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GENETIC VARIABILITIES AND RELATIONSHIPS AMONG SOME EGYPTIAN *ASPERGILLUS* SPP. BASED ON MICROSCOPIC CHARACTERS, ISOZYME PROFILES AND RANDOM AMPLIFICATION OF POLYMORPHIC DNA

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Abstract

Twelve *Aspergillus* spp. were isolated from the Egyptian soils and were studied morphologically and microscopically. Isozyme profiles and random amplified polymorphic DNA (RAPD) were also employed for determination of the genetic relationship among the tested *Aspergillus* spp. Results of RAPD revealed genetic similarity level above 80% between *A. terreus* and *A. aculeatus*. The species *A. niger*, *A. candidus*, *A. terreus* and *A. aculeatus* were joined with a similarity level of 65%. Unique bands were displayed by certain fungi and can be taken as a positive marker for isolate identification. Five isozyme systems; peroxidase, alcohol dehydrogenase, α esterase, β esterase and superoxide dismutase were studied to detect the genetic variabilities among the tested isolates. Subsequently, comparing the three dendrograms which belong to morphological characters, isoymes and RAPD revealed a great relationship between *A. terreus* and *A. niveus* from one side and *A. candidus* and *A. terreus* from the other side**.**

Introduction

The taxonomy of fungal species that belong to Nigri Section is considered a complex process due to the subtle differences between the species. Generally identification of the *Aspergillus* species is based on the morphological characteristics and microscopic studies (McClenny 2005). These traditional methods including colony diameter, color, size and texture of conidia and conidiophore structure (Klich 2002). However, species classification may be difficult due to extensive differences of the above mentioned morphological characters produced by a high level of genetic variability both inter- and intraspecific (Kumeda and Asao 1996).

Recently, molecular marker approaches, such as random amplified polymorphic DNA (RAPD)-PCR (Spesso *et al*. 2013), inter-simple sequence repeat (ISSR)-PCR (Khosravi *et al*. 2012), PCR- restriction fragment length polymorphism (RFLP) (Samuel *et al*. 2013), real time PCR (Bergmans *et al*. 2010) and multiplex PCR assay (Kim *et al*. 2009) were used.

Isozyme analysis is a powerful technique for taxonomic, genetic, and population studies and has been successfully applied for identifying fungal species. Klaas (1998) reported that the isozyme markers can be successfully used in identification of taxa, accessions and individuals, since the assumption of homology can be more accurate than for some genomic DNA markers.

The aim of this study was to determine the relationship among 12 *Aspergillus* spp. isolated from Egyptian soils employing macroscopic, microscopic characters. Moreover, molecular techniques as RAPD analysis of DNA and isozyme profiles were also used for the detection of genetic variabilities of these filamentous genera.

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Materials and Methods

Fungal strains: Twelve *Aspergillus* spp. were isolated from Egyptian soils, Cairo governorate and identified in Taxonomy Department, Ain-Shams University, Egypt. These isolates are; *A. ustus, A. granulosus, A. niger, A. niveus, A. carbonarius, A. glaucus, A. candidus, A. terreus, A. aculeatus,* A. *ochraceous, A. flavus* and *A. crystallinus.*

Microscopic examination and scanning electron microscopy (SEM): The tested isolates were cultured on Czapek-Dox medium for 7 days, microscopic features were studied as size of conidia and vesicle in addition to texture and shape of conidia were examined for species differentiation.

For scanning electron microscopy (SEM), the cultures were fixed in 5% (v/v) glutaraldehyde + phosphate buffer solution for 24 hrs. The samples were then transferred to a graded ethanol dilutions (50, 70, 90 and 100%) for 30 min each and finally to amyl acetate solution (Deo *et al.* 1983). The coated specimens were examined in a Jeol JSM-5600 LV scanning electron microscope.

