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Scientific paper

GENETIC VARIABILITY OF CITRININ-PRODUCING *PENICILLIUM CITRINUM* STRAINS AS OCCUPATIONAL HEALTH HAZARDS IN NORTHERN IRAN

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We evaluated the ability of randomly amplified polymorphic DNA (RAPD) to type citrinin-producing *Penicillium citrinum* (*P. citrinum*) strains recovered from the forest's air in northern Iran. A total of 12 *P. citrinum* strains (P1-P12) were characterised by citrinin production and random amplification of polymorphic DNA (RAPD) technique. All the strains produced citrinin with levels ranging from 1.5 µg mL^{-1} to 39.6 µg mL^{-1} (average value: 12.68 µg mL^{-1}). Of 11 primers tested, eight primers produced polymorphic amplification patterns. These primers generated a total of 105 reproducible RAPD bands, averaging to 13.1 bands per primer. Dendrogram for each primer indicating the distance of the strains to each other was constructed. RAPD results showed that the collected strains constituted four different clusters. The first cluster included two isolates (P1 and P3). The second cluster included seven isolates (P2, P4, P5, P6, P7, P8, and P10). The third and fourth clusters included one isolate (P9) and two isolates (P11 and P12), respectively. We concluded that RAPD analysis might be used in providing genotypic characters for toxigenic *P. citrinum* strains typing in epidemiological investigations and public health related risk assessment.

KEY WORDS: *airborne fungi, genetic variability*, *intraspecific differentiation*, *mycotoxin, random amplification of polymorphic DNA (RAPD)*

Fungi are ubiquitous in the environment and play an important role in decomposing forest litter or dung, fruits or other organic materials. Fruit farms and crops are vulnerable to aerofungal attack and 10 % to 50 % of the world's harvested crops are lost each year due to fungal attack (1). *Penicillium* species are among the most commonly occurring and economically important members of microfungi family. More than 150 different species have been identified in the genus *Penicillium* (2). They are prevalent outdoor airborne fungi that might cause extrinsic bronchial asthma (3). *Penicillium citrinum* (*P. citrinum*) has been reported to be present in different environmental conditions, ranging from permafrost sediments to agricultural fields and forest soils (4). In addition, it is the most prevalent airborne *Penicillium* species reported in different countries (5-7). It is also the most vigorously studied laboratory taxon that gained importance after the discovery of a well-known mycotoxin citrinin. Citrinin is a toxic secondary metabolite of *P. citrinum*, whose natural habitats are air, soil, rhizophere, and water (8). It is also produced by several other species

of *Aspergillus*, *Penicillium,* and *Monascus* (9). It has been implicated in porcine nephropathy (10) and has been found as a natural contaminant of corn, rice, wheat, rye, barley, and oats (11-13). Thus, citrinin is a potentially important mycotoxin that may be ingested by humans and animals and could cause a chronic disease (14). Although much is known about *Penicillium* physiology and mycotoxin chemistry, one of the main challenges is the rapid and reliable identification of *Penicillium* in many areas including community health care, occupational health, and food safety (15, 16). Molecular markers are a useful tool for assessing genetic similarity and resolving species identities. Among these, randomly amplified polymorphic DNA (RAPD) markers typically have high overall variability and can therefore be useful for microbial characterisation among different species. These markers have been widely used for assessing genetic diversity, genome mapping, and molecular diagnostics of many fungal species (17-19). The purpose of this study was to evaluate the ability of RAPD technique to type several citrinin-producing *P. citrinum* strains isolated from the air samples in northern Iran.

MATERIALS AND METHODS

Strains and cultivation

A total of 12 *P. citrinum* strains (P1-P12), isolated from the air of forest area in northern Iran, were chosen from the Collection of Mycology Reference Centre, Faculty of Veterinary Medicine, University of Tehran, Iran. The isolates were cultured on Sabouraud glucose agar with chloramphenicol (0.005 %, Merck Co., Darmstadt, Germany) and incubated at 28 °C for 10 days. The spores and mycelial mats were harvested, washed with sterile distilled water, and stored at -20 °C until use.

