Global Veterinaria 8 (2): 119-123, 2012
ISSN 1992-6197

Intraspecies Molecular Segregation of *Penicillium* Species Isolated from Air in Iran Using Rapid Polymerase Chain Reaction Method

[1]*Azar Sabokbar,* [1]*Amir Bakhtiari,*
[2]*Alireza Khosravi and* [1]*Leila Saeednejad Zanjani*

[1]Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Iran
[2]Department of Mycology Reference Center,
Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

Abstract: The usual method in laboratory diagnosis for fungi is culturing and the morphological features will discern the type of species. To make better sensitivity and specificity for determining of pathogenic and allergenic fungi, the molecular biology have been improved. In this study four species of *Penicillium* (30 isolates) including *Penicillium citrinum*, *Penicillium notatum*, *Penicillium oxalicum* and *Penicillium frequentans* were examined. The goal of this survey was to obtain probable intraspecies differences in their molecular patterns. First, isolates were cultured. Then, DNA of fungi has been extracted and RAPD-PCR was performed with three primers and molecular typing has been done. PCR products have been separated on %1/5 agarose gel. Results revealed that the three primers made bands with all of the isolates except isolates 2 and 25. In regard to intraspecies study with primer1 in *P. citrinum* 2 of the 4 isolates reacted and their patterns were not the same. In *P. oxalicum* 3 of the 6 isolates reacted and had completely similar pattern. In *P. notatum* 2 of the 8 isolates reacted and their patterns were approximately the same. In *P. frequentans* 2 of the 12 isolates reacted and their patterns were totally the same. With primer 2: from 4 isolates of *P. citrinum,* 3 isolates have been reacted and their molecular patterns were the same. In *P. oxalicum* from 6 isolates only one of them didn't react and the other 5 isolates formed multiplied pattern which was similar (but not the same pattern). In *P. notatum* all of the 8 isolates reacted and some of them had the same pattern. In *P. frequentans* 7 of 12 isolates reacted and in these 7 isolates, 2 isolates had the same pattern and the others had some differences in their multiplied pattern. With primer 3: in *P. citrinum* just 2 of 4 isolates reacted and their patterns were somehow similar. In *P. oxalicum*, 6 of 8 isolates reacted and their patterns were the same. In *P. notatum*, 6 of 8 isolates reacted and their patterns were the same. In *P. frequentans* 6 of 12 isolates reacted and among this 6 isolates, 3 of them had totally the same pattern and the other 3 isolates showed approximately the same pattern. It was concluded that intraspecies differences were observed with all of the primers and among these three primers, primer 2 showed the most segregation power. Intraspecies difference in *P. frequentans* was more than the other species and its isolates have shown more genetic differences. In any case all of the isolates from these four species were genetically different.

Key words: Penicillium Isolate · Molecular Typing · Intraspecies Classification · RAPD-PCR

INTRODUCTION

Penicillium species are saprophytic fungi which cause food and clinical contamination [1, 2]. These fungi only work is not food and pharmacological contamination, but they have could obtain antifungal and antibacterial substances, for some of them such as Penicillin which is an antibacterial substance and has been extracted from *P. notatum* and the other is Grisovin which has been made from *P. griseofulvin* [3, 4]. These fungi can cause illness in people with weakened immune system, also they are one of the most important allergic fungi which can cause asthma in atopic individuals [5, 6].

Penicillium species usually can not grow in medium including cycloheximide [7]. *Penicillium* can contaminate the medium immediately and their rate of growth is so fast

Corresponding Author: Azar Sabokbar, Department of Microbiology,
Islamic Azad University, Karaj Branch, Karaj, Iran.

in most of the medium. There are numbers of *Penicillium* species which their identification is base on features of macroscopically and microscopically. These days in order to recognize *Penicillium* species, different methods have been illustrated such as Polymerase chain reaction (PCR) and random amplification polymorphism DNA (RAPD-PCR) [8]. The aim of this survey was to obtain probable intraspecies differences in their molecular patterns.