DNA extraction, amplification and electrophoresis conditions: Genomic DNA of each fungal strain was extracted using glass bead disruption (Yuan *et al.* 1995). DNA amplification was performed in an automated thermal cycler (model Techno 512) according to Williams *et al.* (1990). Amplified DNA fragments were run onto 1.4 % (w/v) agarose gel electrophoresis. The products were detected by staining with ethidium bromide and photographed. The presence/absence of fragments from *Aspergillus* spp. was analyzed using the software package MVSP program of Nei and Li (1979) to estimate the genetic similarity.

RAPD-PCR based procedures: In order to identify the best primers to establish the RAPD profile, 29 arbitrary oligonucleotides from the OPW, OPA and OPX Kits (Invitrogen) were tested, using total DNA from the URM2578 *A. flavus* strain as template. The 5 primers that gave high band number and best reproducibility were; A-01 (5'-CAGGCCCTTC-3'), A-02 (5'-TGCCGAGCTG-3'), A-03 (5'-AGTCAGCCAC-3'), A-04 (5'-AATCGGGCTG-3'), A-05 (5'-AGGGGTCTTG-3').

Data analysis: The software package multi-variant statistical package (MVSP) was used and genetic similarities were computed using the Dice coefficient of similarity of (Nei and Li 1979).

Isozyme profiles of fungal isolates: Enzymes were extracted and 100 µl of the supernatant containing enzymes were mixed with 100 µl of buffer (2.5 mM Tris-HCl (pH 6.8), 10.0 % (v/v) glycerol, 4.0% (w/v) SDS, 0.02% (w/v) bromophenol blue and 10% (v/v) 2-mercaptoethanol). Samples were then loaded directly in the electrophoresis apparatus for isozymes analysis.

Staining of gel and detection of enzymes: The gels were stained for four enzyme systems. The staining protocols were according to Guidkema and Sherman (1980) for peroxidase (E.C.1.11.1.7); for β-esterase (E.C.3.1.1.2), staining protocols were according to Tanksley and Orton (1986), staining protocol for α-aesterase (EC 3.1.1.43) was according to Cheliak and Pitel (1985) and phenylalanine ammonia lyase ((EC 4.3.1.24) staining gel was according to Dickerson *et al.* (1984).

Analysis of data: Multivariate analysis of the isozyme profiles was done and clustering was based on the results of unweighted pair group method using averages (UPGMA) cluster analysis performed on the (Nei and Li 1979) similarity coefficient matrices.

Results and Discussion

Microscopic characters: The microscopic characters of the studied *Aspergillus* spp. are presented in Fig. 1. It was clarified that the tested *A.* isolates have different conidial ornamentation and conidia texture ranged from smooth, rough, echinulate, spinulose and tuberculate. Moreover,

conidial shape was also observed and variated among the species where the majority of the species was characterized by globose to spherical and ellipsoidal shape.

Morphological characters are used as key identifying factors for fungal sp. (Bandh *et al*. 2012). In this respect, Afzal (2013) found that *A. fumigates* showed uniseriates heads, and *A. flavus* var. *columnaris* showed mostly uniseriates heads but a few biseriates heads were also found.