Citrinin determination

Different isolates of *P. citrinum* were cultured on Potato Dextrose Agar with chloramphenicol (0.005 %, Merck Co., Darmstadt, Germany) at 28 °C for 7 days. Spores were suspended and filtered using cheese cloth. Then, they were counted by a hemacytometer and adjusted to a final concentration of 10^5 conidia per mL. An amount of 10 µL from each fungal suspension was inoculated in a flask containing 20 mL of Potato Dextrose Broth and shaken at 200 rpm (8 *g*) at 28 °C for 10 days. Culture filtrates were extracted and estimated for citrinin production based on the Reddy et al. (20) method. Briefly, citrinin was extracted three times with chloroform (1:1). After this, it was pooled and concentrated at 40 °C using a rotator evaporator. Crude extracts were diluted and citrinin level was estimated by thin layer chromatography (TLC) according to the Razak et al. (21) method. Briefly, different volumes (1 µL to 5 µL) of sample extracts were applied to precoated TLC plates (TLC Silica gel 60 F254, Merck, Germany) along with the standard ones (containing citrinin at 0.5 µg mL^{-1}). The plates were developed in toluene: ethyl acetate: formic acid (6:4:0.5) in glass tanks covered with aluminium foil. After development, the plates were dried and observed under long wavelength (365 nm). Citrinin appeared as a fluorescent yellow spot. Finally, the intensity of sample spots was compared with the standard spot and citrinin concentrations were calculated according to Younis and Malik (22).

DNA extraction

A total genomic DNA was extracted and purified using glass bead disruption. An aliquot of fungal material of each isolate was added to 300 µL of lysis buffer [100 mmol L^{-1} Tris, 10 mmol L^{-1} EDTA (pH 8), 1 % SDS, 100 mmol L^{-1} NaCl and 2 % Triton X-100], 300 µL of phenol: chloroform (1:1) and 300 mg of glass beads (0.5 mm in diameter). The samples were strongly shaken for 5 min to perfectly disrupt the cells. Fungal debris was separated by centrifugation at 5000 rpm (5590 *g*) for 5 min, the supernatant was separated, and a volume of chloroform equal to that of supernatant was added. The suspension was centrifuged at 5590 *g* for 5 min once again and the supernatant was separated by adding 0.1 volume of 3 mmol L^{-1} sodium acetate and a volume of 2-isopropanol equal to that of supernatant to precipitate total DNA. Precipitant was washed with 70 % ethanol, air-dried, resuspended in 50 µL of deionised distilled water and stored at -20 °C until use (23).

RAPD-PCR

For RAPD analysis, 11 different primers (*Cinna Gen Co., Tehran, Iran*) were used in this study; P4:5'-GAGCGCCTTG-3', P5:5'-GTGGTGGTGGTGGTG-3', P6:5'-GACAGACAGACAGACA-3', P7:5'-GTGTGTGTGTGTGTGT-3', P8:5'-GATAGATAGATAGATA-3', P10:5'-

CTCTCTCTCTCTCTCT-3', OPU15:5'-ACGGGCCAGT-3', OPD18:5'-GAGAGCCAAC-3', OPα17:5'-GAGCCCGACT-3', OPα04:5'-ACCCGACCTC-3', and R28:5'-ATGGATCCGC-3'. Amplification reactions were done in a final volume of 30 µL. The master mix consisted of 18.6 µL of sterile distilled water, 3 µL of 10x PCR buffer containing 200 mmol L^{-1} $(NH_4)_2SO_4$ and Tris-HCl (pH=8.8), 1 µL of 10 mmol L^{-1} dNTP mix, 2 µL of primer (30 µmol L^{-1}), 3 µL of 25 mmol L^{-1} $MgCl_2$, 0.4 µL of Taq DNA polymerase (5U μL^{-1}, Cinna Gen, Native, without BSA). Reactions were amplified in a programmable thermocycler (Techne, Paya Pajoohesh Pars, Tehran, Iran) for 35 cycles applied for primers P4, OPα04, OPU15, OPα17, OPD18, and R28, 37 cycles applied for primer P5, and 39 cycles for primer P6. The first cycle consisted of 4 min at 94 °C as initial denaturation, 1 min at 31 °C as annealing, and 1 min at 72 °C for extension. The following cycles consisted of 30 s at 94 °C for denaturation, 1 min at 31 °C as annealing, and 1 min at 72 °C, with a final extension at 72 °C for 4 min. The annealing temperature used for primers P5 and P6 was 52 ºC. The PCR products were separated in 1.5 % (W/V) agarose gel electrophoresis in TBE buffer [0.09 mol L^{-1} Tris, 0.09 mol L^{-1} boric acid and 2 mmol L^{-1} EDTA (pH 8.3)], stained with ethidium bromide (0.5 µg mL^{-1}), and then photographed on a transilluminator. DNA marker (100 bp DNA ladder) was used according to the manufacturer's instructions (Cinna Gen Co., Tehran, Iran).