Method of RAPD-PCR with three primers was used for achieving appropriate molecular pattern for intraspecies segregation of *Penicillium* species under study.

MATERIALS AND METHODS

Isolates: Thirty *Penicillium* isolates, obtained from the air of different areas of Iran, were chosen from Fungal Collection of Mycology Research Center, University of Tehran. Table 1 shows the list of these afore fungi.

Mass Production: For preparing DNA, isolates preserved in distilled water were cultured in Sabouraud's glucose agar, after appropriate growth, these fungi were grown in Capek's agar medium at 30°C for 48-72 hours and examined in terms of morphological colony and microscopic examination. For obtaining mass-produce, some fragments of each fungus were transferred to erlens with 600 ml of Sabouraud's glucose broth in condition completely sterile and were stored in shacking incubator at 25°C for 10 to12 days. Fungal colonies were separated from medium by using Whatmann paper, number 1 and were washed with sterile distilled water, three times.

DNA Extraction: For cells disruption, first Freeze-thaw was used and then we did mechanical triturating with glass beads then breaking buffer was prepared: 62/5 moll-1Tris, 1 mmoll-1 Dithioteritol, 0/2 mg ml-1 PMSF, 15%Glycerol, pH=6/8 [9]. Phenol-chloroform method [9] was used for extracting DNA. TES Buffer, 10% SDS, 20 mg/ml proteinase k, saturation phenol, 24/1v Isoamyle alcohol- chloroform, 3M sodium acetate PH=5/2, isopropanol, %70 ethanol and TE Buffer. For perception of the degree of purification of sample DNA, the ratio of absorption of 260 to 280 would be calculated.

RAPD-PCR:

- Three primers were used including:
  Primer 1: 5' GTA TTG CCC T 3'
  Primer 2: 5' GCT GGT GG 3'
  Primer 3: 5' TCA CCC TGC A 3'

Table 1: List of fungi used in RAPD-PCR.

Number	Fungi
1	*P. citrinum* C1
2	*P. citrinum* C2
3	*P. citrinum* C3
4	*P. citrinum* C4
5	*P. oxalicum* O1
6	*P. oxalicum* O2
7	*P. oxalicum* O3
8	*P. oxalicum* O4
9	*P. oxalicum* O5
10	*P. oxalicum* O6
11	*P. notatum* N1
12	*P. notatum* N2
13	*P. notatum* N3
14	*P. notatum* N4
15	*P. notatum* N5
16	*P. notatum* N6
17	*P. notatum* N7
18	*P. notatum* N8
19	*P. frequentans* F1
20	*P. frequentans* F2
21	*P. frequentans* F3
22	*P. frequentans* F4
23	*P. frequentans* F5
24	*P. frequentans* F6
25	*P. frequentans* F7
26	*P. frequentans* F8
27	*P. frequentans* F9
28	*P. frequentans* F10
29	*P. frequentans* F11
30	*P. frequentans* F12

- Master mix(final concentration): 1x PCR Buffer, 3mM Mgcl2,0.5 mM dNTP mix, 20 pmol primer, Taq DNA polymerase 2U, 10ng of DNA and 50µl is the final amount.

Micro tubes containing above materials were transferred to the thermalcycler (Applied biosystem, USA). Amplification was performed for 39 cycles. An initial denaturation 95°C for 5 min, followed by cycle of 94°C for 30 s, 45°C for 30 s and 72°C for 1min, with a final extension at 72°C for 10 min.

Electrophoresis of PCR Products on Agarose gel: PCR products were electrophoresed on %1/5 agarose gel, standard marker was run in parallel and gel was stained with ethidium bromide and Trans Illuminator UV was used for observation of bands.