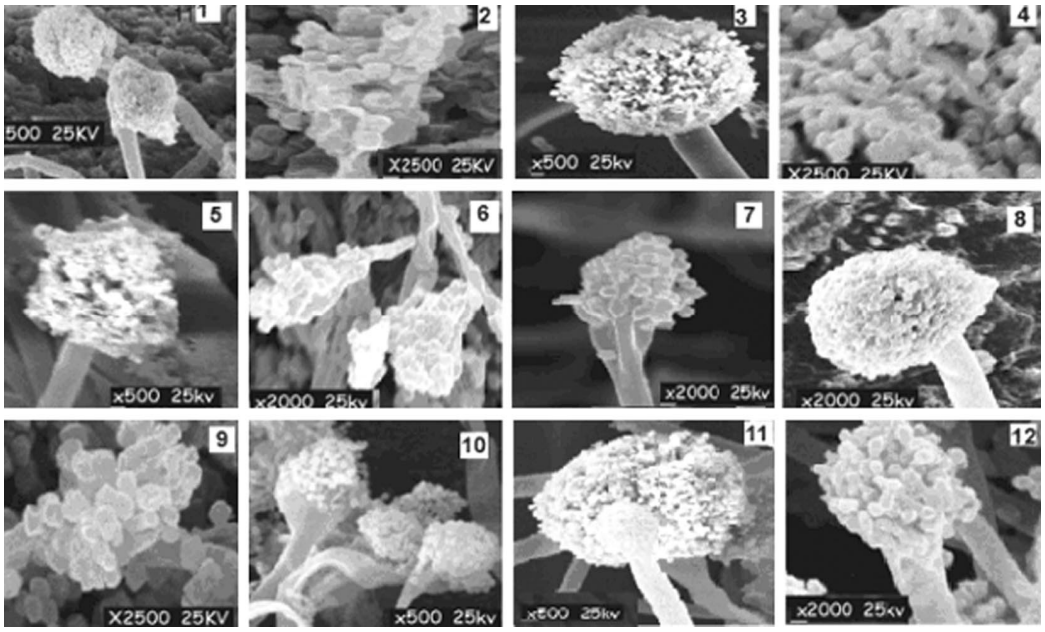

Fig. 1. Scanning electron microscope of the *Aspergillus* spp.

Molecular genetics studies of Aspergillus spp.: The results of RAPD analysis using A-01, A-02, A-03, A-04 and A-05 primers are presented in Fig. 2. It is apparent that there are monomorphic bands which are different in density and accounted for 44.8% of the total bands. While, polymorphic bands showed differences and could be used to examine and establish systematic relationship among the genotypes. In the same manner, Siddiquee *et al.* (2010) stated that RAPD would be the markers of choice, since it offers the advantage of being technically undemanding. Consequently, they usually reveal more polymorphism compared with isozymes or RFLPs, which are always representative of conserved genome regions. The five random primers used resulted in RAPD profile with variable bands for each of the studied *Aspergillus* spp., providing evidence of its high genetic diversity.

This result is similar to the report of Yuan *et al.* (1995) who used RAPD to differentiate between *A. parasiticus* and *A. sojae*, which are similar morphologically. These results support the use of RAPD finger printing for identification of *A. flavus* strains using different primers, as demonstrated previously for different *Aspergilli* strains (Rath *et al*. 2002).

Isozymes electrophoresis: As shown in Fig. 3, the electrophoretic patterns of peroxidase revealed one monomorphic band for all 12 species of the genus *Aspergillus*. However, marked variation in density and intensity were detected. While, the electrophoretic patterns of alcohol dehydrogenase enzyme revealed a marked variation in the occurrence, density and intensity of