Statistical analysis for RAPD

The pattern of RAPD products was separately obtained for each primer. Amplified DNA fragments (bands) reproducible in two to three reactions were scored as zero (fragment absent) and one (fragment present) in a data matrix. A phenogram was constructed by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) after determining the association coefficients by the simple matching method.

RESULTS

In the present study, the data obtained from the citrinin production of seven-day-old culture filtrates (isolates P1 to P12) were as follows: P1 (16.32±0.6) µg mL^{-1}, P2 (19.27±0.1) µg mL^{-1}, P3 (7.31±1.2) µg mL^{-1}, P4 (3.27±0.8) µg mL^{-1}, P5 (9.28±0.9) µg mL^{-1}, P6 (1.5±0.4)µg mL^{-1}, P7 (8.41±0.3) µg mL^{-1}, P8 (5.66±1.4) µg mL^{-1}, P9 (7.49±0.1) µg mL^{-1}, P10 (6.6±0.7) µg mL^{-1}, P11 (27.4±0.8) µg mL^{-1}, and P12 (39.6±1.2) µg mL^{-1}. All *P. citrinum* strains produced citrinin with levels ranging from 1.5 µg mL^{-1} to 39.6 µg mL^{-1}, representing the average value of 12.68 µg mL^{-1}.

RAPD analysis was performed for 12 toxigenic *P. citrinum* strains using 11 primers of arbitrary sequences. These isolates presented differences in band patterns using only eight primers, whereas primers P7, P8, and P10 did not show any molecular pattern. They generated a total of 105 reproducible RAPD bands, averaging to 13.1 bands per primer. The primer OPD18 showed two bands with molecular weights of 818 bp and 376 bp by a high frequency of 100 %, whereas the primers OPU15 and P5 yielded bands of about 449 bp and 504 bp with frequencies of 91.7 % and 75 %, respectively (Figures 1 to 8). Using primers P6 and R28, two isolates of toxigenic *P. citrinum* did not produce any amplified fragment. All primers amplified the fragments ranging from 0.2 kb

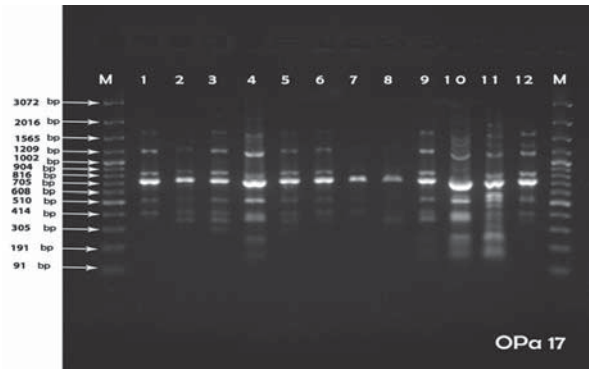

Figure 1 *RAPD-PCR products of 12 isolates of* Penicillium citrinum *with primer OPα17 and 100 bp marker (M: Marker)*

