The four non-unique polymorphic bands that were detected at approximately 1850, 850, 500 and 400 bp represent the most frequently observed bands generated by the primer OP-A15 (Table 3). Monomorphic bands were detected at approximately 1900, 1600, 1300, 1000, 700 and 300 bp. Similarly, these bands provide the potential for further differentiation between the studied genotypes.

Primer OP-A1 generated a total of four monomorphic and five polymorphic bands. One unique polymorphic band was observed in the isolate no. 11 (*Penicillium canescens*), at approximately 1200 bp (Table 3). The four monomorphic bands detected at approximately 1450, 1500, 750 and 600 bp represent the most frequently observed bands generated by the primer OP-A1. The clearest non-unique polymorphic bands generated by the primer OP-A1 are those of the approximate molecular sizes

of 1800, 1600, 1200, 1000 and 400 bp (Table 3). All of the *Penicillium* isolates are characterised by the presence of a band approximately 1000 bp long, except isolate no. 11(*Penicillium canescens*). Isolates no. 1 and 2 of *Penicillium corylophilum* can be identified by one band of approximately 400 bp (Table 3).

In the RAPD profile generated by the primer OP-O19 (Fig. 1), two polymorphic DNA bands were scored out of the total five bands (Table 4). No unique polymorphic bands were observed. The *Penicillium canescens* isolates shown in lanes 13 and 14 were identified by the presence of one polymorphic band (900 bp). The three monomorphic bands that were detected at approximately 1200, 750 and 500 bp represent the most frequently observed bands generated by the primer OP-O19 (Table 3).

Table 3. The presence (1) and absence (0) of DNA in the generated RAPD profiles of the 14 *Penicillium* isolates grown on Czapek Dox's medium

Primer	Molecular weight in bp.	*Penicillium* isolates													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
OPA10	1500	-	+	+	+	+	+	+	-	-	+	-	-	-	-
	1350	+	+	+	+	+	+	+	+	-	+	-	-	-	-
	1000	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	900	+	+	+	+	+	+	-	-	-	+	-	-	+	+
	800	-	-	-	+	+	+	+	+	+	+	+	-	-	+
	700	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	600	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	450	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	300	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	100	-	-	-	-	-	-	-	-	-	-	-	-	+	+
OPA15	1900	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1850	-	-	-	-	-	-	-	-	+	+	+	+	-	-
	1600	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1300	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1000	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	850	+	+	+	+	-	-	-	-	-	-	-	-	+	+
	700	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	500	-	-	-	-	+	+	+	+	-	-	-	-	+	+
	400	-	-	-	-	-	-	-	-	+	+	+	+	+	+
	300	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPA1	1800	+	-	-	-	-	-	-	+	-	+	-	-	-	-
	1450	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1600	+	+	+	+	+	+	-	+	-	-	-	-	-	-
	1500	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1200	-	-	-	-	-	-	-	-	-	-	+	-	-	-
	1000	+	+	+	+	+	+	+	+	+	+	-	+	+	+
	750	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	600	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	400	+	+	-	-	-	-	-	-	-	-	-	-	-	-
OPO19	1200	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1000	+	+	+	+	-	-	-	-	-	-	-	-	-	-
	900	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	750	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	500	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPO12	1300	+	+	+	+	+	+	+	+	+	+	+	+	+	-
	1100	-	-	-	-	+	+	+	+	+	+	+	+	+	-
	1000	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	850	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	800	+	+	+	+	-	-	-	-	-	-	-	+	+	+
	700	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	550	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	300	-	-	-	-	-	-	-	-	-	-	-	-	+	+

A total of three monomorphic and five polymorphic bands were generated by the primer OP-O12 (Table 4). One unique polymorphic band of approximately 550 bp was identified the *Penicillium canescens* isolate shown in lane 14. The clearest non-unique polymorphic bands generated by the primer OP-O12 are those at approximately 1300, 1100, 800 and 300 bp (Table 3). The *Penicillium canescens* isolates in lanes 13 and 14 were identified by the presence of one polymorphic band (300 bp).

