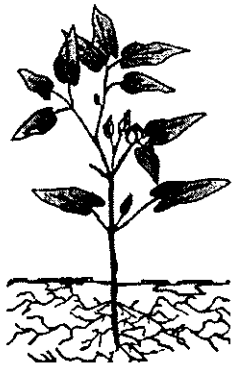

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GENETIC AND METABOLIC VARIATIONS AMONG *Penicillium roqueforti* ISOLATES

[1]

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ABSTRACT

Eleven isolates belonging to *Penicillium roqueforti* were classified from the analysis of their crude extracts by a polyphasic approach with data processing, using the profiles of their fatty acids; secondary and volatile metabolites as taxonomic markers for these isolates, as well as random amplified polymorphic DNA-polymerase chain reaction pattern (RAPD-PCR) technique for the phylogenetic relationship analysis at the intraspecies level using six universal primers. The study showed that with the harmony of all the four investigated markers, about all of the investigated isolates could be classified correctly at the intraspecific level using only the analysis of metabolites produced on one growth medium (YES), except in case of the volatile profile which succeeded as cladogenetic profile but not as a strain marker. The study revealed the ability of RAPD-PCR technique to evaluate the genetic diversity among the investigated isolates at the sub-species level, as well as a rapid and easy method than traditional characterization techniques. Other relations between isolates could be read from the dendrograms and the efficient classification showed the potential of this polyphasic approach identification system.

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INTRODUCTION

Fungi play critical roles in human and animal health, agriculture, food industry and biotechnology. However, until recently, their importance in these areas had not been fully appreciated. As a result of this, there is a pressing need to improve the accuracy and speed of the diagnosis of fungal infections, to identify the sources of individual cases and outbreaks of these infections, and to understand the patterns of genetic variation and evolutionary potentials in populations of pathogenic fungi **(Jianping, 2008).**

Penicillium is one of the most economically important genera among filamentous fungi. Much of their economic impact is deleterious, with food spoilage, mycotoxins production and biodeterioration heading the list, but in fact their potential for economic utility is equally important. It was more difficult to differentiate the species that belonged to the subgenus *Penicillium* **(Smedsgaard *et al* 2004).**

The major industrial uses of *P.roqueforti* are for the production of blue cheeses, flavoring agents, anti-bacterial, polysaccharides, proteases and other enzymes. While, the chief industrial use of it is in the production of Roquefort cheese, stilton and other blue cheeses which has been eaten by human since about 500 years ago. Unfortunately, there is evidence to indicate that most strains are capable of producing harmful secondary metabolites (alkaloids and other mycotoxins) under certain

growth conditions. *P.roqueforti* is considered a Class 1 Containment Agent under the IMI Guidelines for Research Involving Recombinant DNA Molecules (IMI, 2007).

Cellular fatty acid (FA) composition is routinely determined in bacterial systematics (Moss, 1981 and Veys *et al* 1989). Both the type of fatty acid present and its relative concentration are useful characteristics for separating taxa. Until recently, these techniques were only rarely used in fungal taxonomy. Although fewer different fatty acids are produced by fungi than by bacteria (Lechevalier and Lechevalier, 1988), these analyses are increasingly used for differentiating fungi (Augustyn *et al* 1990; Brondz and Olsen, 1990; Amano *et al* 1992; Blomquist *et al* 1992; Stahl and Klug, 1996).

Chemotaxonomic studies of large number of isolates in *Penicillium* have shown that secondary metabolites (SMs) have a potential for the characterization of its species and for phylogenetic relationships. Thus, it complements morphological data to give a fuller description of an important part of the phenotype that may be perceived by other organisms. Chemical analysis of SMs will provide more objective and comparable results than traditional description of color and odor (Smedsgaard and Frisvad, 1997).

The scope of the use of fungal volatile metabolites (VMs) in the detection and classification of fungi is likely to be in the rapid detection of unwanted fungal growth and in the separation of closely related species that are difficult to distinguish by other methods (Zeringue *et al* 1993).

Similarly, it has been demonstrated by Frisvad *et al* (1998) that a large number of related species in genus *Penicillium* could be classified based solely on their profiles of VMs, a finding that may be true for other genera.

Some studies have evaluated a large number of primers to identify only a selected few isolates that can successfully discriminate genetic strain types. Kac *et al* (1999) evaluated fifteen RAPD primers identifying merely one that was highly discriminatory for strains of *Trichophyton mentagrophytes*. So, RAPD technique was evaluated as a reliable tool with good reproducibility of the patterns for each investigated strain as affirmed by Stemmler *et al* (2001). But problems of interpretation due to inconsistent intensity of bands in different PCR runs may arise for less experienced personnel. RAPD analysis can be performed within one working day and needs less DNA compared with RFLP, so, costs will be reduced.

Most of the known established techniques and designing options of fungal taxonomy have been validated for only a few dozen of fungal strains and the lack of efficient genetic engineering strategic forms still an obstacle for a multitude of identifying fungi producing commercially interesting metabolites. To fully explore their biotechnological capacities, these constraints have to be solved (Vera, 2008).

MATERIALS AND METHODS

Fungal strains: all isolates were tested as an Egyptian local isolates from soil that identified by the RCMB, except one single type strain that was purchased from the International Mycological Institute (IMI) culture collections. All isolates were coded here as they were: (38) *P. roqueforti* (IMI 285518), (39) *P. roqueforti* (RCMB0010093), (40) *P.roqueforti* (RCMB0010094), (41) *P.roqueforti* (RCMB0010096), (42) *P.roqueforti* (RCMB0010097), (43) *P.roqueforti* (RCMB0010099), (44) *P.roqueforti* (RCMB00100910), (45) *P.roqueforti* (RCMB001009I), (46) *P.roqueforti* (RCMB001009II), (47) *P.roqueforti* (RCMB001009III) and (48) *P.roqueforti* (RCMB001009IV).