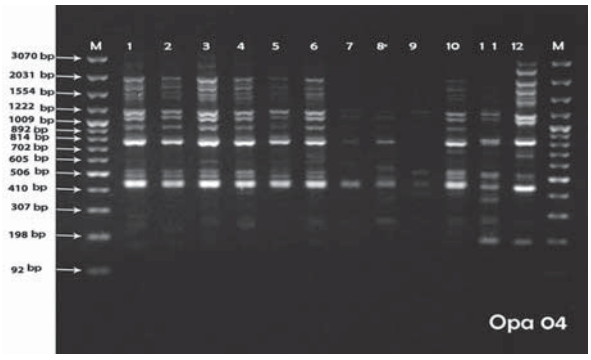

Figure 2 *RAPD-PCR products of 12 isolates of* Penicillium citrinum *with primer OPα04 and 100 bp marker (M: Marker)*

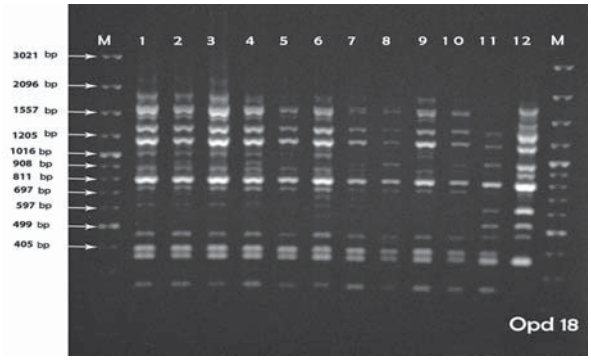

Figure 3 *RAPD-PCR products of 12 isolates of* Penicillium citrinum *with primer OPD18 and 100 bp marker (M: Marker)*

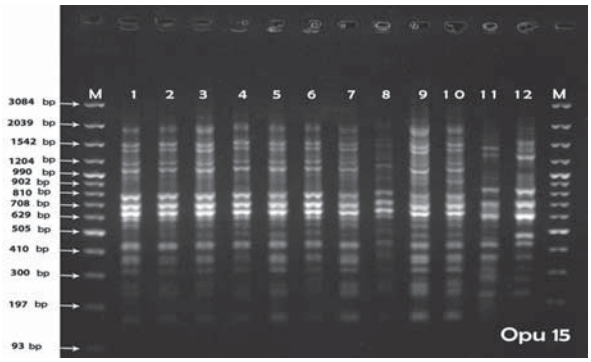

Figure 4 *RAPD-PCR products of 12 isolates of* Penicillium citrinum *with primer OPU15 and 100 bp marker (M: Marker)*

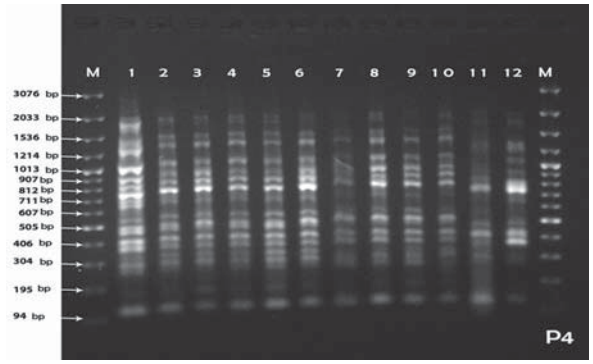

Figure 5 *RAPD-PCR products of 12 isolates of* Penicillium citrinum *with primer P4 and 100 bp marker (M: Marker)*

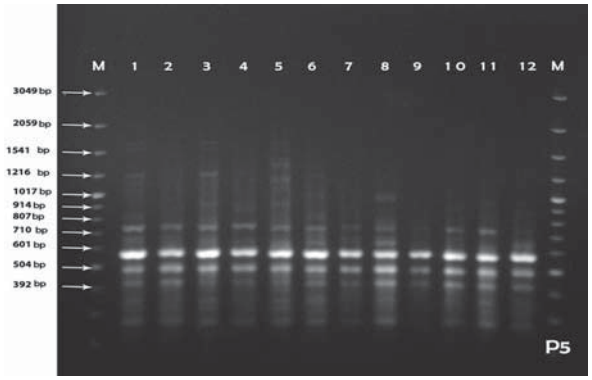

Figure 6 *RAPD-PCR products of 12 isolates of* Penicillium citrinum *with primer P5 and 100 bp marker (M: Marker)*