Table 4. Number and type of the amplified DNA bands as well as the percentage of the total polymorphisms generated by five primers in *Penicillium* isolates grown on Czapek Dox's medium

Primer code	Monomorphic bands	Polymorphic		Total bands	Polymorphic %
		Unique	Non unique		
OP-A10	4	-	6	10	60.0%
OP-A15	6	-	4	10	40.0%
OP-A1	4	1	4	9	55.5%
OP-O19	3	-	2	5	40.0%
OP-O12	3	1	4	8	62.5%
Total	20	2	20	42	-------

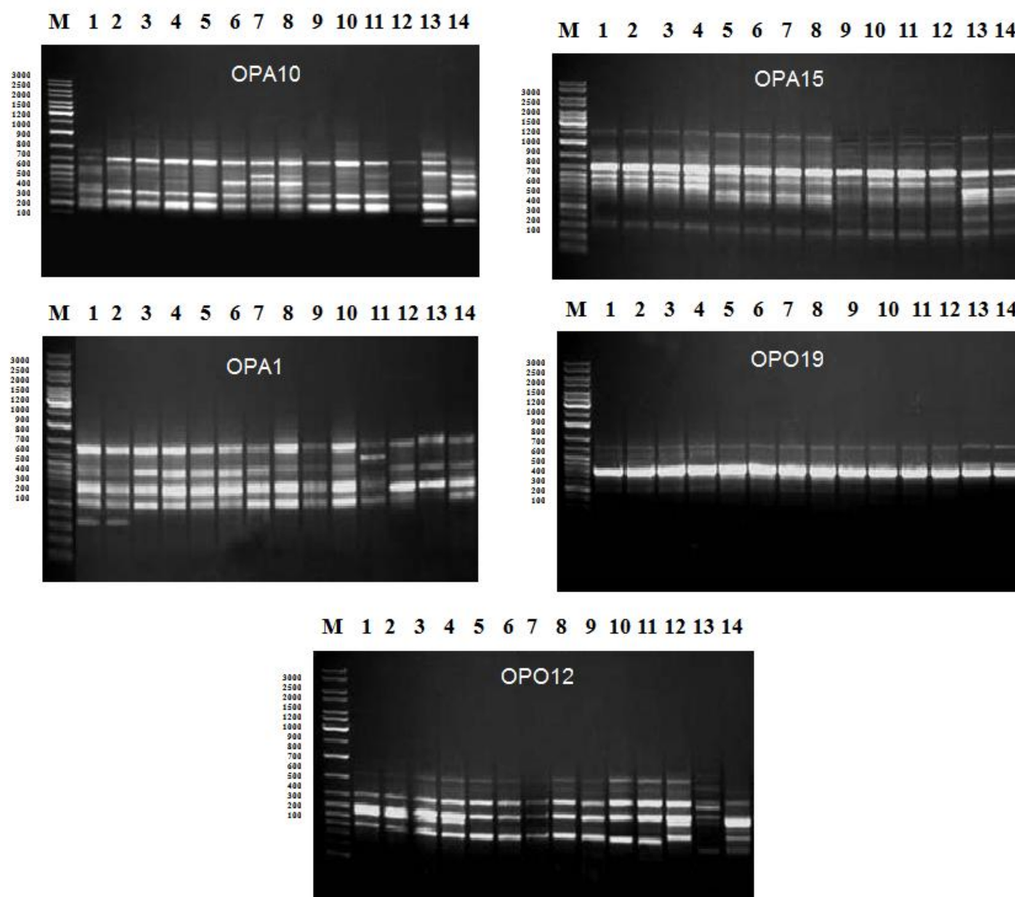

Fig. 1: RAPD fingerprints of the 14 of *Penicillium* isolates grown on Czapek Dox's medium with the primers OPA10, OPA15, OPA1, OPO19 and OPO12.

3.3 RAPD-PCR Analysis of Fungal Isolates Grown on Waksman's Medium

Out of 43 DNA bands detected, 20 were monomorphic and 23 were polymorphic bands. Of the polymorphic bands, no band was unique (Table 6). The five primers revealed considerable variations between the studied species and within genotypes of the same species from the different sources or habitats. The percentage of polymorphisms for these primers varied from 72.7% (primers OP-A10) to 42.9% (primer OP-A1 and OP-O19). All of the primers that were used generated monomorphic bands. The size of the DNA bands ranged from 2300 bp (OP-A19) to 200 bp (OP-A15). The profiles of DNA bands varied with the primer used (Fig. 2).

Eight polymorphic DNA bands were generated by the primer OP-A10, but no unique band was detected (Table 6). The most frequently observed non-unique polymorphic bands that were generated by the primer OP-A10 are those with approximate molecular sizes of 2100, 1800, 1600, 1500, 700, 450, 350 and 300 bp (Table 5). A DNA band at the molecular weight of approximately 700 bp was present in all *Penicillium* isolates except isolate no. 1 (*Penicillium corylophilum*). The *Penicillium canescens* isolates shown in lanes 13 and 14 were identified by the presence of two polymorphic bands (at approximately 350 and 300 bp).