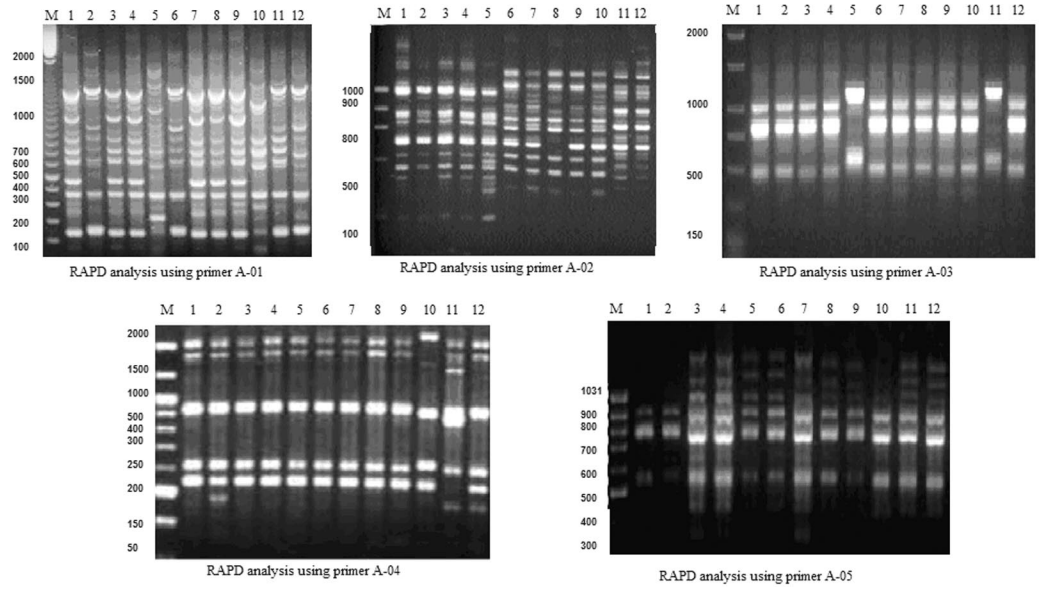

Fig. 2. RAPD band profile obtained with A-01, A-02, A-03, A-04 and A-05 primers of the studied *Aspergillus* spp.

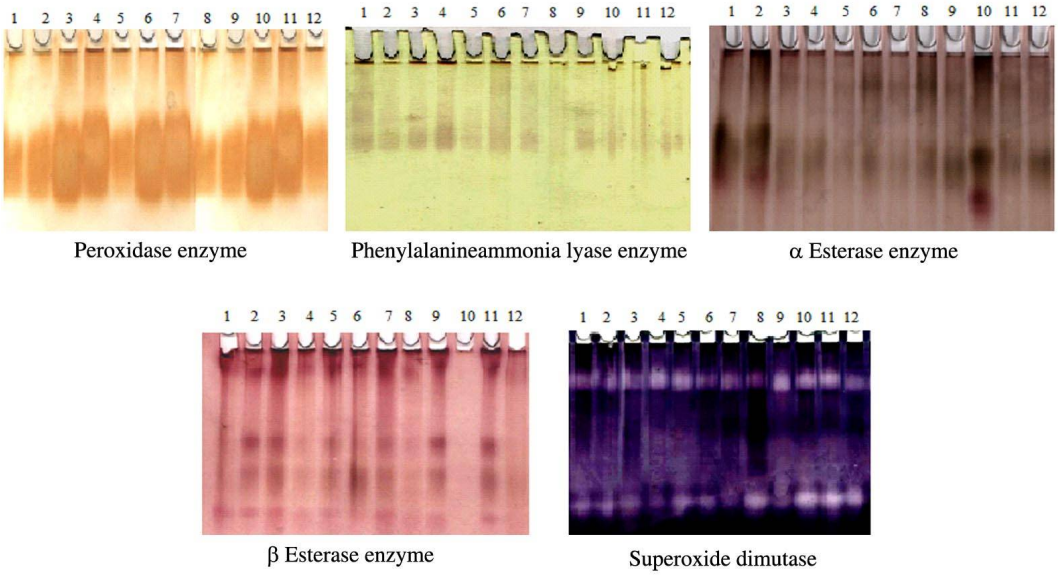

Fig. 3. Isozymes patterns of the studied *Aspergillus* spp.

bands among the studied species where, two bands were detected in *A. ustus*, *A. granulosus*, *A. niger*, *A. niveus*, *A. carbonarius* and *A. aculeatus*, while one band only was appeared in *A. glaucus*, *A. candidus*, *A. terreus*, *A. aculeatus*, *A. flavus* and *A. crystallinus*. Two bands were appeared in α-esterase and superoxide dismutase enzyme patterns for all *Aspergillus* spp., these

bands differ in density and intensity. On the other hand, the electrophoretic patterns of β-esterase enzyme of the tested *Aspergillus* spp. were very much similar.

In this regard, Siddiquee *et al.* (2010) used eight enzymes and single protein pattern systems to identify and study the genetic relationships among 27 *Trichoderma harzianum* isolates, 10 isolates of *Trichophyton aureoviride* and 10 isolates of *Trichophyton longibrachiatum* from Southern Peninsular Malaysia. They found that three isozymes and total protein patterns were useful for the detection of three *Trichoderma* spp.