Media: two types of media were used; Malt Extract Agar (MEA) medium was used for maintenance of the isolates according to Smith and Onions (1983). The other medium type was Yeast Extract Sucrose (YES). This semi-synthetic medium was used in liquid form for the production of intracellular fatty acids form the cultivated isolates (Peter and Michael, 1996), intracellular secondary metabolites (Frisvad and Samson, 2004) and intracellular volatile metabolites (Larsen and Frisvad, 1995; Kristian and Thomas, 2005), as well as for the DNA study (Zhou and Linz, 1999). Media were sterilized by autoclaving at 121°C for 20 min. Mycelia growth from 7 days old cultures on MEA slopes were scraped by using 2 ml of sterile distilled water. Then, 2.0 ml of $4x10^2$ cells/ml spore suspension of each isolate were used to inoculate a 100 ml YES medium in a universal 250 ml flask, and then incubated at 25°C for 7 days, except in case of the DNA analysis that all flasks were incubated with a gentle shaking at 180 rpm. at 25°C for 2 days. Fungal mycelia and pellets were harvested by filtration under aseptic conditions using microcloth and washed thoroughly with sterile distilled water then weighed, decanted in sterile containers and stored at -4°C for further analysis. While for complete DNA analysis, fungal pellets were lyophi-

lized using a freeze dryer system (Heto lyophilizer model Maxi Dry plus). The lyophilized pellets were grounded in a sterile cold mortar using sterile pestle and decanted in a sterile 1.5 ml microfuge tube. It is worthy to mention that all of the experimental work throughout this research was carried out at the Regional Center for Mycology and Biotechnology (RCMB), at Al-Azhar University excepting for the fatty acids analysis which was achieved at the Central Lab of the Ain Shams University.

Volatile Metabolite Analysis: Intracellular volatile metabolites were extracted from fungal mycelia according to **Evans (2002)** then analyzed using Shimadzu QP 5050A GC/MS supported with a Class 5000 software and Whiley mass spectral data base searchable library.

Fatty Acid Analysis: Intracellular fatty acids were extracted according to **Peter and Michael (1996).** Gas chromatographic analysis was achieved using Dani GLC-FID 1000. For the complete identification of the resulted compounds, a fatty acids standard was used. This standard was manufactured by Supelco[tm], containing mixture of 37 fatty acids methyl ester (C_4- C_{24}) dissolved in methylene chloride.

Secondary Metabolite Analysis: Extraction, analysis and identification of intracellular secondary metabolites were carried out using the TLC plate technique of the automatic scanner system (HPTLC Scanner 3 -CAMAG, Switzerland) using griseofulvin as reference standard. The identity of the metabolites was performed by comparing shape, color and R_f values of the recorded spots with those given at **Paterson and Bridge (1994).**

Fungal DNA Extraction: DNA extraction was conducted using DNeasy kit (Qiagen, Germany).

RAPD-PCR: Amplification reaction mixture solution was prepared in a final volume of 50 µl containing: 3 µl (200 ng) of genomic DNA; 1 µl of 50 pmole of each desired primer; 25 µl of the Go Taq Green Master Mixture (Promega Co.) and deionized RNase-DNase free water in sufficient amount to give the total reaction mixture volume of 50 µl. The amplification was performed using Research Programmable Thermal Cycler (gradient Robocycler 96 Stratagene, USA) where the applied program was as follows: universal denaturation cycle (5 min. at 94°C), 45 cycles of annealing/extension reactions (30 sec. at 94°C, 1 min. at an optimum annealing temperature 36°C for each used universal primer and 2 min. at 72°C) and cycle of final extension step (5 min. at 72°C) was followed by soaking at 4°C.The sequence of six oligonucleotide universal primers used in the current search were: primer 1: (5'-GGTGCGGGAA-3'), primer 2: (5'-GTTTCGCTCC-3'), primer 3: (5'-GTAGACCCGT-3'), primer 4: (5'-AAGAGCCCGT-3'), primer 5: (5'-AACGCGCAAC-3') and primer 6: (5'-CCCGTCAGCA-3'). These applied primers were of HPSF grade and obtained from the MGW Biotech. Ag. Co.

Horizontal Gel Electrophoresis: The amplified products were separated by agarose gel electrophoresis using a horizontal submarine gel system (Agagel Maxi, Biometra) as well as agarose (Gibco BRL Life Technologies) at a concentration of 2% (w/v). Electrophoresis was conducted in 0.5XTBE buffer (5.4 g of Tris base, 2.75 g of Boric acid, and 2 ml of 0.5 M EDTA [pH 8.0] in 1 liter of distilled water) at 10 v/cm for various times, depending on the size of the gel unit **(Weising *et al* 1995).** DNA bands were stained with ethidium bromide (10 mg/ml) then visualized and photographed under a UV Transilluminator system using a Gel Doc. 2000 (Bio-RAD).

Statistical Cluster Analysis of the Phylogenetic Relationships: The role of the RAPD-PCR patterns as well as the fatty acid, volatile metabolite and secondary metabolite profiles as useful criteria for studying phylogenetic relationships among the investigated penicillia strains was evaluated by using statistical cluster analysis with joining (tree clustering) being the clustering method. Genetic relationships and divergence between RAPD–PCR patterns of the investigated isolates were calculated from the decimal coefficient using the Quantity One (4.0.3) software and were illustrated in dendrograms constructed using the unweighted pair-group method with arithmetic averages (UPGMA). While, each of fatty acid, volatile metabolite and secondary metabolite profiles were amalgamated by a complete linkage using the Elucidation distance as the distance metric as well as the dice coefficient as the calculation method using the Statistica software for Windows release (4.5 F, State Soft. Inc. 1993).

RESULTS

Volatile Metabolite Profile: The accessible outcomes from the volatile metabolite (VM) profile of the tested *P. roqueforti* isolates, discovered twenty two discrete forms of volatile compounds, which can be demoralized efficiently to clarify special difficulties fixed to the morphological alliance of the considered species, (**Table 1**). Unfortunately, referring to their broadly dispenses in the tested isolates, all of the detected volatiles from *P.roqueforti* isolates couldn't be used as chemotaxonomical

markers for the speciation of these penicillia. In a different way, the demonstration of the VM profile appeared to be impenetrable. Accordingly, the cluster analysis tree (**Figure 1**), revealed the cladogenesis of the investigated isolates into two clades based on their VMs content. The first one included *P.roqueforti* numbers 39, 41, 42, 45, 47 and 48, joining them together at a linkage distance between zero and 1.0, which have this closeness among *P.roqueforti* numbers 39, 41, 45 and 47, fusing all of them as one subspecies at a linkage distance of zero. While, both of *P.roqueforti* numbers 42 and 48 appeared to be liable as the same strain due to the closeness of their similarity matrix value, which was 1.0 to the rest isolates of this clade. Amazingly, the second clade, that represented by *P.roqueforti* numbers 38, 40, 43, 44 and 46, adhered them jointly at a linkage distance of zero for all, which give the impression that all isolates of this group were believed to be one identical subspecies. It is worthy to mention that both groups were a parted from each other by a linkage distance of 4.0. Another point that praise worthy to mention was concerning the only *P.roqueforti* tested type strain (*P.roqueforti* number 38), which declared as a photocopy of either of *P.roqueforti* numbers 40, 43, 44 and 46 that were local isolates thanks to the VM marker.