The primer OP-A15 generated a total of five polymorphic bands in the studied isolates (Table 6). One of these polymorphic bands (at approximately 280 bp) was in the *Penicillium canescens* isolates lanes 13 and 14. No unique polymorphic bands were identified. With respect to the non-unique polymorphic bands generated by the primer OP-A15, the clearest bands generated are those with approximate molecular sizes of 1500, 700, 480, 280 and 200 bp (Table 5). Five monomorphic bands were detected (at approximately 1650, 1200, 1000, 850 and 600 bp) (Table 5). These bands provide the capability to better discriminate among the studied *Penicillium* isolates.

The primer OP-A1 generated a total of seven DNA bands out of which, four monomorphic bands were observed (Table 6). Three non-unique polymorphic bands were detected at approximately 1600, 1200 and 1000 bp and represent the most frequently observed bands generated by the primer OP-A1 (Table 5). A DNA band of a molecular weight of approximately 1000 bp was present in all *Penicillium* isolates, except isolate no. 13 (*Penicillium canescens*). These bands also provide the potential for better differentiation among the studied genotypes.

Primer OP-O19 generated a total of four monomorphic and three polymorphic bands. No unique polymorphic bands were identified. The four monomorphic bands that were detected at approximately 2300, 1500, 900 and 600 bp represent the most frequently observed bands generated by the primer OP-A1. The clearest non-unique polymorphic bands (three bands) generated by the primer OP-O19 are those with approximate molecular size of 1100, 800 and 400 bp (Table 5).

In the RAPD profile generated by the primer OP-O12 (Fig. 2), four polymorphic DNA bands were found, out of a total of eight bands (Table 6). The *Penicillium canescens* isolates found in lanes 13 and 14 were identified by the presence of two polymorphic bands (at approximately 1200 and 900 bp), and the absence of another band that was present in all other *Penicillium* isolates (at approximately 1100 bp). The four monomorphic bands that were detected at approximately 1080, 780, 550 and 300 bp represent the most frequently observed bands generated by the primer OP-O12 (Table 5).

Although, RAPD analysis effectively provided a sufficient number of polymorphisms to discriminate among the studied species, no single primer could differentiate between all of the studied *Penicillium* genotypes. Therefore, when data from five of the primers were combined together with growth on both cultural media, complete identification and discrimination was achieved for all of the studied isolates, as each of the isolates could be discriminated by one or more unique bands or a group of combined class patterns.

The following isolates of *Penicillium* ssp. cultivated on Czapek Dox's medium couled be identified by a unique polymorphic band generated by the five primers: *P. canescens* (no. 11) could be identified by one band (by OP-A1) of an apparent molecular size of 1200 bp (lane 11) and *P. canescens* (no. 14) could be identified by one band (by OP-O12) of an apparent molecular size of 550 bp (lane 14) (Table 3).

Some other isolates of *Penicillium* ssp. that had been cultivated on Waksman's medium could be identified by the absence of a non-unique polymorphic band generated by the five primers. *P. corylophilum* (no. 1) could be identified by the absence of band (by OP-A10) of an apparent molecular size of 700 bp (lane 1), and *P. canescens* (no. 13) could be identified by the absence of a band (by OP-A1) of an apparent molecular size of 1000 bp (lane 13) (Table 5).

Table 5. The presence (1) and absence (0) of DNA in the generated RAPD profiles of the 14 *Penicillium* isolates grown on Waksman's medium

Primer	Molecular weight in bp.	*Penicillium* isolates													
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
OPA10	2100	+	+	+	+	+	+	-	-	-	-	-	-	+	-
	1800	-	+	+	+	+	+	+	+	-	+	-	+	+	+
	1600	+	+	-	+	+	+	+	-	-	+	-	-	-	-
	1500	-	+	+	-	-	-	-	-	-	+	-	+	+	-
	1300	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1100	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1000	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	700	-	+	+	+	+	+	+	+	+	+	+	+	+	+
	450	-	+	+	+	+	+	+	+	+	+	+	+	+	-
	350	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	300	-	-	-	-	-	-	-	-	-	-	-	-	+	+
OPA15	1650	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1500	-	-	-	-	+	+	+	+	+	+	+	+	-	-
	1200	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1000	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	850	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	700	+	+	+	+	+	+	+	+	+	+	+	+	-	-
	600	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	480	-	-	-	-	+	+	+	+	+	+	+	+	+	+
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	200	-	-	-	-	+	+	+	+	+	+	+	+	-	-
OPA1	1650	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1600	-	-	+	+	+	+	+	+	-	+	-	-	-	-
	1500	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1200	+	+	-	-	-	-	-	-	-	-	-	-	+	-
	1000	+	+	+	+	+	+	+	+	+	+	+	+	-	+
	750	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	600	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPO19	2300	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1500	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1100	-	-	-	-	+	+	+	+	+	+	+	+	-	-
	900	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	800	-	-	-	-	+	+	+	+	+	+	+	+	+	+
	600	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	400	-	-	-	-	+	+	+	+	+	+	+	+	+	+
OPO12	1200	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	1100	+	+	+	+	+	+	+	+	+	+	+	+	-	-
	1080	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	900	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	780	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	550	+	+	+	+	-	-	-	-	-	-	-	-	+	+
	400	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	300	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 6. Number and types of the amplified DNA bands as well as the percentage of the total polymorphism generated by five primers in *Penicillium* isolates grown on Waksman's medium

