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**RESEARCH ARTICLE**

# Molecular ecological typing of environmental isolates of *Aspergillusterreus* collected from desert regions in Iraq.

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## *Manuscript Info* *Abstract*

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Most environmental isolates of*A.terreus* reveal phenotypic variation in colonies characters included colony color ,texture and reverse pigments in order to determine if these variations occurred due to environmental response or genetic variation,100 isolates of *A.terreus* were collected from arid region in Iraq in the period 2011- 2012.Ribotyping of ITS region by two universal primer ITS1 -ITS2,ITS1 -ITS4 and specific primer for *A.terreus*. Genotyping of 19 isolates by RAPD -PCR using oligonucleotide primer R108 (5´-GTATTGCCCT-3´) Phylogenetic tree dendrogram (UPGMA)were constructed by using UVI band software, and evaluated the similarity coefficient factor among 19 isolates of *A.terreus*.

The results showed that the morphological characterization had less taxonomic value comparing with molecular characterization which showed powerful keys in the identification of *A.terreus* isolates. The Phylogenetic analysis by RAPD-PCR had been show provide taxonomically useful information created many ecological genotypes , R108 Primer generated a banding patterns composing of one to 5 bands in various sizes ranging from 100 to 1000 bp. Phylogenetic tree based on RAPD –PCR patterns showed variable similarity coefficient values.

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## INTRODUCTION

The genus *Aspergillus* was representative one of largest genera in the fungal kingdom ,which was divided into seven subgenera, and each one was subdivided into several sections with each section comprised of a few to several closely related species. *A.terreus* belongs to the genus *Aspergillus*, sub-genus *Nidulantes*and it was identified in the laboratory by morphological methods. (Raper and Fennell, 1965). It's one of the most common saprophytic fungus inhabit all habitats such as desert soil, grasslands and other habitats. Raper and Fennell recognized *A.terreus* as the only known species within the section *Terrei*, subgenus *Nidulantes*, (Varga*et al.,* 2005) .many of isolates showed morphologically variation in growth criteria such as colonies colors ranged from bright orange to dark brown colonies and variation in surface on culture media(Rath, *et al*., 1999). Most of *A.terreus* isolates produces a variety of secondary metabolites that are drug significant, such as lovastatin drug (Casas L´opez*et al.*, 2003)

Many studies showed that morphological methods were weak characters at species level and genus level, while molecular methods may be powerful tools in species identification (Hong *et al*., 2008).

Ribotyping analysis of ITS region revealed detailed information about molecular analysis. On PCR amplifications ITS region shows monomorphic and polymorphic bands for ITS region in many previous studies (Kurtzman, 1985). Random amplification of polymorphic DNA (RAPD) assay revealed polymorphic DNA patterns of *Aspergillus* spp.( Anderson*et al*.,1995 ) Furthermore, RAPD-PCR assay has been adapted for fingerprinting fungi and bacteria (Aubin*et al*., 1991; Symones, 2000) revealed an accurate and simple method for differentiating among isolates of fungal isolates.

Various genotypic methods have been used successfully fingerprinting typing in many fungi like *A.terreus* , *A. fumigatus*.and*Fusariumsolani* (Crowhurst*et al*.,1991; Loudon *et al*.,1993 ; Nariasimhan*et al*.,2010) Due to high frequency *A.terreus* in desert regions and variations in colony colors ,this led to raised important issue for molecular ecological typing of environmental fungal isolates for any fungus in any district (forest or desert regions) furthermore of their important role of fungi in the arid or rain regions including biodegradation ,drug biosynthesis ,recycle materials, and microbial activities (Amin *et al.*,2010).Few comparable data were available for *A.terreus* in the world .Our study aims to evaluate the macroscopic and microscopic morphological variations, molecular ecological typing of ITS region, and RAPD-PCR of environmental isolates of *A.terreus* which isolated from soil samples in many sites from desert region in Iraq and perform phylogenic analysis.

## MATERIALSANDMETHODS

**1-Soil samples collection and fungal identification**

The source of one hundred forty three isolates were isolated from different soil samples were collected from were from five stations in desert regions in west and west- northern of Iraq provinces(west of Najaf, west of Karbala and Al-Anbar ) in period 2011-2012.Dilution plate method on PDA media was followed for the isolation and purification of *A.terreus*isolates.The plates were incubated at 28C for 4 days and purified *A.terreus* colonies in separated triplicates plates for each isolates.Preliminary identification followed (Raper and Fennel, 1965). Microscopic examination was performed by attached tape on the margin of fungal colony and mountain inoculums on drop of cotton blue lacto phenol stain and examined by microscope.