The studied VM profile acquired another importance as a differentiator factor between the groups of the studied species. The phylogenetic affiliations of the considered penicillia were reliable in some cases and detracted in others. Hence, this marker lonely couldn't be applied for the chemotaxonomical purpose of the considered species and in favor of supplementary affirmation, fatty acid (FA) profile as a chemotaxonomical marker had been employed.

Fatty Acid Profile: The presented results from the fatty acid (FA) profile of an eleven *P.roqueforti* isolates, declared nineteen different types of fatty acids, which studied to resolve certain problems associated with the morphological classification of the considered species, (**Table 2**). Fortunately, each of arachidic, elaidic, capric, lauric, γ-linolenic and arachidonic acids were seemed to be successful chemotaxonomic markers according to their minor and restricted allocations among the tested isolates. Additionally, the demonstration of the FA profile looked confusing. So, the phylogenetic tree (**Figure 2**), revealed the grouping of the investigated isolates into three clades. The first clade embraced *P.roqueforti* numbers 38, 39, 40, 43, 44 and 45, joining them together at a linkage distance from 1.4 to 4.0. While, the closest two isolates through this group were *P.roqueforti* numbers 38 and 43 that tied to each other as an identical subspecies at a linkage distance of 1.4. The second clade included *P.roqueforti* numbers 42 and 46 joining them together like a one and the same strain at a linkage distance of 1.7. As the same time, the third clade gathering either of *P.roqueforti* numbers 41 and 47 to the same extent as a replicates at a linkage distance of 2.2. The last point was the separation of *P.roqueforti* number 48 alone a part from the rest clades at a linkage distance of 12.0, which considered so far, indicating the speciation of this strain as a separate subspecies making its own group.

Convincingly, the results of the fatty acid profile as a marker confirmed the correlations between the *P.roqueforti* numbers 38, 40, 43 and 44, as well as among *P.roqueforti* numbers 41 and 47 owing to the VM marker, while both markers argued about the rest isolates; *P.roqueforti* numbers 39, 42, 45, 46, and 48 being located in different groups as illustrated before. So, it seemed extra encouraging allowing for the use of the FA profiling in penicillia speciation, but it wouldn't be likely to corroborate such associations without supplementary consideration using other chemotaxonomical marker such as the compliance of the secondary metabolite (SM) profile.

Secondary Metabolite Profile: The chemical diversity of the in hands secondary metabolite (SM) profile findings of the investigated *P.roqueforti* isolates, revealed twenty two different chemical compounds, (**Table 3** and **Figure 3**). Neither of the detected SMs could be considered as a useful taxonomic marker due to their extensively scattering among the tested *P.roqueforti* isolates, except for the two unidentified SMs with the R_f values of 21 and 40 that were regained from two different isolates, as well as the three unknown SMs with R_f values of 5, 27 and 44 that were found in three different isolates. The last five unknown compounds were the most secondary metabolite compounds of interest thanks to their constrained existence in the investigated *P.roqueforti* isolates. Hence, they could be considered as excellent taxonomical markers throughout the studied *P. roqueforti* isolates and they will be characterized in further studies later on. Moreover, the previous representation of the SM outline seemed to be confuses, particularly for unspecialized scientists in taxonomy. So, it was recommended to use the cluster analysis for this purpose which represented as the phylogenetic relationships (**Figure 4**) of the studied isolates according to their SM content revealed the alliance of the investigated isolates into two clades. The first one included *P.roqueforti*

Table 1. Volatile metabolite profile of the investigated *P. roqueforti* isolates

Compound	*P. roqueforti* isolate numbers										
	38	39	40	41	42	43	44	45	46	47	48
Isobutanol	*	*	*	*	*	*	*	*	*	*	*
Isopentanol	*	*	*	*	*	*	*	*	*	*	*
3-Octanone	*	*	*	*	*	*	*	*	*	*	*
1-Octene	*		*			*	*		*		
Isopentyl Acetate	*		*			*	*		*		
Isobutyl Acetate		*		*	*			*		*	*
Citronellene	*		*			*	*		*		
β-Myrecene	*		*			*	*		*		
p-Cymene	*	*	*	*	*	*	*	*	*	*	*
Limonene		*		*	*			*		*	*
1,8-Cineol		*		*	*			*		*	*
Linalool		*		*	*			*		*	*
(+)Aristocholene	*		*			*	*		*		
β-Patchoulene	*		*			*	*		*		
β-Elemene	*	*	*	*	*	*	*	*	*	*	*
Geosmine	*		*			*	*		*		
β-Caryophillene	*	*	*	*	*	*	*	*	*	*	*
Eremophillene		*		*	*			*		*	*
α-Selinene	*	*	*	*	*	*	*	*	*	*	*
Methoxy methyl benzene	*		*			*	*		*		
1,2-Dimethyl benzene		*		*	*			*		*	*
3-Methyl Furan	*	*	*	*	*	*	*	*	*	*	*

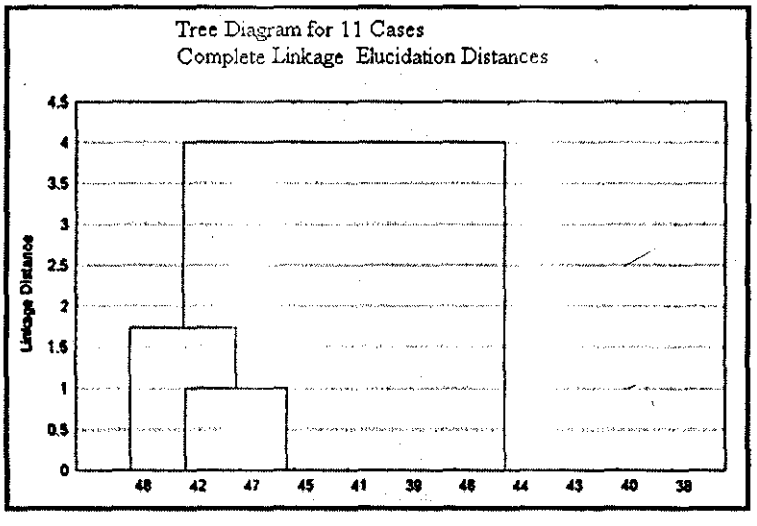

Figure 1. The phylogenetic tree of the considered *P. roqueforti* isolates based on their similarity matrix values of volatile metabolite profile