Primer code	Monomorphic bands	Polymorphic		Total bands	Polymorphic %
		Unique	Non unique		
OP-A10	3	-	8	11	72.7%
OP-A15	5	-	5	10	50.0%
OP-A1	4	-	3	7	42.9%
OP-O19	4	-	3	7	42.9%
OP-O12	4	-	4	8	50.0%
Total	20	-	23	43	-----

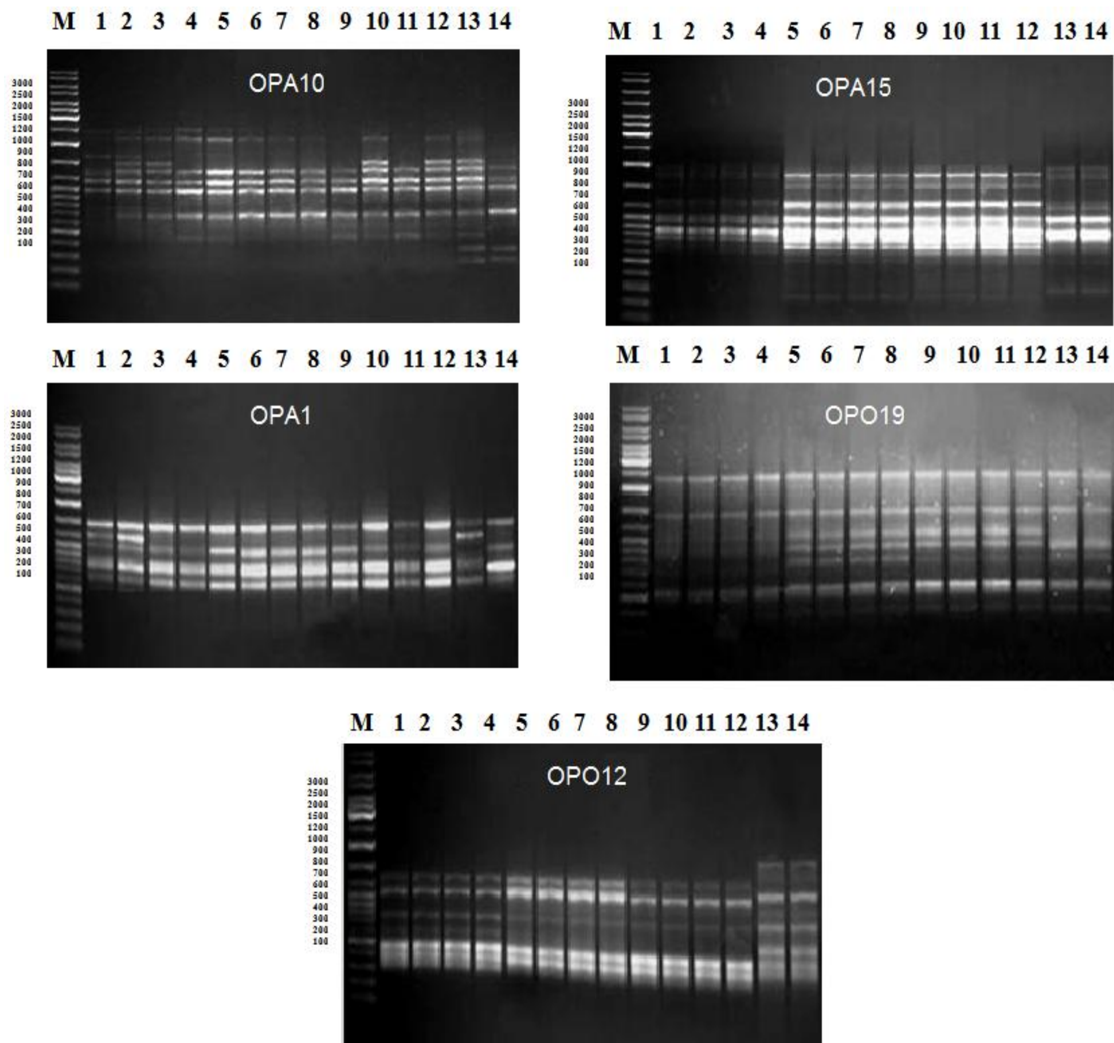

Fig. 2: RAPD fingerprints of the 14 of *Penicillium* isolates grown on Waksman's medium with the primers OPA10, OPA15, OPA1, OPO19 and OPO12.

3.4 Numerical Analysis of the RAPD-PCR Data

The obtained RAPD-PCR data for each isolate were pooled (85 attributes) and coded to create the data matrix used for computation, where "0" represented the absence of a band and "1" represented the presence of a band in each species (Tables 7 and 8). *Penicillium* isolates grown on Czapek Dox's medium 42 attributes and the same isolates grown on Waksman's medium exhibited 43 attributes. The relationships between the studied isolates, expressed by the similarity coefficient, are presented as a phenograms in Fig. 3 (A, B & C).

Table 7. Amplified fragments with all primers obtained from *Penicillium* isolates grown on Czapek Dox's medium

No;	***Penicillium* isolates (OTU'S)**													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1	0	1	1	1	1	1	1	0	0	1	0	0	0	0
2	1	1	1	1	1	1	1	1	0	1	0	0	0	0
3	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	0	0	0	1	0	0	1	1