Dendrograms analysis: Phylogenies generated in this work revealed that taxa possessing similar characters may not necessarily be phylogenetically related. The phenogram (Fig. 4) demonstrating the relationships among the studied *Aspergillus* spp. which based on diameter of conidia (μm) and diameter of vesicles (mm) have yielded two clusters (Fig. 4). The first cluster included *A. carbonarius* only, however, the second cluster included all the studied *Aspergillus* spp. except *A. carbonarius* showing that this strain is not closely related to the other spp. Also, It was noticed that there was no distance between *A. terreus and A. niveus*, this revealed the very strong relationship between them. Additionally, *A. crystallinus* and *A. ustus* are correlated at major distance of 1.8%; this revealed the high association between these two species.

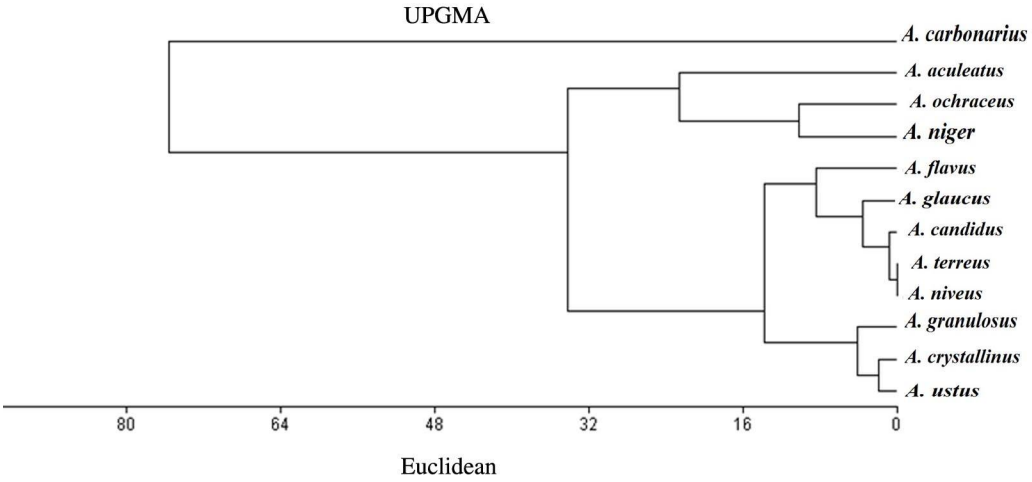

Fig. 4. Phenogram demonstrating the relationships among the studied *Aspergillus* spp. based on diameter of conidia (μm) and diameter of vesicles (mm).

The phenogram (Fig. 5) demonstrated the relationships among the studied *Aspergillus* spp. based on texture and shape of conidia yielded three clusters. This third cluster is subdivided into two subclusters in each of them there was a strong association of 100% between its members while, *A. ochraceus* and *A. candidus* are far away of each other.

In this respect, Samson *et al.* (2004) found that although, the morphological and microscopic characterization are popular methods but it is time consuming and are not sufficient to differentiate different fungal species due to their intra- and interspecific morphological divergences.

The dendrogram (Fig. 6) using the similarity matrix produced with the banding patterns obtained with the above mentioned primers showed the formation of a main group consisting exclusively of *Aspergillus* spp. It is clear from this dendrogram that there is a similarity level above 78% between *A. terreus* and *A. aculeatus*, while, 68% similarity between *A. ustus* and *A. niveus*. Morever, *A. candidus*, *A. terreus* and *A. aculeatus* were joined with *A. niger* at a similarity

level of 65%, while, *A. niger* joined at 51% similarity near *A. crystallinus*. *A. carbonarius* was joined at 46% similarity near *A. flavus* and *A. crystallinus*.

