

## **Detection the genetic relation between several fungal isolates by using a universal ITS primers**

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### **Summary**

The goal of this study is to evaluate the internal transcribed spacer regions (ITS) of the rDNA to detect a phylogenetic relationships based on PCR technique between four isolates of *Trichoderma viride*, *Chaetumium elatum* and tow isolates (pathogen and non-pathogen strains) of *Rhizoctonia solani* obtained from tomato plant rhizosphere, rice plant rhizosphere and root rotted tomato plants fields of Al-Najaf province, Iraq.

Internal transcribed spacer regions (ITS) of the ribosomal DNA was amplified by polymerase chain reaction (PCR) technique using a set of common universal primers for ITS1, ITS3 and ITS4 regions. Two of three different random primers examined (primers 3 and 4) reflected a specific amplicons within *Rhizoctonia solani*(1), *Chaetumium elatum* , and *Trichoderma viride*, while the primer 1 did not give any PCR end product with all of tested isolates, while *Rhizoctonia solani*(2) isolates did not revealed any band detected in PCR technique with all primers, also data showing a different degree of genetic similarity depending on PCR data analysis using UVIBand softwear.

Results of this study indicate that ITS set of primers can used to find a genetic relation between fungi.

### **دراسة العلاقة الوراثية بين عدد من العزلات الفطرية باستخدام بادانات لمنطقة المستنسخات الداخلية**

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#### **ملخص:**

الهدف من هذه الدراسة هو تقييم مناطق الاستنساخ الداخلية (ايتس) من الدنا الرايبوزي للكشف عن العلاقات الجينية بينها على أساس تقنية تفاعل البلمرة المتكرر الى أربع عزلات هي

*Trichoderma viride*, *Chaetumium elatum* and tow isolates (pathogen and non-pathogen strains) of *Rhizoctonia solani*

عزلات *Rhizoctonia solani*, *Trichoderma viride*, *Chaetumium elatum* تم الحصول عليها من نبات الطماطم ريزوزفير، نبات الأرز ريزوزفير و الجذور النباتات الطماطم المتضررة من حقول محافظة النجف، العراق.

تم تضخيم المناطق الاستساخ الداخلية من الحمض النووي الريباصي بواسطة تقنية تفاعل البوليميراز المتسلسل باستخدام مجموعة من الاشعال العالمية الشائعة لمناطق ITS1 و ITS3 و ITS4. اثنان من ثلاثة الاشعال عشوائية مختلفة فحص (الاشعال 3 و 4) تعكس أمبليكونس محددة داخل ريزوكتونيا سولاني (1)، تشيتوميوم إلاتوم، و تريتشوديرما فيريد، في حين أن التمهيد 1 لم يعط أي المنتج النهائي ير مع جميع العزلات اختبارها، في حين ريزوكتونيا سولاني (2) لم يكشف عن أي فرقة تم الكشف عنها في تقنية ير مع جميع الاشعال، كما تظهر البيانات وجود درجة مختلفة من التشابه الجيني ديباندينغ على تحليل البيانات ير باستخدام لينة أوياند لينة. وتشير نتائج هذه الدراسة إلى أن مجموعة إيتس من الاشعال يمكن أن تستخدم للعثور على علاقة وراثية بين الفطريات.

## Introduction

It is well known that *Trichoderma* species have been used as biological control agent (BCAs) for many years (Hjeljord and Tronsmo, 1998), but it is lately become a commercially available product (United Nations Environment Programme, 1995) used for eradicate group of microorganisms. The data about competitors or antagonists effects of these fungal species is important for their effective use since thier mode of action is difere depends on many factors (Jeffries and Young, 1994).