Table 2. Fatty acid profile of the investigated *P. roqueforti* isolates

Fatty Acid	*P. roqueforti* isolate numbers										
	38	39	40	41	42	43	44	45	46	47	48
Butyric	*	*	*	*	*	*	*	*	*	*	
Caproic	*	*	*	*		*	*		*		*
Caprilic	*		*			*	*		*	*	*
Capric	*		*			*	*				
Laurlc	*		*			*	*				
Tridecanoic	*		*			*	*		*		
Myristic					*		*	*	*	*	
Pentadecanoic	*	*	*		*	*			*		*
Palmitic	*	*			*		*	*	*		
Palmitoleic		*	*	*	*	*	*	*	*	*	*
Margaric	*				*	*	*	*	*		*
Cis-Heptadecenoic	*	*	*		*	*	*	*	*		
Stearic	*	*	*	*	*	*	*	*	*		*
Oleic	*	*	*	*	*	*	*		*	*	*
Elaidic								*		*	
Linolelaidic	*	*			*	*		*	*		*
γ-Linolenic	*	*					*	*			
Arachidic							*				
Arachidonic					*				*		

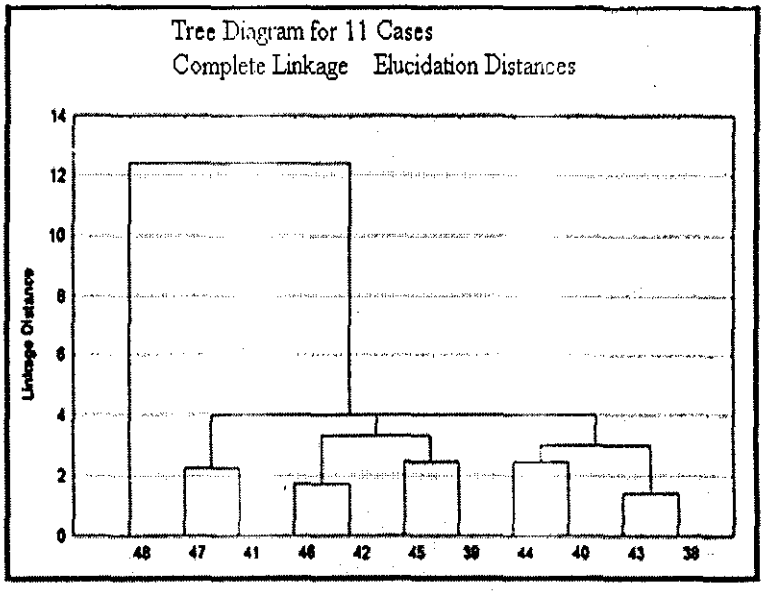

Figure 2. The phylogenetic tree of the considered *P. roqueforti* isolates based on their similarity matrix values of fatty acid profile

Table 3. Secondary metabolite profile of the investigated *P. roqueforti* isolates

Compound Name	*P. roqueforti* isolate numbers										
	38	39	40	41	42	43	44	45	46	47	48
(-)Flavoskyrin	*		*		*	*	*		*	*	*
Brevianamide A	*	*	*	*	*	*	*	*	*	*	*
Citrinin	*	*	*	*	*	*	*	*	*	*	*
Cytochalasin C		*		*		*		*		*	
Dehydrocarolic Acid	*	*	*	*	*				*	*	*
Fulvic Acid	*	*	*	*	*	*	*	*	*	*	*
Hevalonic Acid	*	*	*	*	*	*	*	*	*	*	*
Mycophenolic Acid	*	*	*	*	*	*	*	*	*	*	*
Palitantin	*			*	*	*	*	*	*		*
Pareitin	*	*	*	*	*	*	*	*	*	*	*
Penicillic Acid	*	*	*	*	*	*	*	*	*	*	*
Roquefortine B	*	*	*	*	*	*	*	*	*	*	*
Roquefortine C	*	*	*	*	*	*	*	*	*	*	*
Rugulovasin A	*	*	*		*	*			*	*	*
Viridicatin	*	*	*	*	*	*	*	*	*	*	*
Xanthocillin	*	*	*	*	*	*	*	*	*	*	*
PR Toxin	*		*		*		*		*		*
Unknown (R_f of 27)									*		
Unknown (R_f of 21)							*		*		
Unknown (R_f of 5)							*				
Unknown (R_f of 40)									*		*
Unknown (R_f of 44)											*

Figure 3. TLC plate of *P. roqueforti* isolates secondary metabolites under long UV. Lane G; griseofulvin, lanes 38-48; *P. roqueforti* isolates

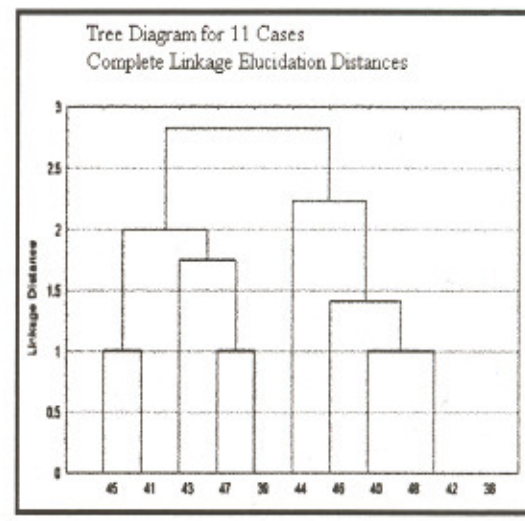

Figure 4. The phylogenetic tree of the considered *P. roqueforti* isolates based on their similarity matrix values of secondary metabolite profile

numbers 39, 41, 43, 45 and 47, joining them together at a linkage distance ranged from 1.0 to 2.0, in which each of *P.roqueforti* numbers 39, 47, 41 and 45 were closely adhered to each other as the same subspecies at a linkage distance of 1.0. While, *P.roqueforti* number 43 was likely to be the same subspecies also due to its closeness to the remainder isolates at a linkage distance of 2.0. Additionally, the second clade which included *P.roqueforti* numbers 38, 40, 42, 44, 46 and 48, combining them together at a linkage distance from zero to 2.2, appearing to join closely the *P.roqueforti* numbers 38 with 42 and 48 like a single matching subspecies at a linkage distance of zero. It is worthy to mention that both clades were connected not far from each other at a linkage distance of 2.8.