RESULTS

In this study, three primers were used. In all of the isolates with primers 1, 2 and 3 except isolates 2 and 25, 2 to 17 bands have been observed (Figures 1, 2, 3, 4). With primer 1 among 30 fungal isolates, 9 isolates reacted with 1 to 2 bands. 510 bp (500- 550 bp) bands have been observed with frequency 16.6% and 700 bp band with frequency 26/6% (Table 2). In *P. citrinum*, 2 of 4 isolates (C3 and C4) reacted with primer 1 which had not the same pattern (Table 2), ~510 bp band in C3 isolate has been appeared and 700 bp band in C4. In *P. oxalicum*, 3 of 6 isolates (O3, O4 and O5) reacted with primer 1 showing the same pattern and both of the bands (~ 510 bp and 700 bp) (Table 2). In *P. notatum*, 2 of 8 isolates (N1 and N2) have reacted with primer 1 having approximately the same pattern, 700 bp band with 25% frequencies and ~ 510 bp band have been observed. In *P. frequentans*, 2 of 12 isolates (F9 and F10) reacted with primer 1 which had totally the same pattern and the only band which has observed was 700 bp (Table 2). With primer 2: all of the isolates except 7 of them have reacted with 1 to 17 bands. The bands were: 250, 250-300 (~280), 350-400 (~380), 500, 500-550 (~510), 600, 700, 800, 900, 950-1000 (~980), 1000, 1100, 1200, 1300, 1400, 1500 and 1600 bp. According to intraspecies studies when we used primer 2, 3 of 4 isolates of *P. citrinum* (C1, C3 and C4) have reacted, which had the same molecular pattern and the only band which has been observed in them was ~280bp which had %75 frequency (Table 2). In *P. oxalicum*, just 1 of 6 isolates (O4) did not react and the other 5 isolates formed similar multiplied pattern (but not the same pattern). Bands in *P. oxalicum* were: ~280, 600, 700, 800, 900, 1000, 1200, 1300, 1400, 1500 and 1600 bp that the bands with 600, 800, 1000, 1200, 1300, 1400, 1500 and 1600bp were observed in 4 isolates O5, O6, O7and O10 (Tables 2). In *P. notatum*, all the isolates have reacted with N1 and N2 having the same pattern and N6 and N7 also had the same pattern (Table 2). In this species the following bands were observed; ~280, ~380, 600, 700, 800, 900, ~980, 1000, 1100, 1200, 1300, 1400, 1500 and 1600bp with ~280bp band was observed in all of the isolates and ~980bp has been just observed in N3. 600, 1200 and 1500bp bands observed in N11, N12, N13, N15, N16 and N17 and 900, 700 and 1000bp observed in N1 and N2 were observed. In *P. frequentans*, 7 of 12 isolates reacted which among them F6 and F11 had the same pattern and the others had some differences in their multiplied pattern (Table 2). 250, ~280, ~380, 500, ~510, 600, 700, 800, 900, ~980, 1000, 1100, 1200, 1300, 1400, 1500 and 1600bp bands have been observed in this species. ~280bp band has been observed just in F1 isolates, 900bp band in F2 and 800bp just in F12. 500 and 700bp bands observed in F2 and F12 and 1500 and 1600bp bands with frequency %16/6 in F1 and F2 have been observed. Among bands there are some bands which have the same frequency but they have not been observed in the same isolates (Table 2). With primer 3: 19 of 30 fungal isolates

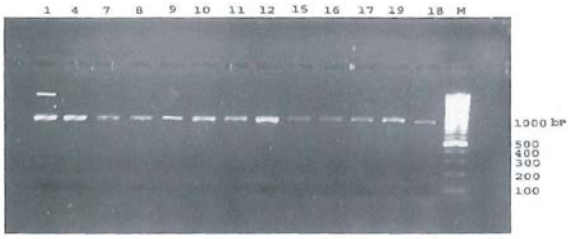

Fig. 1: Results of RAPD-PCR on gel (primer 3)

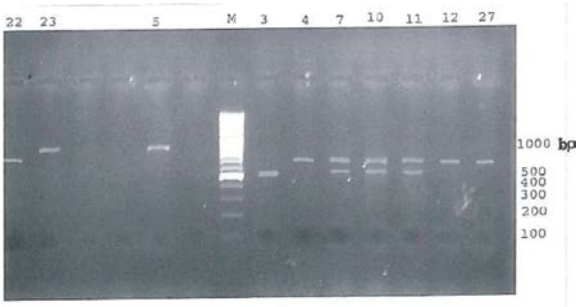

Fig. 2: Results of RAPD-PCR on gel (Isolates 22- 23- 5 with primer 3, isolates 3- 4- 7-10- 11- 12 and 27 with primer 1).