**2-DNA extraction**

The mycelia of 19 representative isolates for all isolates collections of *A.terreus* (nos. 1-19))were freeze -20°C for overnight , harvested tiny portion of mycelia mats of mycelia approximately 1gm(fresh weight) and dropped in a 1.5 ml of Eppendrof tube.

The harvested mats were mixed the with 400 ul extraction buffer by vortex for 5 min. and added 10 ul proteinase K and incubated at55°Cin water bath for 60 minutes. Mixed gently, phenol: chloroform: Isoamyl alcohol (25:24:1) was added and mixed.The tubes were centrifuged 7000 rpm. For 3 minutes. The aqueous supernatant was transferred to a new tube. An equal volume of cool isopropanol was added and mixed gently many times and centrifuged at 1000rpm. for 10 min. The supernatant was poured out. The pellet containing DNA was rinsed with 70% ethanol. It was air dried, pellets were allowed to re-suspend with 75ul TE and placed at 70°Cin water bath, 6 ul of RNase A was added and incubated the tubes for 30min. at 37C. The tubes were centrifuged 5000 rpm. for 2minutes and the supernatant was trans to new tube and freeze at -20C until uses. (Saghai-Maroof*et al.*, 1985; Edwards *et al.*, 1991).

**3-Simple PCR assays:**

PCR amplification of ITS region was performed with two universal primers pairs ITS1/ITS4 and ITS1 /ITS2 for amplified theITS1–5.8S–ITS2. and molecular diagnostic for*A.terreus* by specific primer pairs*:* ATE1:CTA TTG TAC CTT GTT GCT GGCG, ATE2 :AGT TGC AAA TAA ATG CGT CGG CGG ,(Logothet*i et al*.,2009) . The reaction was carried out in a thermal cycler (LABENAT, USA) .12.5 ul master mix PCR buffer ,with each primer 1.2 ul (20 pemole ) and 0.8 ul of genomic DNA and water adjusted to a final volume of 20 ul. The PCR protocol consisted of initial denaturation at 95°C for 5 min; 35 cycles of 94°C for 1 min, 59°C for 1 m, and 72°C for 1.5 min; and a final elongation of 72°C for 5 min.

**4-RAPD-PCR ecological typing**

The primer R108 (5´-GTATTGCCCT-3´) described by Aufauvre-Brown *et al*.,1992. was used for RAPD-PCR typing for 19 environmental isolates of *A.terreus* .Amplification reactions were fulfilled in a final volume of 20µl containing 1.5 µl of genomic DNA, 1µL Primer (50 pmole) , and 12.5 ul master mix PCR buffer. Water adjusted to final volume reaction .PCR was carried out in a thermal cycler (LABENAT, USA) with the following temperature profile: 1 cycle of 5 min at 94°C , followed by 35 cycles of 45s at 94°C, 45S at 36°C and 1min at 72°C and a final extension step at 72°C for 10 min. 10 µl of amplification products were loaded onto 2% agarose gel was premixed with ethidium bromide stain (0.5µg/ml) and TBE running buffer for 1.30 h at 80 V at room temperature. The products were visualized under a UV transilluminator and photographed (Lass-Flor*let al.*, 2007).

**5-Phylogenetic analysis:**

Phylogenetic tree Dendrogram (UPGMA) for 19 environmental isolates of *A.terreus* were constructed by using UVI band software, and evaluated the similarity coefficient factor according to Ute *et al.,* 1994.

## RESULTS

**1-Macroscopic and Microscopic characters of *A.terreus***

The fungus *A.terreus* grew fast on PDA medium after 5-7 days of incubation. Macroscopic characters included colonies characters of examined isolates were showed difference in their colors from pale-yellow to dark yellow, while the reverse pigments ranged from yellow to dark gray color. The colonies texture raised surfaces velvety, tough, Yellowish, powdery. Microscopic characters not revealed more variations among isolates under interest; conidiophores were typically long, hyaline and smooth giving rise to sub-spherical vesicles that were biseriate. Conidia smooth walled, slightly elliptical.

**2-Molecular ecological typing**:

Molecular diagnosis of *A.terreus* isolates

The specific pair primers for *A.terreus*ATE1 and ATE2, was successfully annealed and amplified their targeted region of 19 environmental isolates of *A.terreus*, the PCR product showed monomorphic bands 450 bp. in length (primers included) (Figure 1). These results were confirmed the diagnosis of all isolates under interest belonged to *A.terreus*.