5	0	0	0	1	1	1	1	1	1	1	1	0	0	1
6	1	1	1	1	1	1	1	1	1	1	1	1	1	1
7	1	1	1	1	1	1	1	1	1	1	1	1	1	1
8	1	1	1	1	1	1	1	1	1	1	1	1	1	1
9	0	0	0	0	0	0	0	0	0	0	0	0	1	1
10	0	0	0	0	0	0	0	0	0	0	0	0	1	1
11	1	1	1	1	1	1	1	1	1	1	1	1	1	1
12	0	0	0	0	0	0	0	0	1	1	1	1	0	0
13	1	1	1	1	1	1	1	1	1	1	1	1	1	1
14	1	1	1	1	1	1	1	1	1	1	1	1	1	1
15	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16	1	1	1	1	0	0	0	0	0	0	0	0	1	1
17	1	1	1	1	1	1	1	1	1	1	1	1	1	1
18	0	0	0	0	1	1	1	1	0	0	0	0	1	1
19	0	0	0	0	0	0	0	0	1	1	1	1	1	1
20	1	1	1	1	1	1	1	1	1	1	1	1	1	1
21	1	0	0	0	0	0	0	1	0	1	0	0	0	0
22	1	1	1	1	1	1	1	1	1	1	1	1	1	1
23	1	1	1	1	1	1	0	1	0	0	0	0	0	0
24	1	1	1	1	1	1	1	1	1	1	1	1	1	1
25	0	0	0	0	0	0	0	0	0	0	1	0	0	0
26	1	1	1	1	1	1	1	1	1	1	0	1	1	1
27	1	1	1	1	1	1	1	1	1	1	1	1	1	1
28	1	1	1	1	1	1	1	1	1	1	1	1	1	1
29	1	1	0	0	0	0	0	0	0	0	0	0	0	0
30	1	1	1	1	1	1	1	1	1	1	1	1	1	1
31	1	1	1	1	0	0	0	0	0	0	0	0	0	0
32	0	0	0	0	0	0	0	0	0	0	0	0	1	1
33	1	1	1	1	1	1	1	1	1	1	1	1	1	1
34	1	1	1	1	1	1	1	1	1	1	1	1	1	1
35	1	1	1	1	1	1	1	1	1	1	1	1	1	0
36	0	0	0	0	1	1	1	1	1	1	1	1	1	0
37	1	1	1	1	1	1	1	1	1	1	1	1	1	1
38	1	1	1	1	1	1	1	1	1	1	1	1	1	1
39	1	1	1	1	0	0	0	0	0	0	0	1	1	1
40	1	1	1	1	1	1	1	1	1	1	1	1	1	1
41	0	0	0	0	0	0	0	0	0	0	0	0	0	1
42	0	0	0	0	0	0	0	0	0	0	0	0	1	1

Table 8. Amplified fragments with all primers obtained from *Penicillium* isolates grown on Waksman's medium