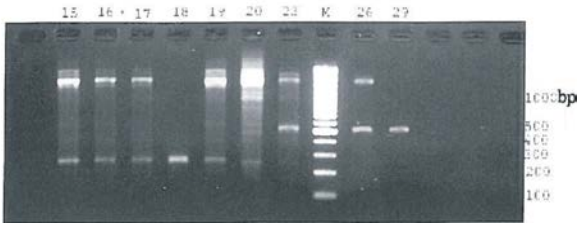

Fig. 3: Results of RAPD-PCR on gel (primer 2).

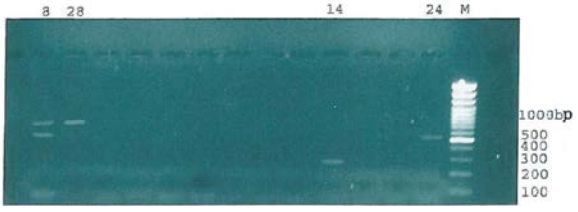

Fig. 4: Results of RAPD-PCR on gel (Isolates 8 and 28 with primer 2, isolates 14 and 24 with primer 2)

Table 2: Results of RAPD-PCR method using 3 primers in *Penicillium* isolates understudy.

	Reacting bands (Bp)																													
	Primer1			Primer 2																				Primer3					
Number					(~280)	(~380)		(~510)						(~980)																
of isolate	500-550	700	Total	250	250-300	350-400	500	500-550	600	700	800	900	950-1000	1000	1100	1200	1300	1400	1500	1600	Total	500	700	800	1000	1500	Total
1	-	-		-	+	-	-	-	-	-	-	-	-	-	-	-					1			-	+	+	2
2	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	+	-	1	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
4	-	+	1	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	+	-	1
5	-	-		-	+	-	-	-	+	-	+	+	-	+	-	+	+	+	+	+	10	-	-	-	+	-	1
6	-	-		-	+	-	-	-	+	-	+	-	-	+	-	+	+	+	+	+	9	-	-	-	-	-	-
7	+	+	2	-	+	-	-	-	+	+	+	-	-	+	-	+	+	+	+	+	10	-	-	-	+	-	1
8	+	+	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	1
9	-	-		-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	+	-	1
10	+	+	2	-	+	-	-	-	+	+	+	+	-	+	-	+	+	+	+	+	11	-	-	-	+	-	1
11	+	+	2	-	+	-	-	-	+	+	+	+	-	+	-	+	+	+	+	+	11	-	-	-	+	-	1
12	-	+	1	-	+	-	-	-	+	+	+	+	-	+	-	+	+	+	+	+	11	-	-	-	+	-	1
13	-	-		-	+	-	-	-	+	-	+	-	+	-	+	+	+	-	+	-	8	-	-	-	-	-	-
14	-	-		-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
15	-	-		-	+	+	-	-	+	-	-	-	-	+	+	+	+	+	+	-	9	-	-	-	+	-	1
16	-	-		-	+	+	-	-	+	-	-	-	-	-	+	+	+	+	+	-	8	-	-	-	+	-	1
17	-	-		-	+	+	-	-	+	-	-	-	-	-	+	+	+	+	+	-	8	-	-	-	+	-	1
18	-	-		-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	+	-	1
19	-	-		-	+	+	-	-	+	-	-	-	-	+	+	+	+	+	+	+	10	-	-	-	+	-	1
20	-	-		+	-	-	+	-	+	+	-	+	-	+	+	+	+	+	+	+	12	-	-	-	-	-	-
21	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	2
22	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	1
23	-	-		+	-	+	-	+	-	-	-	-	-	+	+	-	+	+	-	-	7	-	-	-	+	-	1
24	-	-		-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
25	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	-	-		-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	-	-	3	-	-	-	-	-	-
27	-	+	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	2
28	-	+	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	1
29	-	-		-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
30	-	-		+	-	+	+	-	-	+	+	-	-	+	+	+	-	-	-	-	8	-	-	-	-	-	-
Total	5	8		3	17	6	2	4	12	6	8	5	1	11	8	13	14	13	12	8		1	1	3	16	1	
%	16/6	26/6		10	56/6	20	6/6	13/3	40	20	26/6	16/6	3/3	26/6	36/6	43/3	46/6	32.5	40	26/6		3/3	3/3	10	53/3	3/3	