*Trichoderma* strains can be used for production group of extracellular enzymes (Haran *et al.*, 1996), antifungal like substances (Ghisalberti and Rowland 1993), they may play a competitive role against group of pathogenic fungi (Simon and Sivasithamparan, 1989), growth promoters for some plants (Inbar, *et al.*, 1994), in addition to their ability to induce a resistance in some plants (De Meyer *et al.*, 1998, Zimand *et al.*, 1996). The commercial uses of *Trichoderma* strains as BCAs must be preceded by an accurate detection, adequate information, and synergistic effects mechanisms to interstand ther biocontrol strategies. The manifestation of taxonomic variation multiplicity of the biological control includes *Trichoderma* species, which have the deffirent species with very wide spreading in worldwide (Bissett, 1991; Rifai, 1969). Therefore the *T. harzianum* S.I. involves the importante strains which

used as biological control agents (Ospina-Giraldo *et al.*, 1999) and comprises *Th1* and *T. inhamatum* (Kuhls *et al.*, 1997; Kindermann *et al.*, 1998).

*Rhizoctonia solani* was noticed as causative agent on soybean crops that causes economic loss throughout the world (Naito *et al.*, 1995; Embrapa, 1999). *R. solani* caused several infection diseases on soybean plants which include damping-off seedlings, roots rots, web and aerial blight (Naito *et al.*, 1995; Embrapa, 1999). In several countries especially in brazil, foliar blight disease led to decrease the production of soybean which range between 32% to 61% (Meyer and Yorinori, 1999).

The incredible diversity in morphology, physiology and pathogenicity of *R. solanistrains* directed to a special classification system based on anastomosis grouping which supported by a molecular confirmation (Carling and Kuninaga, 1990; Gonzales *et al.*, 2001).

Sequence analysis of the (ITS) rigons has been used to deduce the taxonomic and phylogenetic relationships between different *R. solani* and *Rhizoctonia* spp. anastomosis groups (Carling *et al.*, 2002), and grouping on a PCR techniques correlated with fungal virulence (Kuninaga *et al.*, 1997).

*Chaetomium* spp. has also been recognized as one of the biological control agent's fungi (Aggarwal *et al.*, 2004). Despite the numerous phynotypic and genetic studies to arrange this group of

fungi into a suitable classification scheme but still hardly to classify because of limited number of morphological characters (Arx *et al.*, 1986).

Internal transcribed spacer (ITS) segments are two segments, ITS1 segment located between the 18S rDNA and 5.8S rDNA while ITS2 segment located between 5.8S rDNA and 28S rDNA, which has been established as an important molecular indicator in the study of fungi because of its variation and uniqueness in each fungal species. (Bryan *et al.*, 1995; Sherriff *et al.*, 1995).

We have made use of ITS region polymorphisms originated by hybridization of rDNA with universal primer. This study describes the rDNA ITS1-ITS2 analysis based on PCR technique to find out a genetic relationship between group of economic important fungal species included in this study.

#### **Materials and Methods:**

##### **Fungal species:**

Fungal species were routinely isolated in this study as following *Rhizoctonia solani* was isolated from root rotted tomato plants cultivated in Al-Najaf province. *Chaetumium elatum* was isolated from rice plant rhizosphere located in Rice research institute and identified at the Plant protection department, Agriculture College, Kufa University, *Trichoderma viride* was isolated from tomato plant rhizosphere, Babylon province and identified at the Plant protection department, Agriculture College, Kufa University, Al-Najaf province, Iraq. The fungus were identified according to approved taxonomic keys (Barnett, 1965; Moustafa, 1982; Domsch *et al.*, 2003). The samples were cultured on to Potato dextrose agar for 5-7 days at 25°C. The consequential fungal isolates were identified by microscopic and culture techniques. In the present study, four fungal isolates were examined by the PCR.

These included *T. viridi* (1 isolates), *Ch. elatum* (1 isolates), *R. solani* (two isolates).