**3-Ribotyping ITS region for environmental isolates of*A.terreus*:**

Amplified of targeted rDNA (ITS1 -5.8S-ITS2 region) of 19 isolates with primer pairs: ITS1/ITS4 that amplifies produced amplicon length approximately 550 -600 bp. was obtained for all tested isolates of *A.terreus* (Figure 2). and ITS1/ITS2 primer that amplify the ITS1 region, produce amplicon length approximately 280-380 bp.(Figure 3).

**4-RAPD-PCR ecological typing**

Several bands (1 to 5 bands) in various sizes ranging from about 100 to about 1000 base pair were obtained by using primer R108.This primer was generated and composing RAPD-PCR patterns allow a distinction of very closely related environmental isolates, but incidental similarities among the typing of distantly related isolates may occur. This study used the UPGMA algorithm for analyses the results was reliable method, by which bands strongly discriminative of distinguishing the isolates were as important as markers occurring occasionally. We point out that similar typing patterns bands correspond to the same erotological genotype had same locus or loci and generated similar or different patterns with identical band sizes in different environmental isolates of *A.terreus* (Figure 4).

**5-Phylogenetic tree of *A.terreus* isolates based on RAPD-PCR.**

The results of molecular ecological typing of 19 isolates of *A.terreus*showed wide rang degrees of similarity among environmental isolates of *A.terreus* : J and L isolates showed 100% similarity coefficient value(0% distance coefficient) ,C and D and showed 80% similarity ,both S, T and N,O 68% similarity , H,I isolates showed 50% similarity coefficient value while R isolate showed distinct ecological genotype has 0% of similarity coefficient values. Figure 5 showed others similarity coefficient values.

**Figure 1:Agarose gel electrophoresis of PCR products for *A.terreus* isolates amplified by pair primers ATE1 andATE2. Lane M= Molecular marker100bp; lanes 1- 19 isolates of *A.terreus*.**

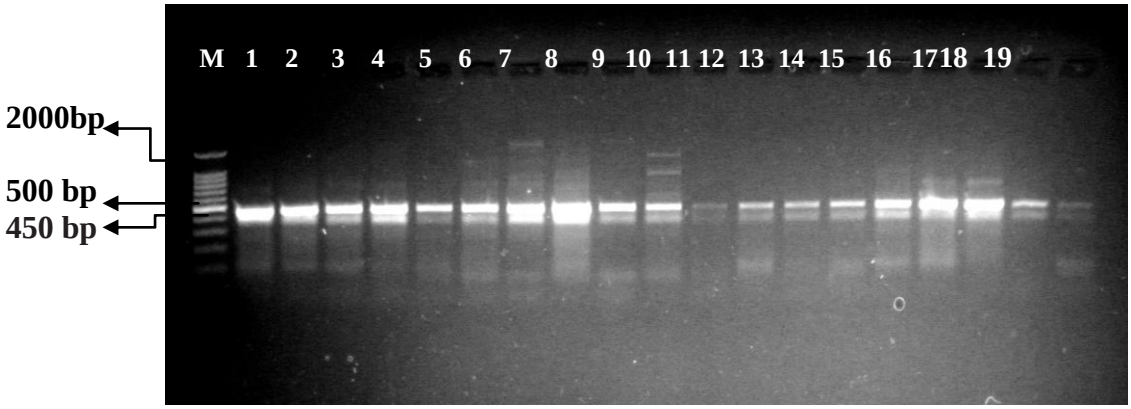


**Figure 2:Agarose gel electrophoresis of PCR products for *A.terreus* isolates amplified by pair primers 1TS1 - ITS4. Lane M= Molecular marker100 bp; lanes 1- 19 isolates of *A.terreus*.**