have reacted with 1 to 5 bands. These bands were: 500, 700, 800, 1000 and 1500bp. In *P. citrinum* only (C1 and C4) have reacted with primer 3 and their patterns were similar (not completely the same). 100bp band and 1500bp band have been observed (Table 2). In *P. oxalicum,* 5 of 6 isolates reacted with primer 3 (O1, O3, O4, 05 and O6) which had completely the same pattern and just 1000bp band has been appeared (Table 2). In *P. notatum*, 6 of 8 isolates reacted with primer 3 (N1, N2, N5, N6, N7 and N8) which had the completely same pattern and just 1000bp band has been appeared (Table 2). In *P. frequentans*, 6 of 12 isolates (F1, F3, F4, F5, F9 and F10) reacted with 3 isolates (F1, F5 and F10) had the same pattern and they only had 1000bp band and the other 3 isolates had approximately the same pattern, they had 500, 700 and 800bp bands (Table 2). In any case intraspecies differences have been observed with all of the primers. We can use primer 1 and 3 for separating *P. citrinum* and primer 2 for *P. oxalicum* and primer 1 and 2 for *P. notatum* and primer 2 and 3 for *P. frequentans*. Intraspecies difference in *P. frequentans* was more obivous than the other species and its isolates have shown more genetically differences. Totally all four species isolates had genotypic differences with each other and were not the same.

DISCUSSION

There are many saprophytic fungi in the environment which their spores usually are distributed in the air, so human and animal are in contact with them through inhalation [10, 11]. *Penicillium* also has a high variety in nature. These fungi can grow on the ruining food stuffs and vegetables and spoil them; also its conidia can enter to lung through inhalation and make infection in especial condition [12-14]. In recent years molecular methods have been dramatically used to discern the fungal infection and its species [8, 15]. PCR which based on the use of DNA polymerase enzyme is a method invented in 1985 and became up to date in 1988. This method is one of the most sensitivity and specificity methods for recognition of important medical fungi and diagnosis of fungus infections [9, 16 and 17].One type of PCR is RAPD-PCR which involves random and particular rather multiply of

genome segments. Primers will band with location which have the highest degree of determined homology according to PCR condition. Recently this method is applied for molecular diagnosis studies of some microorganisms because this method can make different patterns that are used for classification of the microorganisms [18].

In the present study molecular typing of 30 isolates, obtained from different regions of air in Iran has been done with RAPD-PCR. In this 3 primers were used. Among 30 isolates of *Penicillium* 2 of them reacted with none of primers. (Isolate 2 related to *P. citrinum* and isolate 25 related to *P. frequentans)*. Intraspecies differences have been observed with all the primers which in 2 recent surveys based on molecular pattern segregation of isolates such as *Aspergillus* and *Candida* according to RAPD-PCR method, intraspecies differences was less than interspecies [9, 17]. The results of RAPD-PCR using three primers produced different products and indicated intraspecies differences. In accordance with intraspecies differences, different kind of *Penicillium* species were separated with different primers; *P. citrinum* with primers 1 and 3, *P. oxalicum* with primer 2, *P. notatum* with primer 1 and 2 and *P. frequentans* with primer 2 and 3. Intraspecies difference in *P. frequentans* was more than the other species and its isolates have shown more genetic differences. Totally all the isolates of the four studied species had genotypic differences, so they were not equal. In regard to the conclusion, if different multiple primers and more *Penicillium* isolates are used, we can hope to use the RAPD methodology for fingerprinting of *Penicillium* different isolates.