##### **Isolation culture and harvest:**

To determine the genotypes of four fungal isolates recovered from out of many investigated samples. A portion of sample was cultured on PDA by spot inoculation technique. The cultures were incubated at 25 ± 2 °C until visible fungal growth or (3-5) day-old for early PCR analysis. The genomic DNA was extracted by using liquid nitrogen for initial breaking up of the mycelia by grinding. Final DNA extraction was achieved by EZ-10 spin column fungal genomic DNA mini-preps kit (BioBasic Incorporation /Korea). The amount of (5 µl) of DNA extract was used as a template in the PCR technique; the amplicons were checked and quantified on 2% agarose gel which was run at 80 volts for 1 hour.

##### **Isolation and Purification of Genomic DNA:**

A specific EZ-10 spin column fungal genomic DNA mini-preps kit (BioBasic Incorporation /Korea), was used for purification of fungal DNA, the implication of genes was carried out according to the experimental protocol of Accu power TLA PCR Premix tub under employing the following cycling for each gene amplification consisted of an initial denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 4 min;. by assessing primers in PCR it were possible to select several with potential for identification and differentiation of fungal species these included primers ITS1 (5-TCC GTA GGT GAA CCT GCG-3), ITS3 (5-GCA TCG ATG AAG AAC GCA GC-3), and ITS4 (5-TCC TCC GCT TAT TGA TAT GC-3), targeting the conserved regions of 18S, 5.8S, and 28S rDNAs

respectively (White *et al*, 1990). The PCR reaction mixture was prepared as 5µl of Premix Bioneer corporation USA, which is supplied in a ready to use in 100 µl PCR tube, followed by 5 µl templet DNA extract, 2µl of 10 pmol/µl primers solution, the volume was completed to 20 µl with deionized distilled water, the tube were mixed with vortex to dissolved the lyophilized blue pellet, and briefly spin down.

#### **Gene tool and phylogenetic analysis**

The electrophoresis results was detected by using Vision-gel documentation system and analyzed by gene tool analysis (UVI band software), gel was photographed using Vision-Gel documentation system. Phylogenetic tree dendrograms and other variables were constructed by using UVI band software, also similarity coefficient factor were evaluated in the same way according to Timothy and Christopher (1992); Ute *et al* (1994); Nei *et al* (1985) formula

### **Results**

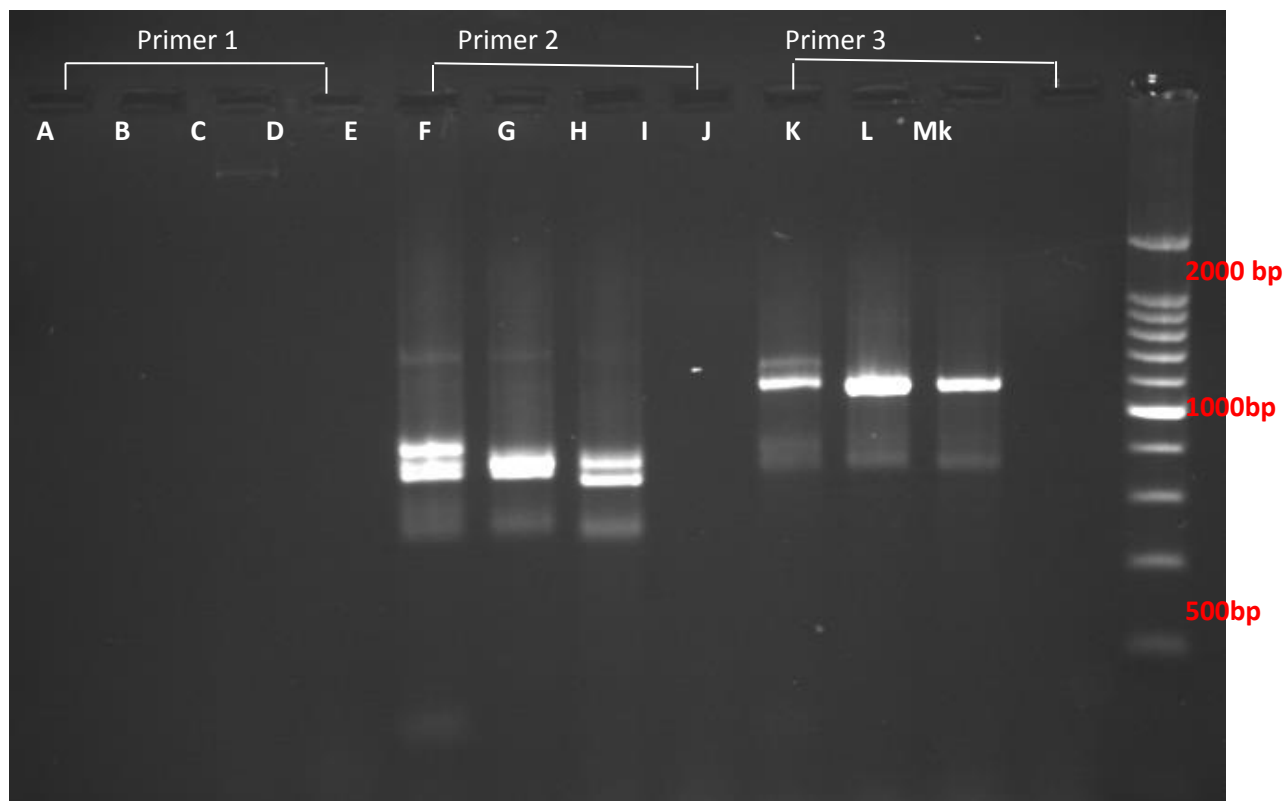
#### **Fungal isolates and Genotype analysis**