No;	***Penicillium* isolates (OTU'S)**													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1	1	1	1	1	1	1	0	0	0	0	0	0	1	0
2	0	1	1	1	1	1	1	1	0	1	0	1	1	1
3	1	1	0	1	1	1	1	0	0	1	0	0	0	0
4	0	1	1	0	0	0	0	0	0	1	0	1	1	0
5	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1	1	1	1	1	1
7	1	1	1	1	1	1	1	1	1	1	1	1	1	1
8	0	1	1	1	1	1	1	1	1	1	1	1	1	1
9	0	1	1	1	1	1	1	1	1	1	1	1	1	0
10	0	0	0	0	0	0	0	0	0	0	0	0	1	1
11	0	0	0	0	0	0	0	0	0	0	0	0	1	1
12	1	1	1	1	1	1	1	1	1	1	1	1	1	1
13	0	0	0	0	1	1	1	1	1	1	1	1	0	0
14	1	1	1	1	1	1	1	1	1	1	1	1	1	1
15	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17	1	1	1	1	1	1	1	1	1	1	1	1	0	0
18	1	1	1	1	1	1	1	1	1	1	1	1	1	1
19	0	0	0	0	1	1	1	1	1	1	1	1	1	1
20	0	0	0	0	0	0	0	0	0	0	0	0	1	1
21	0	0	0	0	1	1	1	1	1	1	1	1	0	0
22	1	1	1	1	1	1	1	1	1	1	1	1	1	1
23	0	0	1	1	1	1	1	1	0	1	0	0	0	0
24	1	1	1	1	1	1	1	1	1	1	1	1	1	1
25	1	1	0	0	0	0	0	0	0	0	0	0	1	0
26	1	1	1	1	1	1	1	1	1	1	1	1	0	1
27	1	1	1	1	1	1	1	1	1	1	1	1	1	1
28	1	1	1	1	1	1	1	1	1	1	1	1	1	1
29	1	1	1	1	1	1	1	1	1	1	1	1	1	1
30	1	1	1	1	1	1	1	1	1	1	1	1	1	1
31	0	0	0	0	1	1	1	1	1	1	1	1	0	0
32	1	1	1	1	1	1	1	1	1	1	1	1	1	1
33	0	0	0	0	1	1	1	1	1	1	1	1	1	1
34	1	1	1	1	1	1	1	1	1	1	1	1	1	1
35	0	0	0	0	1	1	1	1	1	1	1	1	1	1
36	0	0	0	0	0	0	0	0	0	0	0	0	1	1
37	1	1	1	1	1	1	1	1	1	1	1	1	0	0
38	1	1	1	1	1	1	1	1	1	1	1	1	1	1
39	0	0	0	0	0	0	0	0	0	0	0	0	1	1
40	1	1	1	1	1	1	1	1	1	1	1	1	1	1
41	1	1	1	1	0	0	0	0	0	0	0	0	1	1
42	1	1	1	1	1	1	1	1	1	1	1	1	1	1
43	1	1	1	1	1	1	1	1	1	1	1	1	1	1

The constructed phenogram based on all of the attributes obtained by RAPD-PCR analysis of the 14 isolates of fungi belonging to the genus *Penicillium* is shown in Fig. 3C. This phenogram demonstrates that the examined isolates (OTU's) possess a similarity coefficient level of approximately 1.93. At this level, two isolates of *Penicillium canescens* Sopp. (13 and 14) (2/6) from agricultural soil and wheat grains were split off from the remaining isolates, and could then be distinguished from each other at the level of a 1.25 similarity coefficient. The remaining isolates at the same level (of approximately 1.93) were then divided into GROUP I (composed of four

isolates) and GROUP II (composed of eight isolates). GROUP I (at the level of a1.10 similarity coefficient) included two isolates of *Penicillium corylophilum* (2/3) (1 and 2), the only isolate of *Penicillium rubrum* (3) and one isolate of *Penicillium citrinum* (4) (1/2). GROUP II was divided into two sub-group (A) and (B) at the level of a 1.05 similarity coefficient. Sub-group (A) included the two isolates of *Penicillium canescens* (11 and 12) (2/6) and *Penicillium crustosum* (9), while sub-group (B) included two other isolates of *Penicillium canescens* (8 and 10) (2/6), *Penicillium corylophilum* (5), *Penicillium citrinum* (6) and *Penicillium* sp. (7).