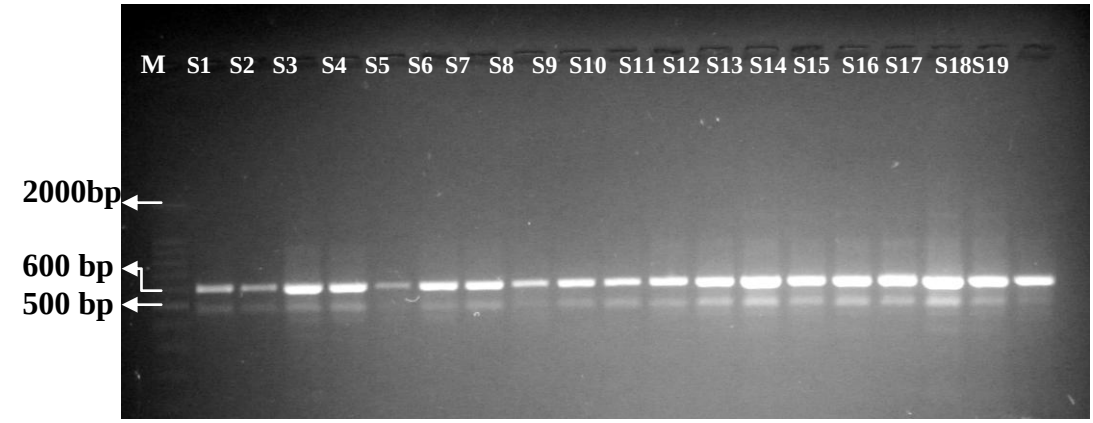



**Figure 3:Agarose gel electrophoresis of PCR products for environmental *A.terreus* isolates amplified by pair primers 1TS1 -ITS2. Lane M= Molecular marker100 bp; lanes S1- S19 isolates of *A.terreus*.**

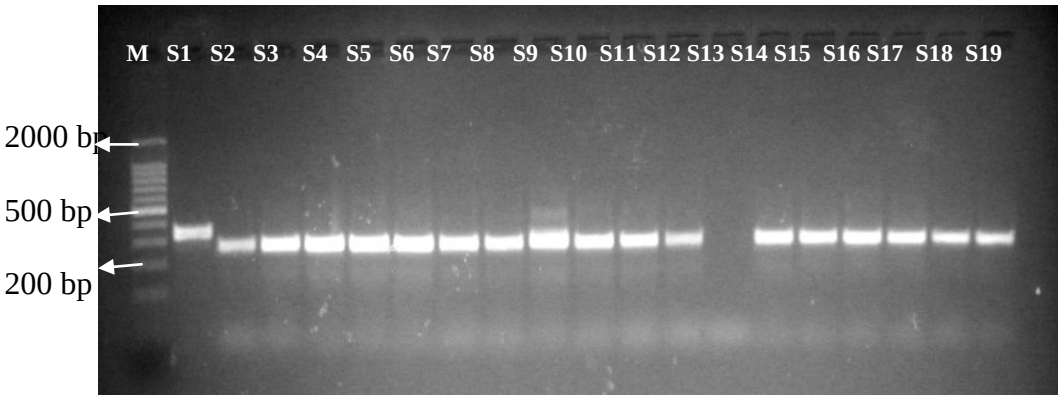



**Figure 4:Agarose gel electrophoresis of RAPD-PCR products for environmental isolates of *A.terreus* by primer ( R108). The PCR products of isolates were analyzed in 2% agarose gel. A=Moleculer marker 100bp, ecological isolates were B-T.**

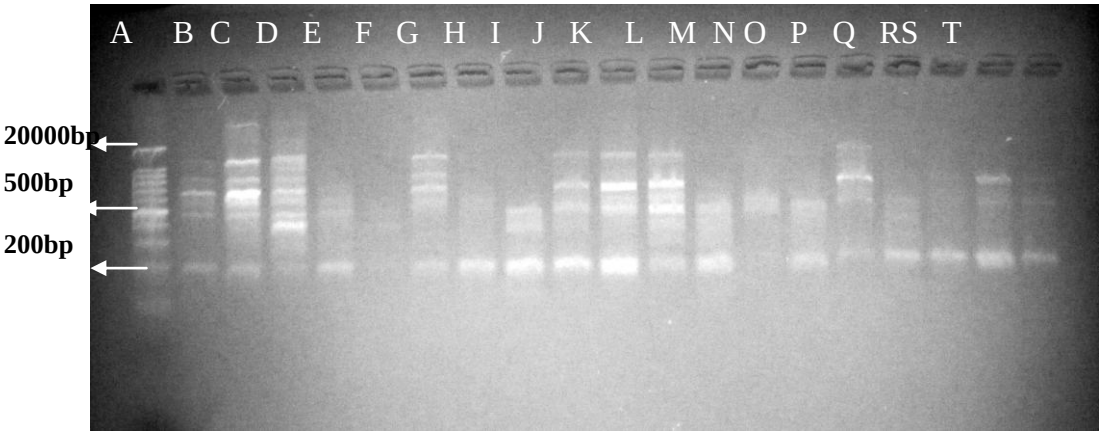
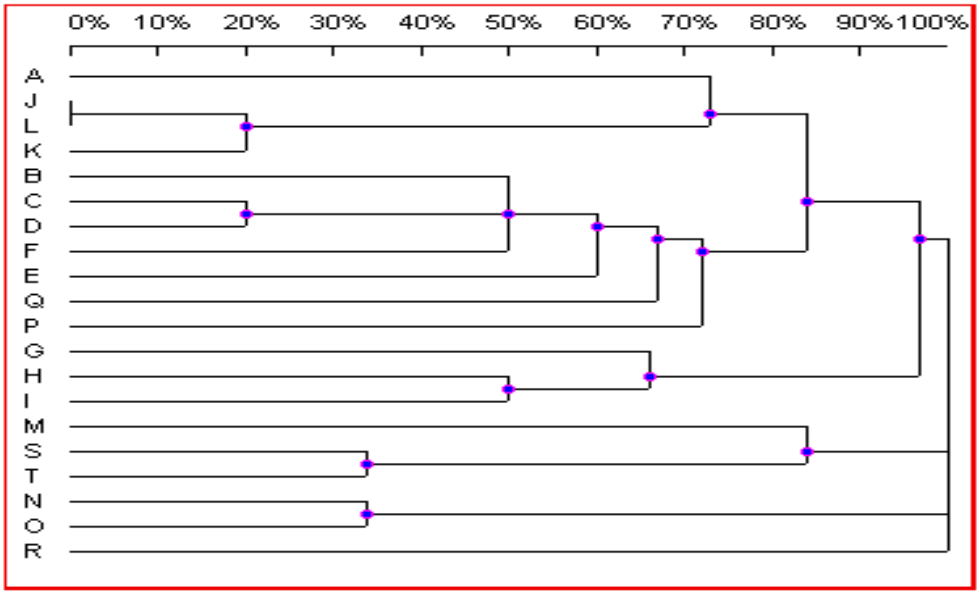