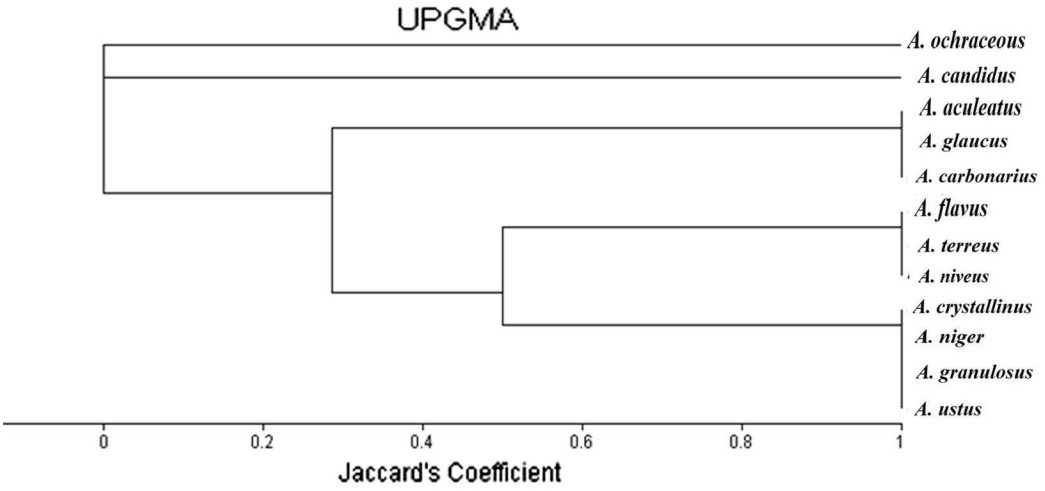

Fig. 5. UPGMA-phenogram based on texture and shape of conidia of the studied *Aspergillus* spp. (UPGMA: Unweighted Pair-Group Method for Arithmetic average of analysis).

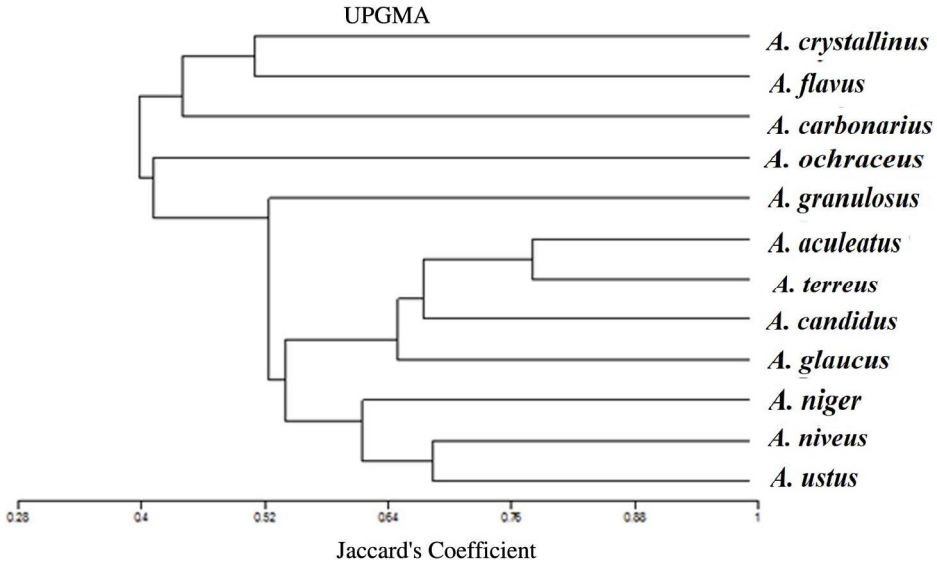

Fig. 6. Dendrogram obtained by UPGMA method using Jaccard (J) similarity coefficient calculated from PCR amplification banding of RAPD with A-01, A-02, A-03, A-04 and A-05 primers of the studied *Aspergillus* spp.

From the previous results of the RAPD fragment sizes it was noticed that; *A. carbonarius*, *A. ochraceous*, *A. flavus* and *A. crystallinus* exhibited low similarity, while *A. terreus*, *A. aculeatus* and *A. ustus*, *A. niveus* exhibited high similarity level. Subsequently, comparing the previous three dendrograms, a great relationship existed between *A. terreus* and *A. niveus* from one side and *A. candidus* and *A. terreus* from the other side. Similar studies were also carried out

recently by Godet and Munaut (2010) in differentiating *Aspergillus flavus, A. parasiticus, A. tamari* and *A. nomius* by PCR-RAPD techniques.

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