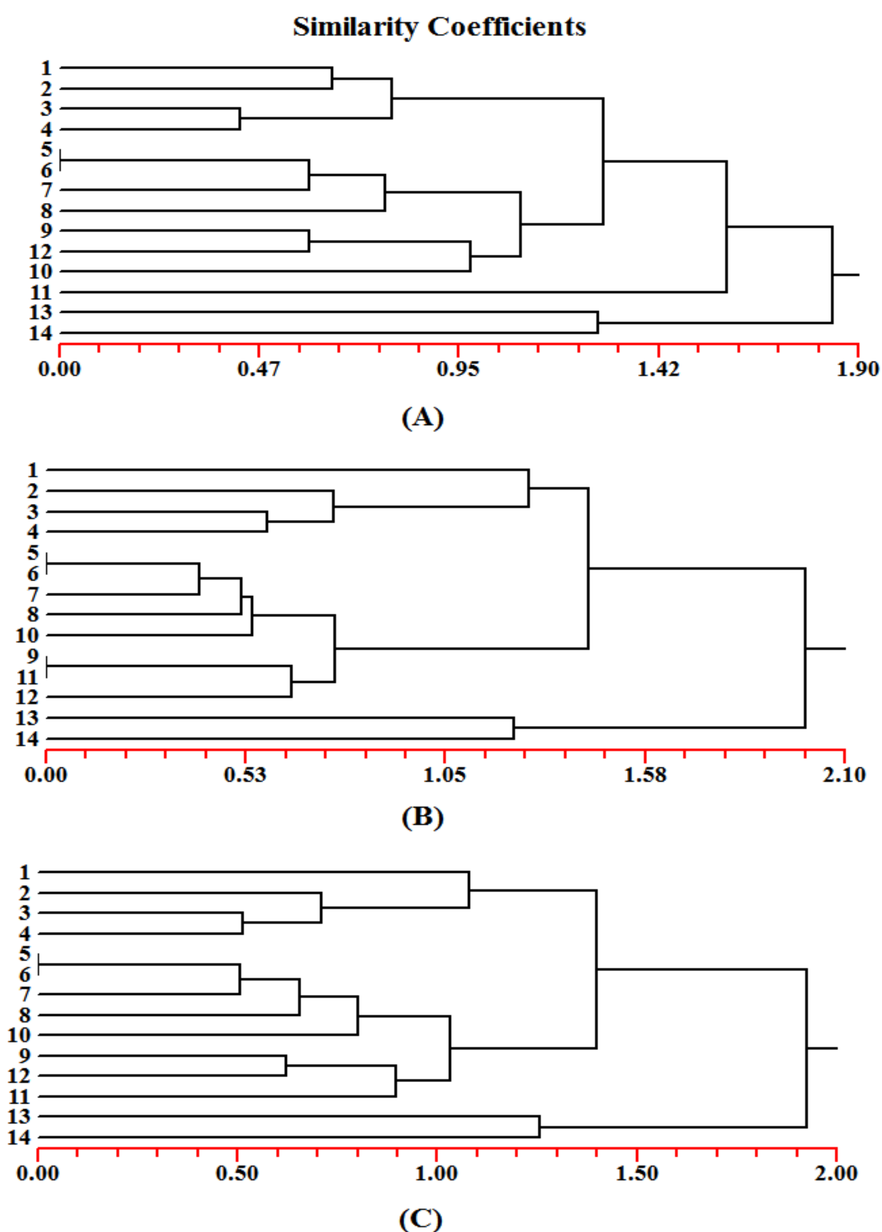

Fig. 3: UPGMA-phenogram constructed from the 14 isolates of *Penicillium* species grown on the following: (A) Czapek Dox's medium, (B) Waksman's medium and (C) both media, based on amplified fragments obtained with all primers.

Recently, techniques that use the polymerase chain reaction have allowed a more representative assessment of genetic variation in fungi through the screening of multiple loci distributed throughout the genome. The analyses reveal sufficient polymorphisms to allow for the examination of fine-scale genetic differences among individuals.

In the present studies only five out of the ten primers succeeded in generating polymorphic and reproducible amplification products. Many authors have reported the use of a large number of primers to identify and characterize many fungal genotypes but the present study, a limited number of primers succeeded in generating distinct and reproducible profiles with sufficient polymorphism.

The genus *Penicillium* is one of the largest and most widely distributed fungal genera described to date. As a result, its taxonomic classification and species discrimination has become complicated [11]. The genus *Penicillium* is known worldwide for the production of secondary metabolites and extracellular enzymes of commercial value, including the pectinases, which are used in the fruit juice industry during the stage of pulp maceration, juice liquefaction or depectinisation [24].