It seemed clearly that both of the VM and SM markers were more or less resembling each other referring to the grouping of the tested *P.roqueforti* isolates into two clades that matched in most of isolates (*P.roqueforti* numbers 38, 40, 44, 46 of the first clade and *P.roqueforti* numbers 39, 41, 45, 47 of the second one), but conflicted in the rest isolates (*P.roqueforti* numbers 42, 43, 48) that located in different clades as illustrated before.

Similarly, both of the FA and SM markers agreed in grouping each of *P.roqueforti* numbers (38, 40, 44), (42, 46) and (41, 47) in three separate clades based on FA profile and in two clades according to SM profile, which gathering all of *P.roqueforti* numbers 38 with 40, 44, 42 and 46 as several copies of one subspecies in the same clade.

Apparently, the results obtained from the SM marker confirmed what gained from both of the VM and FA markers concerning the grouping of *P.roqueforti* number 38 as a type strain with *P.roqueforti* numbers 40 and 44 as local isolates like one identical subspecies.

Also, the SM marker results resolved some of the clue concerning what both of the VM and FA markers argued about; which was the speciation of *P.roqueforti* numbers 39, 42, 45, 46, 47 and 48 as said before. So, the SM marker gathered each of *P.roqueforti* numbers 42, 46 and 48 to the group of *P.roqueforti* numbers 38, 40 and 44 as the same subspecies, but each of *P.roqueforti* numbers 39, 45 and 47 still remaindered as a problem that needed to solve.

Conclusively, it was more helpful allowing the use of the secondary metabolite profiling in *P.roqueforti* speciation, which was not an effortless mission.

The succession of each of the FA and SM profiles in resolving some morphological clues associated with *P.roqueforti* at the intraspecific level was noticed. While, the VM profile was failed at this level but considered as a good strain profile. The succession of the chemotaxonomic marker depends on the use of a lot of isolates (70%) of the same species.

RAPD-PCR pattern: Initially, six oligonucleotide primers were tested for their ability to generate RAPD-PCR fingerprinting pattern from the genomic DNA of the eleven investigated *P.roqueforti* isolates. **Figures (5, 7, 9, 11, 13 and 15)** entail the DNA bands of the eight examined *P.roqueforti* isolates that amplified by all primers, which explained as the tree dendrograms represented at **Figures (6, 8, 10, 12, 14 and 16)**. The interactions among *P.roqueforti* isolates developed by primer six were more or less not far from that aroused from the all mentioned primers. Since; all of the studied primers agreed in grouping the eleven examined *P.roqueforti* isolates into two clades a part from primer one that grouped them into three clades. Remarkably, it was noticed that each of *P.roqueforti* numbers 38 along with 40 and 41 were thought to be the same subspecies due to the harmony of the all tested primers apart from primers five and six. Given that *P.roqueforti* numbers 38, 39 and 41 were gathered by primer five, while; only *P.roqueforti* numbers 38 and 39 were gathered by primer six.

Correspondingly, without primers two and three, each of *P.roqueforti* numbers 43, 44 and 45 might be considered as identical duplicates of one subspecies. Additionally, both of *P.roqueforti* numbers 46 and 47 were matched copies of the same subspecies owing to the union of all the studied primers excluding merely primer six. Hence, RAPD-PCR pattern as a chemotaxonomical marker succeeded to a large degree in classifying and differentiating among the investigated *P.roqueforti* isolates.

DISCUSSION

In recent years, there has been substantial progress in the development of innovative methods to analyze fungi (and other organisms) at the molecular level as well as at biochemical level **(Jianping, 2008).**

Considering the current results of using the volatile metabolite (VM) as a chemotaxonomical marker for identifying the eleven isolates of *P. roqueforti*, there was no volatile compound that considered being unique to any isolate of the

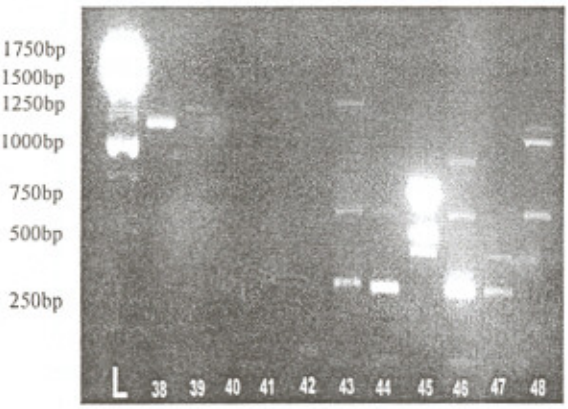

Figure 5. Banding pattern of RAPD-PCR of *P. roqueforti* isolates using primer-1. Lane L; molecular weight marker 250bp ladder; lanes 38-48 *P. roqueforti* isolates

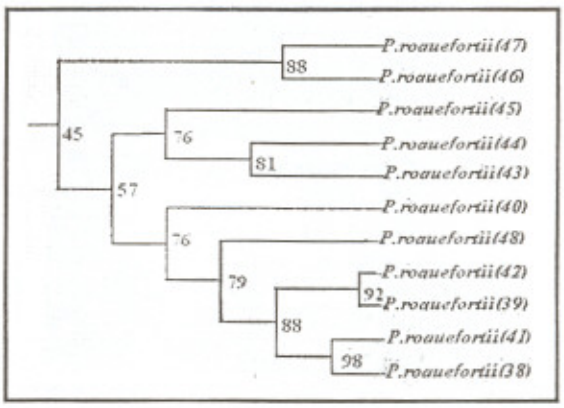

Figure 6. The phylogenetic tree developed from the similarity matrix values of the *P.roqueforti* isolates RAPD-PCR pattern using primer-1

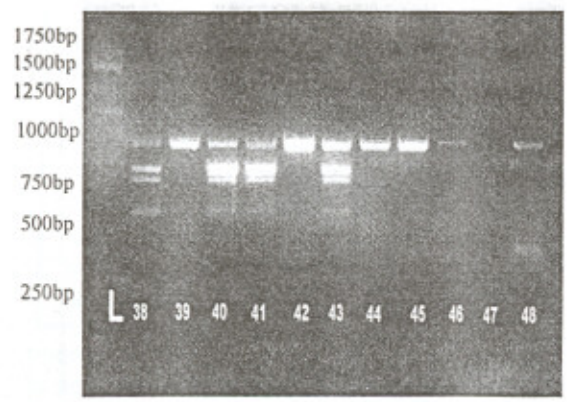

Figure 7. Banding pattern of RAPD-PCR of *P. roqueforti* isolates using primer-2. Lane L; molecular weight marker 250bp ladder; lanes 38-48 *P. roqueforti* isolates

