Sequence analysis of rDNA ITS of clinical Fusarium species

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Abstract—The study on molecular structure of *Fusarium proliferatum* clinically isolated can provide the basis for accurate diagnosis and treatment of *F. proliferatum* infection. The rDNA ITS gene of 6 strains that had been identified as *Fusarium proliferatum* based on morphological were cloned and sequenced. The molecule phylogenetic tree was constructed based on rDNA ITS sequences, and the 6 genus were divided into two groups. Group Ⅰ was *F.proliferatum*, including strains of CSUFT201, CSUFT204, CSUFT205. Group Ⅱ was F. *moniliforme*, including CSUFT202,CSUFT203,CSUFT206. The results of this study showed that 3 strains of *F. moniliforme* were confirmed out of the 6 strains with the molecular methods.

Keywords-Fusarium; ITS gene; clone; phylogenetic; sequence analysis

I. INTRODUCTION

Fusarium spp. are common plant pathogens and causative agents of superficial and systemic infections in humans. Infections caused by Fusarium spp. are collectively referred to fusariosis. This fungus is the most common cause of mycotic keratitis. Fusarium produces very harmful toxins, especially in storage of infected crops. *Fusarium moniliforme* produces mycotoxins called fumonisins. Eating grains contaminated with these toxins may give rise to allergic symptoms or be carcinogenic in long-term consumption.

Recently, with the increasing of immunodeficiency disease and the use of broad-spectrum antibiotics, the human diseases caused by Fusarium were increasing(Musa MO,et al,2000), such as keratitis, skin infections,arthritis, etc(Rosa RH,et al,1994). Because they all have microconidia born in chains both in *F. proliferatum* and *F. moniliforme*, it is limited to identify Fusarium spp by their morphological characters. This study's aim is to use molecular method to identify over again those 6 strains which have been identified morphologically as *Fusarium proliferatum*. Amplified the rDNA ITS regions, cloned and sequenced, and then by blast search and construction phylogenetic tree to analyze the difference of them, it will lay a foundation for quickly accurate diagnosis and treatment of *F. proliferatum* infection in clinical.

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II. MATERIALS AND METHODS

A. Materials

1) Strains:

6 strains of *Fusarium proliferatum* (isolated and identified by CSUFT Microbiology lab), numbered CSUFT201,CSUFT202,CSUFT203,CSUFT204,CSUFT205,CSUFT206.The isolates originated from Corneal secretions in Hunan, China.

2) Primers:

fungal universal primers ITS1,ITS4, the sequences as follows: ITS1: TCCGTAGGTGAACCTGCGG, ITS4: TCCTCCGCTTATTGATATGC (synthesized by Shanghai Sangon Biological Engineering Technology & Services Co.Ltd).

3) Equipment

5415D-high speed centrifuge (eppendorf company, Germany); 9700-PCR instrument (ABI); Gel electrophoresis (Bejing); U410-86 ultra low temperature refrigerator (England); gel imagine system (Tianneng company, Shanghai).

B. Methods

1) DNA isolation

Total genomic DNA was isolated from fresh mycelium according to a miniprep protocol described by Cenis (1992). Briefly, 500 µl of liquid potato dextrose medium was inoculated with fungal hyphal threads and cultivated it at room temperature for 72 h. The resulting mycelial mat was pelleted by centrifugation at 13,000 rpm for 5 min and was washed with 500 µl of Tris-EDTA (pH 8.0). The mat was then homogenized by hand in 300 µl of extraction buffer (200 mM Tris-HCl [pH 8.5], 250 mM NaCl, 25 mM EDTA, and 0.5% sodium dodecyl sulfate) for 5 min. One hundred and fifty micro liters of 3 M sodium acetate (pH 5.2) was added, and the mixture was cooled to 20°C for 10 min. Fungal debris was pelleted by centrifugation at 13,000 rpm for 5 min, the supernatant was transferred to a fresh tube, and an equal volume of isopropanol was added. DNA was then pelleted by centrifugation at 13,000 rpm for 10 min. Excess salt was removed by washing with 70% ethanol, and DNA was resuspended in Tris-EDTA (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

2) PCR amplification of rDNA ITS regions :