Most *Penicillium* species are considered ubiquitous, opportunistic saprophytes. Nutritionally, they are supremely undemanding, able to grow in almost any environment with a sprinkling of mineral salts and any but the most complex forms of organic carbon, and can grow in a wide range of physical-chemical environments, including variations in aW, temperature, pH and redox potential. The taxonomy of this genus is difficult, as its classification is based mainly on conidiophore and conidia structure, although the colony diameter after incubation under standardised conditions has greater relevance for classification [3].

The RAPD technique is useful in for establishing genetic fingerprinting for the Penicillium genotypes. The RAPD technique can detect enough polymorphisms in the studied genotypes to distinguish each genotype from all of the others by at least one unique band or a group of combined class patterns. The polymorphism information obtained through RAPD analysis may also help to identify polymorphic primers for further studies in other Penicillium genotypes. Additionally, by comparing the two phenograms of each group, the basic relationships that were found between the studied isolates revealed that there is remarkable correlation and variations between the geographical origin (sources) of isolates and the DNA profiles of the studied isolates.

Dupont et al. [25] developed a rapid and accurate molecular tool to discriminate species of white industrial Penicillia. They applied three different Polymerase Chain Reaction (PCR) based techniques (Sequencing of the ITS region of the rRNA gene unit and of the 5' end of the beta tubulin gene yielded 1.2% and 5.8% nucleotide variability, respectively) to distinguish *Penicillium camembertii* and *Penicillium nalgiovense*. They reported that, polymorphic restriction sites were found in both sequences and these may be used in diagnostic PCR-RFLP analysis to rapidly distinguish the two *Penicillium* species [25].

Random amplified polymorphic DNA (RAPD) markers were also useful to distinguish these two species, but no polymorphisms were found at the sub specific level, which indicated a high level of homogeneity of the isolates studied. Using these three techniques, the real identities of the industrial strains of *Penicillium chrysogenum* and *P. nalgiovense* could be demonstrated. The comparison of these isolates with type strains of the two species suggested that the former is identical to *P. nalgiovense*. The genetic relatedness between *P. naglovense* and *Penicillium dipodomyis* was also confirmed.

Boysen et al. differentiated between species on the basis of ribosomal DNA sequences and secondary metabolite profiles from the *Penicillium roqueforti* group associated with spoiled animal feed [5].

In 2007, Bakri et al. [8] reported that random amplified polymorphic DNA (RAPD) analysis was a useful tool to distinguish *Penicillium* species. They used the technique to evaluate genetic diversity among 13 soil *Penicillium* strains originating from widely dispersed areas. Their results demonstrate a high level of diversity of RAPD markers among the strains. All the strains could be identified by their characteristic amplification profile, using selected random primers and they suggested that RAPD analysis is a useful and reliable assay for characterising the species of *Penicillium* genus [8].

Cardoso et al. differentiated between two species from the genus *Penicillium*: *Penicillium expansum* and *P. griseoroseum* (Brasilian isolates) based on morphological and molecular assays (the molecular characterization was based on RAPD markers, telomeric fingerprinting and ITS sequencing) [9].

Redondo et al. [11] studied 52 isolates belonging *Penicillum* genus using two DNA-based methods: (i) analysis of the nucleotide sequences of internal transcribed spacers in ribosomal DNA and (ii) analysis of DNA fingerprints that were generated by polymerase chain reactions using specific primers for enterobacterial repetitive intergenic consensus (ERIC) and repetitive extragenic palindromic (REP) sequences and BOX elements. They concluded that, *Penicillium* species could be distiguished from other fungal genera using both methods. Furthermore, *Penicillium* species used as biocontrol agents, such as *P. glabrum, P. purpurogenum*, and *P. oxalicum*, could be distinguished from other *Penicillium* species using these techniques [11].

They also confirmed that a polyphasic approach including analysis of the nucleotide sequences of ribosomal DNA and detection of highly conserved, repeated nucleotide sequences can be used to determine the genetic relationships among different *Penicillium* species. Based on their results they resulted it could be used as a starting point to develop a strategy to monitor the environmental presence of particular strains of *Penicillium* species when they are used as biocontrol agents.

From one studies it can conclude that random amplified polymorphic DNA (RAPD) analysis is a very useful to differentiate isolates and species of the genus *Penicillium*.

4. CONCLUSION

RAPD Technique is a very useful tool to differentiate isolates and species of the genus *Penicillium*. Also, it could be used to achieve the environmental stress on particular strains of *Penicillium* species which grown in different habitats when they are used as biocontrol agents.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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