Two out of three different random primers examined (ITS primers 3 and 4)

reflected specific amplicon within *Rhizoctonia solani* (1), *Chaetomium elatum*, *Trichoderma viride* and *Rhizoctonia solani*(2), while the ITS primer 1 did not give any end product of PCR bands with all tested isolates (Figure 1), on the other hand *R. solani* isolates did not revealed any band detected in PCR technique with all primers.

The ITS region amplified from the species *Rhizoctonia solani*, *Chaetomium elatum* and *Trichoderma viride* with primer 2 were (~341-387, ~358 and 329-366~ bp) in length respectively, while the ITS region amplicons from primer3 were (~600, ~574 and ~587 bp) respectively (Table 1 and Figure 1,2).

Tolerance distance and dendrogram phylogentic tree using UVIBand softwear (Figure 2) showing a complete identity between *Rhizoctonia solni* and *Trichoderma viridi* strains while 50% similarity between these two strains and *Chetomium elatum* strain when using ITS primer 3 sequence, while a complete identity between *Rhizoctonia solni*, *Chetomium elatum* and *Trichoderma viridi* strains notesed on data analysed based on ITS primer 3 sequence. (Figure 2)



**Figure 1:** Agarose gel electrophoresis of fungal species DNA products generated through the PCR with the primers for ITS region. Lane (Mk): Size marker (100bp); Lane A.E.I., DNA products amplified from *Rhizoctonia solni* (1) isolates; Lane B.F.J., *Chetomium elatum* isolates; Lane C.G.K. *Trichoderma viridi* isolates ;and Lane D.H.L., *Rhizoctonia solani* (2) isolates A.B.C.D (primer 1); E.F.G.H (primer 2);I.J.K.L (primer 3), (2% Agarose gel, 80 volts for 2 hour).