REFERENCES

1. Burge, H.A., 1989. Airborne - allergenic fungi. Immunol. Allergy Clin. North Am., 9: 307-319.
2. Portnoy, J., J. Chapman, H. Burge, M. Muieleberg and W. Solmon, 1987. *Epicoccum* allergy, skin reaction patterns and spore/ mycelium disparities recognized by IgG and IgE ELISA inhibition. Ann. Allergy, 59: 39-43.
3. Adejuwon, O.O., 2011. Synthetic Production of Amylase from *Penicillium* species Isolated from Apple Fruit. World Appl. Sci. J., 13: 415-418.
4. Akhavan Sepahy, A., A. Navabzadeh, M. Larypoor and B. Navabzadeh, 2011. Isolates of Halotolerant *Penicillium* Strains from the Howz Soltan Lake to produce α-amylase. Middle-East J. Sci. Res., 7: 407-412.
5. Shen, H.D., M.F. Tam, H. Chou and S.H. Hwa han, 1999. The importance of serine proteinases as aeroallergens associated with asthma. Int. Arch. Allergy Immunol., 119: 259-264.
6. Salvaggio, J.E. and L. Aukrust, 1981. Mouldinduced asthma. J. Allergy Clin. Immunol., 68: 327-46.
7. Murphy, J.W., H. Friedman and M. Bendinelli, 1993. Fungal infections and immune responses. Plenum press, New York and London, pp: 379-391.
8. Tiwari, K.L., S.K. Jadhav and K. Ashish, 2011. Morphological and Molecular Study of Different *Penicillium* Species. Middle-East J. Sci. Res., 7: 203-210.
9. Rath, P.M. and G. Marggraf, 1995. Use of phenotypic and genotypic finger printing methods in the strain identification *Aspergillus fumigatus*. Mycoses, 38: 429-434.
10. Horner W.E., A. Helbling, J.E. Salvaggio and S.B. Lehrer, 1995. Fungal allergens. Clinical Microbiology Reviews, 8: 161-179.
11. Lehrer, S.B., L. Aukrust and J.E. Salvaggio, 1983. Respiratory allergy induced by fungi. Clin. Chest Med., 4: 23-41.
12. Arshad, S.H., 1997. Development of allergic disease in children. Clinical and Experimental Allergy, 27: 1231-1233.
13. Kurup, V.P., H.D. Shen and B. Banerjee, 2000. Respiratory fungal allergy. Microbes Infect., 2: 1101-1110.
14. Saeednejad, L., A. Sabokbar, A. Khosravi, M. Bayat and A. Bakhtiari, 2010. Determining Protein Patterns for Three Fungus Species *Aspergillus fumigatus*,*Asp. Flavus* and *Asp. Niger*, Obtained from Outdoor Air in Iran. Global Veterinaria, 4: 130-134.
15. Shantha, R., K. Sarayu and S. Sandhya, 2009. Molecular Identification of Air Microorganisms from Municipal Dumping Ground. World Appl. Sci. J., 7: 689-692.
16. Renhsueh, P.O. and L.E.E. Jeneteg, 2000. Molecular evidence for strain dissemination of *Penicillium marneffei*. J. Infect. Dis., 181: 1706-1712.
17. Thanos, M., G. Schonian, W. Meyer, C. Schweynoch, Y. Graser, T.G. Mitchell, W. Presber and H.J. Tietz, 1996. Rapid identification of *Candida* species by DNA fingerprinting with PCR. J. Clin. Microbiol., 34: 615-621.
18. Ajello, L. and R.J. Hay, 1998. Medical Mycology. Volume 4, Ninth Edition, Oxford University press, Inc: New York, pp: 133-157.