**Figure 5: Phylogenetic tree Dendrogram for 19 environmental isolates of *A.terreus* were constructed by using UVI band software on the bases of RAPD-PCR bands. A=Molecular marker, J-R= isolates of *A.terreus*, Scale 0%-100% =distance coefficient (UPGMA).**




## Discussion

Our results showed wide range of variability in phenotypic characters then all isolates were determinate belonged to*A.terreus* genotype confirmed diagnosis by specific primer pair, ATE1 / ATE2 and remove any delusive confuse with other species those reveal the yellow colour of colonies .This results came coincidence with results of Logothet*i et al*.,(2009), Ribotyping of the main conservative and high frequency(ITS)region showed approximately monomorphic bands, these results emphasize that these isolates had the same length of ITS regions , then the variations in phenotypic characters may raise due to the concept ofgene-environment correlation (Smith and Kruglyak.2008)which can arise by multi-mechanisms **(**Geoffrey *et al* ., 2013**).** may be effects of sun light , salinity and soil ecology or raised by natural selection ,genetic drift and gen flew and gene mutation which are representative the domain source of genetic variations (Carlile, *et al*., 2001; Fe´ral ,2002 ; Lass-Flor*et al*., 2007) .

Molecular typing based on RAPD-PCR patterns for19 isolates of *A.terreus*showed distinctive patterns among environmental isolates. This result agree with results of Lass-Flor*et al*., 2007) which showed type of molecular typing for clinical isolates of *A.terreus* , based on RAPD-PCR patterns . These variations in the patterns may contribute in explanation of the sources of variation and shared in the solution of several phenotypic variations in colonies colors and difference in colors of reverse pigments of *A. terreus*. Phylogeny tree based on RAPD-PCR profile was sufficient in genotyping of *A.terreus* isolates collected from arid regions of Iraq and showed variable degrees of similarity among 19 isolates of *A.terreus* and divided them into many genotypes; only two isolates were show 100% similarity coefficient value to each other. While other isolates were showed 0-80% similarity coefficient value (Figure 5).Our results agree with the results of Lasker( 2002) who succeed in genotyping A .*fumigatus* isolates and with results of Raclasky*et al*., 2006 and Nariasimhan, *et al.*,2010.

RAPD-PCR patterns of *A.terreus*environmental isolates were emphasized that the monomorphic Ribotyping results not sufficient for ecological genotyping. And there requirement for fingerprinting by oligonucleotides primer to determine the homology of *Aspergillus* isolates (Loudon,*et al*., 1993;Symones,*et al*.,2000).Finally the ecological genotyping results was emphasize a way for finding minor differences among isolates at species level as at genus level. Birch *et al*.,(1995).

## Conclusion

This study may encourage future researches in a way ecological genotypic closely related isolates. And showed highly discriminatory profiles of RAPD –PCR because they showed genetic diversity among closely related isolates in the same species. The molecular genotyping of *A.terreus* based on ITS region was reliable but still required for

RAPD -PCR typing and at the same time was proven useful tools in taxonomical studies ,and gave precise, rapid results with low cost and not consuming long time . This study confirmed the efficient genotyping trend in solution of fungal ecological diversity problems.

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