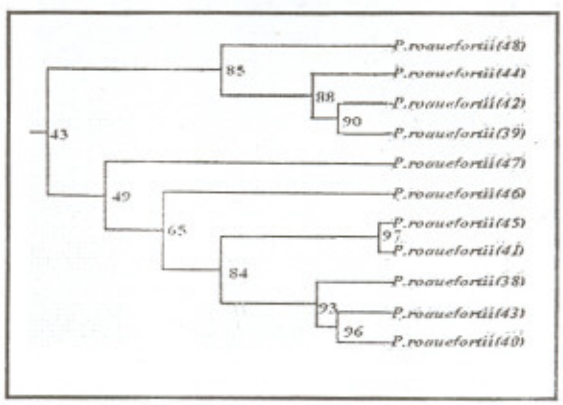

Figure 8. The phylogenetic tree developed from the similarity matrix values of the *P. roqueforti* isolates RAPD-PCR pattern using primer -2

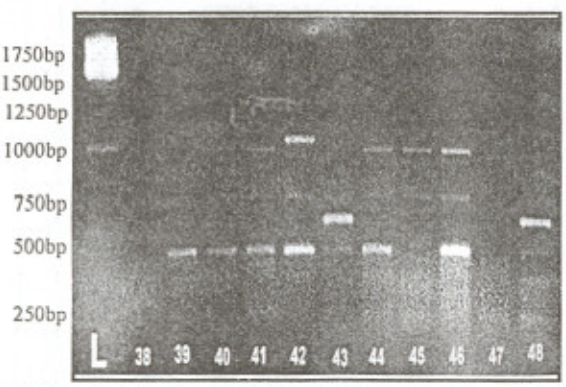

Figure 9. Banding pattern of RAPD-PCR of *P. roqueforti* isolates using primer-3. Lane L; molecular weight marker 250bp ladder; lanes 38-48 *P. roqueforti* isolates

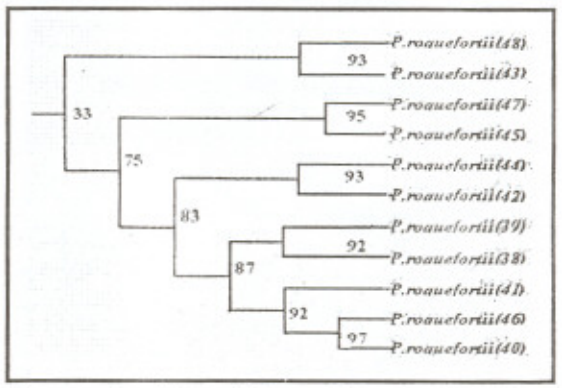

Figure 10. The phylogenetic tree developed from the similarity matrix values of the *P.roqueforti* isolates RAPD-PCR pattern using primer -3

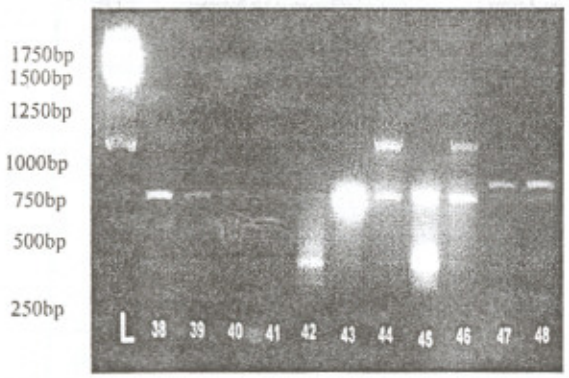

Figure 11. Banding pattern of RAPD-PCR of *P. roqueforti* isolates using primer-4. Lane L; molecular weight marker 250bp ladder; lanes 38-48 *P. roqueforti* isolates

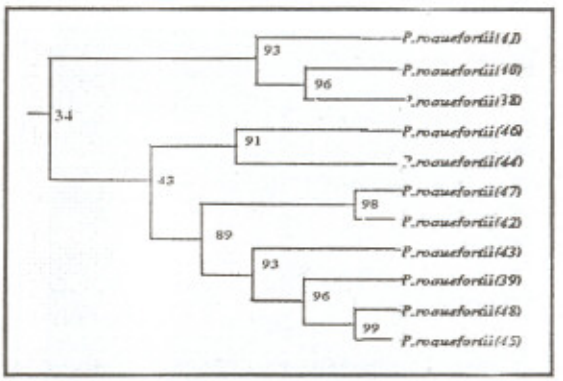

Figure 12. The phylogenetic tree developed from the similarity matrix values of the *P.roqueforti* isolates RAPD-PCR pattern using primer-4.

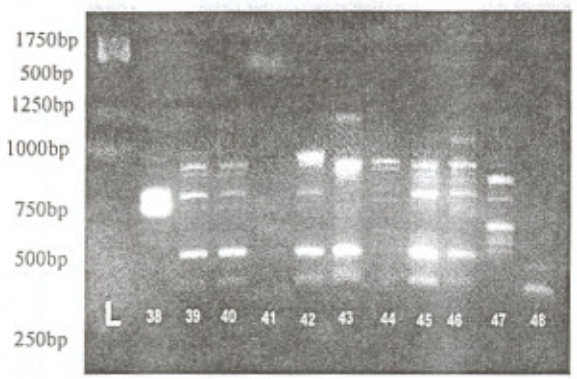

Figure 13. Banding pattern of RAPD-PCR of *P.roqueforti* Isolates using primer-5. Lane L; molecular weight marker 250bp ladder; lanes 38-48 *P. roqueforti* isolates

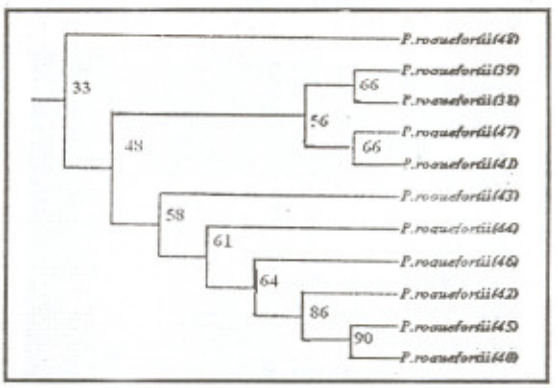

Figure 14. The phylogenetic tree developed from the similarity matrix values of the *P.roqueforti* Isolates RAPD-PCR pattern using primer-5.