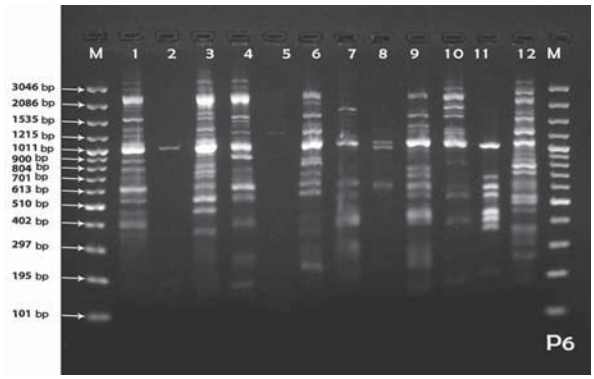

Figure 7 *RAPD-PCR products of 12 isolates of* Penicillium citrinum *with primer P6 and 100 bp marker (M: Marker)*

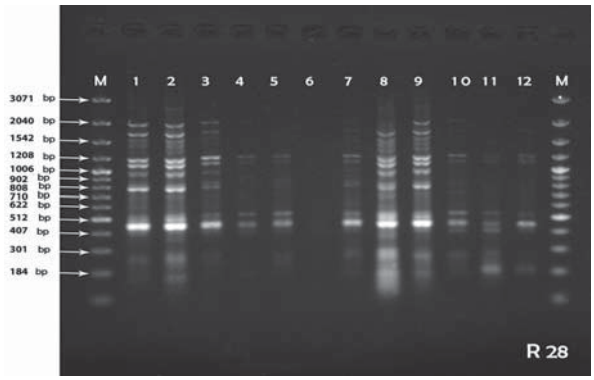

Figure 8 *RAPD-PCR products of 12 isolates of* Penicillium citrinum *with primer R28 and 100 bp marker (M: Marker)*

to 2.7 kb, but primer P6 revealed the fragments above 3.5 kb (3619 bp related to P12 and 3853 bp related to P3 and P4).

The dendrogram and similarity indices constructed from the pair-wise similarity among all *P. citrinum* isolates demonstrated that the tested isolates were categorised into four distinct clusters (Figure 9 and Table 1): the first cluster was formed by isolates P1 and P3 with 75.5 % similarity, the second one was formed by isolates P2, P4, P5, P6, P7, P8, and P10 with 76 % similarity, the third one was formed by isolate P9, and the fourth one was formed by isolates P11 and P12 with 69 % similarity.