The ITS1 and ITS2 and the inverted 5.8S coded rDNA were amplified by PCR using the primers ITS1 and ITS4 as described by White et al. (1990). Each PCR reaction mixture contained 5-10 ng of genomic DNA, each of the primers ITS1

and ITS4 is 1μM , reaction buffer (50 mM KCl, 50 mM Tris-HCl; [pH8.3]0.1mg/ml bovine serum albumin), 3mM $MgCl_2$, 200μM each of dNTP and 2.5 U of Taq DNA polymerase in a total volume of 50 μl. The PCR profile was enetration at 95°C for 3 min, followed by 36 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min, then a final extension at 72℃ for 10 min. PCR product detection: admixed 4μL reaction solution and 1μL spotting solution(16×bromophenol blue) then performed 1.0% agarose gel electrophoresis(contain 1% EB, 100v, 1 hour).

3) PCR product cloning:

PCR product was purified and cloned it into PMD18-T plasmids and transformed it into electrocompetent cells of *Escherichia coli*, maked positive clones selection by the white-blue-plaque-plate method(contain X-Gal,IPTG,Amp LB), liquid cultivated it in LB culture medium.

4) PCR product sequence:

Then PCR product was sequenced by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd, the primer was ITS1.

5) rDNA ITS sequence analysis:

Acquired ITS sequences were compared with non-redundancy nucleotides database by BLASTN to determine the homology of ITS gene sequences (http://www. ncbi. nlm. Nih/blast). Multiple sequences alignment was done by the software of CLUSTAL W, and phylogenetic tree was constructed by neighbor-joining method in software of MEGA (Version4.1). The gap caused by sequence length polymorphism was deemed to missing. Test confidence level of each branch (1000 repeats) was by bootstrap.

III. RESULT AND ANALYSIS

1) Amplification of rDNA ITS:

The amplified product(Internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; 18S and 28S ribosomal RNA gene, partial sequence.) was electrophoresized on 1.5% agarose gel stained with EB, and then directly visualized under UV. Except lane 1 using H_2O as template, each group (lane 2-7) was found to have an obvious specific amplified band and with small difference (Figure 1).

2) Screen clones of PCR product:

The colony in plate culture medium shows that electrocompetent cells of E. coli have been transformed into exogenous plasmid vectors; The fact that most of them are white in these colony shows that exogenous gene fragment has been inserted into plasmid vector; and The fact that little are blue proves that plasmid vector does not have exogenous gene fragment. After have been cultivated several time in liquid LB culture medium, eliminated false positive reaction, and sequenced by Shanghai Sangon Biological Engineering Technology & Services Co.Ltd, the portion result of PCR product cloned follows as figure 2.

3) Search results of homologous sequences of ITS region

Blast search of ITS region sequences of Fusarium shows that, in 6 strains of *F. proliferatum*, identified morphologically there were actually only 3 strains of *F.proliferatum* (CSUFT201,CSUFT204,CSUFT205), and the others were *F.moniliforme* (CSUFT202,CSUFT203,CSUFT206). (Sexual phase of *F.moniliforme* is *Gibberella moniliformis*).

4) Sequence analysis

The total length of ITS1, 5.8S and ITS2 regions of CSUFT201,CSUFT204 and CSUFT205 is 457 bp(ITS1=134 bp,5.8S=158 bp, ITS2 =165 bp),there are no variable sites in the ITS and 5.8S region ingroup.The total length of ITS1, 5.8S and ITS2 regions of CSUFT202,CSUFT203 and CSUFT206 is 458bp(ITS1=147 bp,5.8S=158 bp, ITS2 =153bp),there are no variable sites in the ITS and 5.8S region ingroup.

Downloaded related representative ITS region sequences of Fusarium and arranged with the acquired 6 strains, *Colletotrichum gloeosporioides* as outgroup. Phylogenetic tree was constructed with MAGE software neighbor-joining method, and test confidence level of each branch (1000 repeats) by bootstrap. Through the phylogenetic tree based on the rDNA ITS sequences we concluded that this genus can be divided into two groups (species). Group Ⅰ is *F. proliferatum*, including strains of CSUFT201,CSUFT204,CSUFT205. Group Ⅱ is F. *moniliforme*, including CSUFT202,CSUFT203,CSUFT206. It shows the difference because of the far genetic distance between *F. proliferatum* and *F. moniliforme*. This identification species method is based on the rule of phylogenetic tree, it accords with the standard of identification phylogenetic species by phylogenetic species recognition(Taylor,et al,2000).