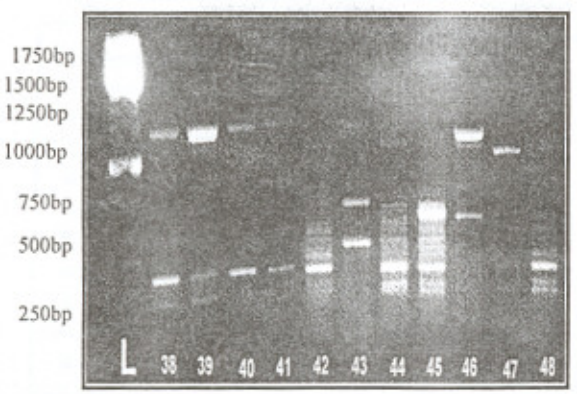

Figure 15. Banding pattern of RAPD-PCR of *P. roqueforti* isolates using primer-6. Lane L; molecular weight marker 250bp ladder; lanes 38-48 *P. roqueforti* isolates

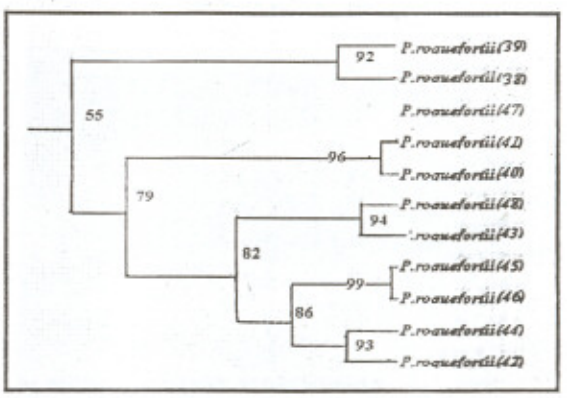

Figure 16. The phylogenetic tree developed from the similarity matrix values of the *P. roqueforti* isolates RAPD-PCR pattern using primer -6.

tested species. These results could be due to the theory stated by **Borjesson (1993)** who concluded that "if few isolates used, it found to be misidentified, and then false taxonomic conclusions might be made". As well it may be in agreement of the topical study by **Frisvad and Samson (2004)**, they declared that a major reason why some literatures reports question the use of fungal metabolites in taxonomy is probably the use of only one or very few isolates of the species studied. Furthermore, some of these few isolates might be incorrectly identified. However, the studied VM profile acquired another importance as a differentiator factor between the groups of the studied species as stated formerly. Volatile profile from different species such as *P.roqueforti* and *P.commune* could easily be distinguished by the studies of **Lund and Frisvad (1994 and 1995)**. Likewise, a total of twenty four isolates *of P.roqueforti* and *P.paneum* from the *P.roqueforti* group differentiated by VM profiling primarily of sesquiterpenes produced on YES medium, some of which they have in common and some of which are unique for the two species **Kristian and Thomas (2005)**. Regarding the only *P.roqueforti* tested type strain (*P.roqueforti* number 38), which declared as a photocopy of either of *P.roqueforti* numbers 40, 43, 44 and 46 that were local isolates thanks to the VM marker. This fallout was in concurrence with what recommended by **Fischer *et al* (2000)** in that isolates of some species in their study were morphologically compared to reference strains from the CBS culture collection (Fungal Biodiversity Centre; Centraalbureau voor Schimmelcultures, Baaren-Nietherland) to confirm proper identification.

With reference to the succession of the fatty acid (FA) profile as a chemotaxonomical marker for the speciation of the inspected eleven *P.roqueforti* isolates, this was in agreement with the conclusion made by **Ricardo *et al* (2001)** who investigated the chemical composition of FAs in eighteen strains of two *Cunninghamella* species, which had shown the presence of four groups. The average percentage of fatty acids of the species *C.elegans* and *C.bertholletiae*, showed variations in linolenic and stearic acids, suggesting the possibility of differentiation between the two species using the FA profile. Remarkable outcomes at the present work concerning each of arachidic, elaidic, capric, lauric, γ-linolenic and arachidonic acids as they all were seemed to be successful chemotaxonomical markers thanks to their minor and restricted allocations among the tested *P.roqueforti* isolates. Convincingly, the results of the FA profile as a marker confirmed the correlations between the *P.roqueforti* numbers 38, 40, 43 and 44 as four copies of the same strain as illustrated by the VM marker results.

Since, the secondary metabolites (SM) profile of the studied *P.roqueforti* isolates at the current work introduced very interested results such as, the detection of unique compounds of very restricted distribution (i.e. the five unknown SM compounds with different R_f values) which consequently could be used as an excellent chemotaxonomical markers at the strain level for the examined species, these were in consistency with that concluded by **Lund and Frisvad (1994)**, they stated that "compounds of restricted distribution might be considered as taxonomic markers for the rapid identification of the species, however to ensure their species specificity, a number of isolates belonging to the same species needed to be analyzed". Fungal extract mostly contains compounds from the secondary metabolism, but also some primary metabolites. Not all of these compounds can be used for chemotaxonomic and classification purposes, even though they are of great importance to the fungus. The profile is based on all the secondary metabolites that have differentiation power. Other compounds that are produced in response to the abiotic environments, like cell wall constituents, accumulated different polyols or lipids **(Kock and Botha, 1998)**, can be included in chemical profiling if they have differentiation power, even though they are not secondary metabolites. It is not unusual that different fungal species have one or more secondary metabolites in common **(Frisvad *et al* 2008)**. The in hands results of the SM profiling can be used successfully to resolve certain morphological systematic problems associated to these penicillia. It seemed clearly that both of the VM and SM markers were more or less resembling each other, similarly, both of the FA and SM markers agreed in some cases and disagree in others as illustrated before, therefore in that concern **Frisvad and Samson (2004)** stated that "some of the similar and dissimilar features of the studied species show why it is important to combine a suite of characters in order to classify or identify these fungi correctly". Based on a smaller subset of these characters many species could be placed in synonymy, but taken as a whole the species are indeed very different. Apparently, the results obtained from the SM marker confirmed by what gained from both of the VM and FA markers concerning the grouping of *P.roqueforti* number 38 as a type strain with *P.roqueforti* numbers 40

and 44 as local isolates like one identical subspecies. Referring to all of the SMs that were established at every single one of the investigated *P.roqueforti* isolates, they couldn't be considered here to be useful chemotaxonomical markers due to wide spreading among the testes strains, such conclusion was coherently accepted with what suggested by **Ciegler *et al* (1973)** that"production of similar metabolic products does not provide an adequate basis for recognition of a new taxon", based on the advice of **Frisvad and Filtenborg (1990)** whom was the first to suggest that "only unique extrolites could be used directly in *Penicillium* taxonomy" and this was followed up by a study on many of the species in subgenus *Penicillium* by **Frisvad *et al* (1998)**, where it was shown that only restricted extrolites were of particularly high value in the taxonomic sense.