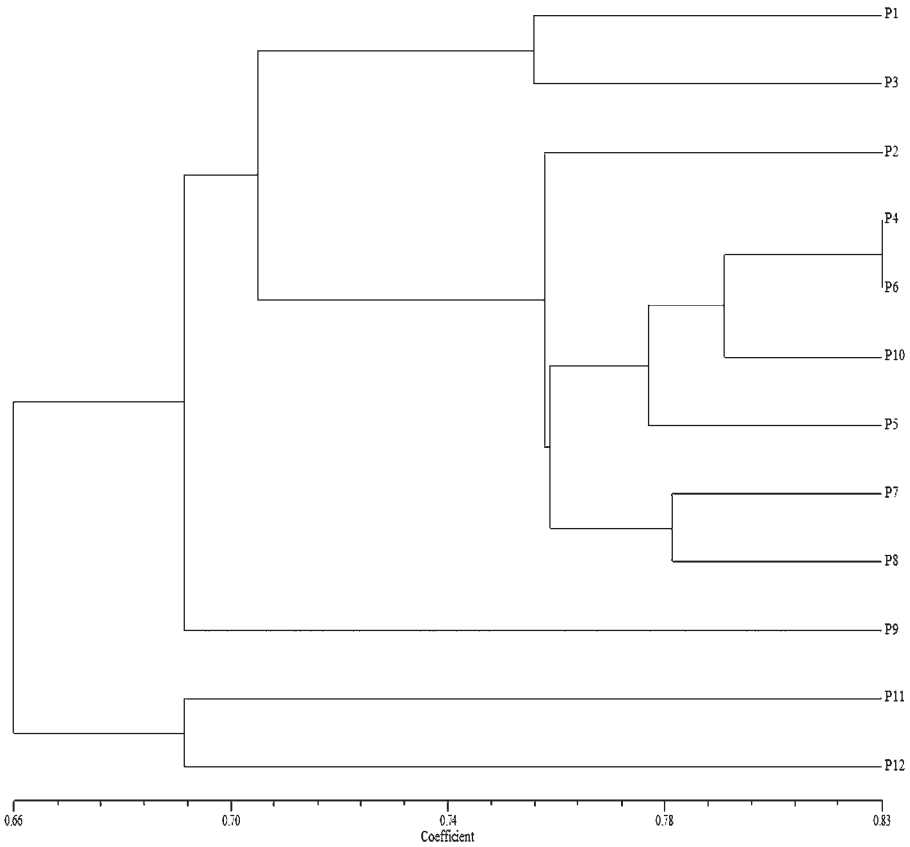

Figure 9 *Phenogram obtained by UPGMA depicting relationships among 12 operational taxonomic units of* Penicillium citrinum *isolates using 34 characters.*

Table1 *Similarity indices among 12 genotypes of* Penicillium citrinum *(P1-P12 isolates)*

Genotypes	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
P1	1											
P2	0.690	1										
P3	0.758	0.683	1									
P4	0.731	0.766	0.793	1								
P5	0.690	0.779	0.766	0.793	1							
P6	0.738	0.759	0.759	0.828	0.759	1						
P7	0.676	0.752	0.628	0.738	0.752	0.731	1					
P8	0.628	0.772	0.662	0.745	0.786	0.779	0.786	1				
P9	0.621	0.683	0.710	0.697	0.683	0.703	0.710	0.703	1			
P10	0.690	0.738	0.724	0.821	0.793	0.772	0.779	0.786	0.696	1		
P11	0.607	0.600	0.614	0.669	0.655	0.676	0.738	0.703	0.669	0.697	1	
P12	0.690	0.579	0.621	0.662	0.634	0.738	0.648	0.655	0.607	0.676	0.690	1

DISCUSSION

The taxonomy of *Penicillium* has always been complex due to its great number of species (nearly 250), which have very few differences. This fact complicates researchers' understanding of their ecology, diversity, and consequently its exploration by industry. Citrinin produced by *Penicillium* species, in particular *P. citrinum*, has been found as a natural mycotoxin contaminant in food- and feed-stuffs (12). Citrinin was found in 100 % of the samples with levels that ranged from 1.5 μg mL^{-1} to 39.6 μg mL^{-1},

representing the average value of 12.68 μg mL^{-1}. The citrinin levels were consistent with those found by Boca et al. (24) and Martins et al. (25), but lower than those reported by Mossini and Kemmelmeier (26). On the other hand, higher levels of citrinin production have been reported by Bailly et al. (27), who analysed *P. citrinum* isolates from different sources.

The genetic diversity of some *Penicillium* species was previously studied by DNA method (28, 29). In this study, eight out of 11 primers tested resulted in the appearance of polymorphic and reproducible PCR products. In PCR amplification with the oligonucleotide primers, 2 to 20 bands, averaging to 13.1 bands per primer, were observed on agarose gel in the region of 0.2 kb to 3.9 kb. This study showed that 105 reproducible RAPD bands were detected among toxigenic *P. citrinum* isolates, 103 of which were polymorphic (98.1 %), while the remaining two bands were common to all samples and could be considered as specific bands for the toxigenic isolates. Primers P5 and OPU15 resulted in the lowest, and primers P6 and R28 in the highest level of variation. Although the genetic variations were shown among different toxigenic *P. citrinum* strains analysed, there was considerable uniformity of banding patterns among these strains. These results are in agreement with the finding reported by both of Abd-Elsalam et al. (30) and El-Fadly et al. (31) who confirmed that molecular markers could be useful in the development of phylogenetic relationships among fungal species. Previously reported data indicated that molecular techniques based on PCR had been used as a tool for genetic mapping, molecular taxonomy, evolutionary studies, and diagnosis of several fungal species (32).