IV. DISCUSSION

A. Traditional fungus classification is mainly based on visual form of fruit body or microcosmic morphological and structural characteristic, but phenotypic characteristics are greatly affected by environmental factors, it often makes the same fungus in different circumstance with different morphological variations, it is difficult for classification and identification. Fusarium spp. is classified into Fungi, Ascomytota, Ascomycetes, Sordariales, Hypocreales, Gibberella(Kirk PM,et al,1995). Fusarium spp is widely distributed in soil, aboveground part and underground part of plant in nature, and it has a very close relationship with human.

B. With the quickly development of molecular biotechnology and bioinformatics, many acquired sequences of rDNA ITS regions of fungi have been submitted to three database such as Genbank, it make identification fungi come true by searching sequences of rDNA ITS regions from database, and it has been widely used in the study and identification of interspecific, intergeneric molecular systematics(Gu J ， et al，1994； W ang J B，et al,1999).

C. Gene which coding ribosomal nucleic acid of eukaryotic is an repeated transcription units ,it is rRNA-ITSI-5.8 S rRNA-ITSII-28S rRNA, about 100～200 copies, including coding region and noncoding region, ITS region is noncoding region. Coding region is relatively conservative, but evolution speed of ITS sequence is fast and often occurs variation, even in the same interspecific or intraspecific there are differences of different degree, so it is significant to identify strains by rDNA ITS sequence.

D. This study cloned, sequenced and analyzed the rDNA ITS sequences of 6 strains which have been identified morphologically as *Fusarium proliferatum*, it claims that there are actually 3 strains identified as *F. moniliforme* in 6 strains of *F. proliferatum*. In order to make an accurate diagnosis of the infection caused by *F. proliferatum* or *F. moniliforme* in clinical, It is significant to take effective treatment to cure the disease caused by the pathogens.

E. Figures and Tables

Figure.1.PCR products of rDNA ITS of *Fusarium*
M: ladder； Lane 1: H_2O; Lane2：CSUFT201 ；
Lane5：CSUFT204；Lane6：CSUFT205 ；
Lane3：CSUFT202；Lane4：CSUFT203 ；
Lane7：CSUFT206.

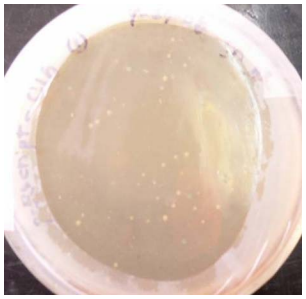

Figure 2. The clones of ITS gene

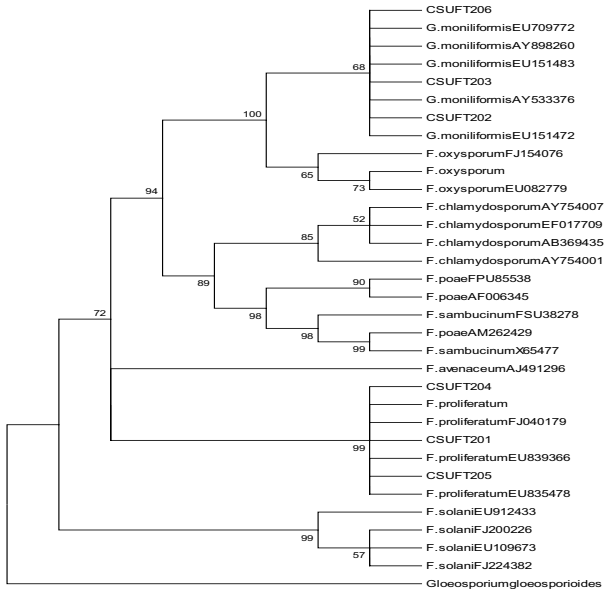

Figure 3. Phylogenetic tree based on the rDNA ITS sequences .Sexual phase of *F.moniliforme* is *Gibberella moniliformis*

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