RAPD pattern produced a profile of bands that allowed the identification of intraspecific polymorphisms among the investigated penicillia. Each primer yielded a strong distinctive pattern for the studied isolates, while the number and the size of the generated fragments were entirely differed from each other. Results of the current study declared that the RAPD-PCR pattern as a chemotaxonomical marker succeeded to a large extent in classifying and differentiating among the investigated *P.roqueforti* isolates. High polymorphism among ten *Penicillium* species was reported by **Pereira *et al* (2002)**, in which the species *P. roqueforti, P. oxalicum, P. griseofulvum, P. brevicompactum, P. camembertii* and *P. charlesii* was demonstrated by the amplifications using molecular characterization based on the RAPD marker among them. Referring to the *P.roqueforti* results, the tree dendrogram of the RAPD-PCR pattern decoded the tested *P.roqueforti* isolates into three clades using primer one and into two clades owing to the rest five primers. The associations among the *P.roqueforti* isolates developed by the entire six studied primers looked near to each other with the exception of primer one. Random primed methods are particularly useful to determine relationships below the level of species as demonstrated by **Hadrys *et al* (1992)**, but depending on the length of the primers and the recognized taxonomic diversity of the group under study, the method may help to discriminate species. The use of different primers for the identification of the microorganisms increased the level of confidence of the identification, but in practice, one primer is usually sufficient **(Pinto *et al* 2004; Foschino *et al* 2004)**. Like wisely, **Bergmann *et al* (2007)** stated that primers with arbitrary sequences give different banding patterns with the same DNA even by applying on the same individual genotypes of species which may be obtained due to the recombination generating species. These results agreed with those of others **(Cadez *et al* 2002; Vasdinyei and Deak, 2003; Fadda *et al* 2004)** which have shown that RAPD-PCR methods performed with different oligonucleotide primers basically generated consistent patterns, with several shared fragments unique to each species. In another study that reported by **Gomes *et al* (2007)**, a remarkable polymorphism was detected among *P.expansum* and *P.griseoroseum* by RAPD analysis that utilized by an adequate number of primers. Finally, RAPD-PCR represented a powerful technique that could be alone used in the discrimination at the intraspecies level under the conditions of using a lot number of universal primers as well as a lot number of isolates. The study of **Frisvad and Samson (2004)** showed that about 70% of the *Penicillium* species can be classified correctly into species level using only the analysis of metabolites produced on one growth medium; such as YES medium. This work demonstrated that with the harmony of all the four investigated markers; the profiles of the fatty acid, secondary and volatile metabolites, as well as RAPD-PCR pattern, about all of the investigated isolates could be classified correctly at the intraspecific level using only the analysis of metabolites produced on one growth medium (YES), except in case of the volatile profile which succeeded as cladogenetic profile but not as a strain marker.

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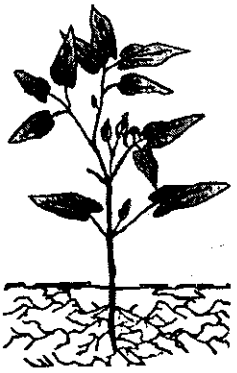
مجلد(٥٤)، عدد (١)، ٣-١٧، ٢٠٠٩



التنوع الجينى و الأيضى بين عزلات *Penicillium roqueforti* [١]

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٢- المركـــز الأقليمـــى للفطريات وتطبيقاتهـــا - جامعـــة الأزهـــر- مدينـــة نصر - القاهـــرة

الموجـــز

اهتم هذا البحث بدراسة احدى عشر عزلـــة مـــن جـــنس *Penicillium* والتـــى تنتمـــى الـــى نـــوع *P.roqueforti* والتى صنفت من خلال تحليل محتواها من المركبات الأيضية المتطايرة والأحماض الدهنية وكذلك المحتوى الأيضي الثانوى بالإضافة إلى تحليل العلاقات الوراثية بين هذه العزلات باســـتخدام تقنيـــة التضخم العشوائى عديد الأوجه المعتمد على تفاعـــل البلمرة المتسلسل (RAPD-PCR) وذلك بتطبيق ســـتة أنواع متابينة من البادئات عشوائية التتابع لاستخدامهم كنموذج بصمة الإصبع للتفرقة بين العـــزلات محـــل الدراسة ، وذلك لمعرفة مدى الاســـتفادة مـــن هـــذه الخصائص كدلالات تصنيفية لتعريف هذا الجنس على وجه العموم وهذه السلالات علَى وجه الخصوص.

وقد اظهرت الدراسة انه باتفـــاق ألأربـــع دلالات التصنيفية محل البحث قد امكن تصنيف كل العزلات المدروسة بنجاح على مستوى السلالات وذلك عنـــد تنميتها على بيئة غذائية موحدة وهى بيئة مـــستخلص الخميرة والسكروز (YES) فيما عدا محتـــوى هـــذه العزلات من المركبات الأيـــضية المتطـــايرة والتـــى نجحت فقط على مستوى المجموعات ولـــيس علـــى مستوى السلالات وذلك نظراً لقلة عـــدد العـــزلات المتاحة من هذا النوع.

هذا وقد كشفت هذه الدراسة عـــن قـــدرة تقنيـــة التضخم العشوائى عديد الأوجه المعتمد على تفاعـــل البلمرة المتسلسل (RAPD-PCR) على تقيـــيم مـــدى التنوع الجينى بين هـــذه العـــزلات علـــى مـــستوى السلالات وذلك كوسيلة سريعة وسهلة مقارنة بالطرق التقليدية الأخرى. وقد امكن كذلك استنباط العلاقـــات بين هذه العزلات من خـــلال الرســـومات الـــشجرية التخطيطية الناتجة عن تحليـــل محتـــوى الـــدلالات التصنيفية الأربعة المدروسة والتى أظهرت فعالية هذه الدلالات كنظام تصنيفى تعريفى لهذه العزلات.

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أ.د اجلال عبد الله غنيمى