The 12 *P. citrinum* isolates were classified into four clusters, based on the principal coordinate analysis of the similarity index data from RAPDs. Cluster I mostly comprised toxigenic isolates P1 and P3 with 75.5 % similarity, cluster II comprised isolates P2, P4, P5, P6, P7, P8, and P10 with 76 % similarity, cluster III comprised isolate P9 and cluster IIII comprised isolates P11 and P12 with 69 % similarity. In fact, Baba et al. (33) observed very distinctive *Penicillium* species RAPD patterns among the strains isolated from the air origin, indicating dispersal by more than one isolate. In the present study, the strain P9, which was isolated from the same source, also showed very different RAPD profiles suggesting that it probably belonged to different colonies.

We also investigated the relationship between the ability of citrinin production and genetic fingerprinting profile among 12 *P. citrinum* strains. The results represented an RAPD pattern similarity of 69 % for producers P11: 27.4 μg mL^{-1} and P12: 39.6 μg mL^{-1}, whereas the other producers showed different degrees of genetic diversity. Our findings were in agreement with Tran-Dinh et al. (34), who compared the genetic relationship between the toxigenic and non-toxigenic strains of *Aspergillus flavus* and *A. parasiticus* by RAPD, and detected no association between RAPD genotypes and potency of toxin production. Other studies indicated no relationship between RAPD profiles and severity of toxicity (35-37). In contrast, identical RAPD patterns were demonstrated in patulin-producing *Paecilomyces variotii* strains (38), ochratoxin A-producing *Aspergillus* species (39), and aflatoxins-producing *A. flavus* strains (40). We concluded that RAPD analysis can be used in the intraspecific differentiation of toxigenic *P. citrinum* strains obtained from air samples. These variations in the genotype of *P. citrinum* have important implications for the study design when looking at the epidemiology of airborne toxigenic fungi for air quality monitoring.

Acknowledgement

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Sažetak

GENSKA VARIJABILNOST CITRININ-TOKSINOGENIH SOJEVA *PENICILLIUM CITRINUM* KAO IZVOR OPASNOSTI ZA ZDRAVLJE PROFESIONALNO IZLOŽENIH POPULACIJA U SJEVERNOM IRANU

Istražili smo sposobnost tehnike nasumično umnožene polimorfne DNA (RAPD, od engl. *Random Amplified Polymorphic DNA*) za tipiziranje sojeva *Penicillium citrinum* (*P. citrinum*) koji tvore citrinin, izoliranih iz šumskog zraka u području sjevernog Irana.
Na osnovi tvorbe citrinina i primjenom RAPD-tehnike karakterizirano je ukupno 12 sojeva *P. citrinum* (P1-P12). Svi sojevi tvorili su citrinin u koncentracijama od 1,5 μg mL^{-1} do 39,6 μg mL^{-1} (srednja vrijednost: 12,68 μg mL^{-1}). Od 11 testiranih početnica njih 8 dalo je polimorfne uzorke umnožavanja. Te početnice generirale su ukupno 105 reproducibilnih RAPD-vrpca, prosječno 13,1 vrpcu po početnici. Na osnovi podataka za svaku je početnicu konstruiran dendrogram iz kojega su vidljive međusobne udaljenosti sojeva. Rezultati dobiveni primjenom RAPD-tehnike pokazuju da se prikupljeni sojevi dijele u četiri različita klastera. U prvom su klasteru dva izolata (P1 i P3). U drugom je klasteru sedam izolata (P2, P4, P5, P6, P7, P8 i P10). Treći klaster sadržava jedan izolat (P9), a četvrti njih dva (P11 i P12). Zaključili smo da se RAPD-analiza može primijeniti u istraživanju genotipskih značajki toksinogenih sojeva *P. citrinum,* epidemiološkim istraživanjima i procjeni rizika povezanog s javnim zdravstvom.

KLJUČNE RIJEČI: *genska varijabilnost, gljive u zraku, intraspecifična diferencijacija, mikotoksin, nasumično umnožena polimorfna DNA*

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