**Table 1: Molecular weight of PCR band for fungal isolates using UVIBand soft wear**

Lane Mk	Lane L	Lane K	Lane J	Lane I	Lane H	Lane G	Lane F	Lane E	Lane D	Lane C	Lane B	Lane A*	
2000		587.021	574.087	600		366.396	358.123	387.39					No 1
1000						329.98		341.85					No 2
900													No 3
800													No

													4
700													No 5
600													No 6
500													No 7
400													No 8
300													No 9
200													No 10
100													No 11

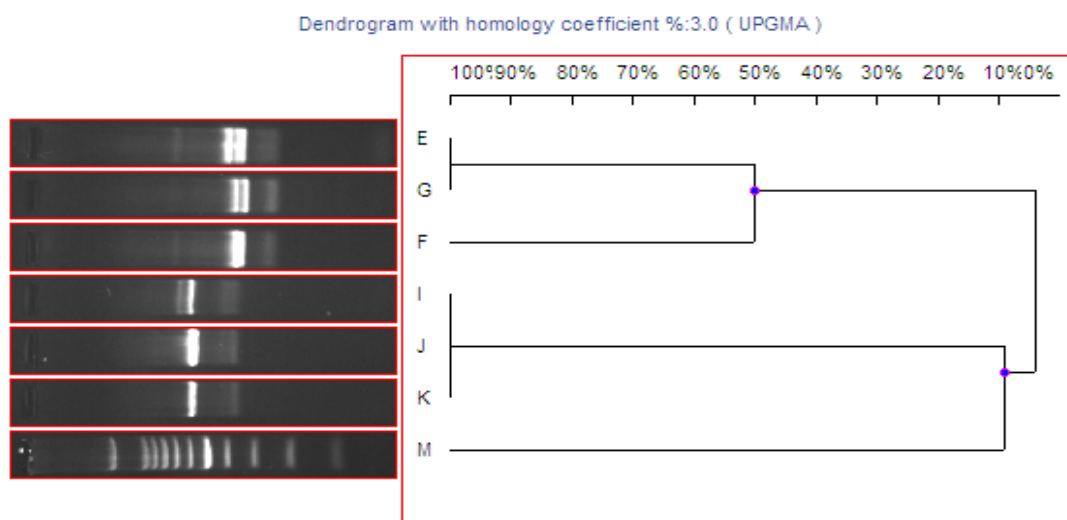
\* Lane A.E.I., DNA products amplified from *Rhizoctonia solni* isolates

Lane B.F.J., *Chetomium elatum* isolates

Lane C.G.K. *Trichoderma viridi* isolates

Lane D.H.L., *Rhizoctonia solani* isolates

**Figure (2):** Tolerance distance and dendrogram phylogentic tree of fungal isolates on the basis of the random primers.



## Discussion

The study was conducted to determine the genetic diversity and genotypes of several fungal plant pathogens collected from root rotted tomato plants, rice plant rhizosphere and tomato plant rhizosphere using ITS set of primers for PCR technique. The fungal pathogen of plants are in great numbers, therefore our attention was paid to the several fungal species (e.g. *Rhizoctonia solani*, *Chaetomium elatum*, *Trichoderma viridi* and *Rhizoctonia solani* isolates) isolated from an infected plants in local fields.

Internal transcribed spacer (ITS) primers (primers 1, 3 and 4) showing different degree of efficiency, ITS primer 3 and 4 showing an amplification product in most study fungi while ITS primer 1 failed to amplify the specific region on rDNA. The correct genetic structure and functions of the criteria of amplified band from isolates using ITS primer-1 (5-TCC GTA GGT GAA CCT GCG-3) is not obvious at this study.

Phylogenetic data analysis prove that the ITS primers give a type of genetic correlation between study isolates it can be used to find a genetic relationship between different fungi, it is probable that the analyzes of the nucleotide base sequences of this bands will shed more accurate information into the molecular basis of genetic relationship among different fungal species and result in further improvement in PCR-based assays for the diagnosis of fungal plant pathogen in terms of specificity and direct application to practical samples.

We conclude from this study that we can able to differentiate some economically important genera of fungal by using ITS primer 3,4 set, and we can used this set to find a genetic relation between fungi and used these primers

directly on the isolate samples to assist the diagnosis of other fungal plant infections.

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