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Analysis of the rDNA internal transcribed spacer region of the *Fusarium* species by polymerase chain reaction-restriction fragment length polymorphism

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Abstract. The *Fusarium* species are a widely spread phytopathogen identified in an extensive variety of hosts. The *Fusarium* genus is one of the most heterogeneous fungi and is difficult to classify. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis is a useful method in detection of DNA polymorphism in objective sequences. The aim of the present study was to identify the phylogenetic associations and usefulness of the internal transcribed spacer (ITS) region as a genetic marker within the most clinically important strain of the *Fusarium* species. A total of 50 strains of *Fusarium* spp. were used in the study, including environmental, clinical and reference isolates. The primers ITS1 and ITS4 were used in the study. Two restriction enzymes, *Hae*III and *Sma*I, were assessed for the digestion of PCR products. A PCR product of ~550-base pairs was generated for each *Fusarium* species. The digested products with *Hae*III and *Sma*I demonstrated that the bands generated for the medically significant *Fusarium* species, including *F. solani*, *F. oxysporum*, *F. verticillidea*, *F. proliferatum* and *F. fujikuri*, have different restriction enzyme patterns. In conclusion, it appears that the PCR-RFLP method used in the present study produces a sufficient restriction profile for differentiation of the most medically significant *Fusarium* species.

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Introduction

The genus *Fusarium* members are ubiquitous fungi frequently found in soils and plants (1). *Fusarium* species have been distinguished as a cause of localized infections (1,2). Due to bone marrow grafts and immunosuppressive treatment, invasive *Fusarium* infections have increased during the last few decades. The host immunological status and the level of the infection are the most significant aspects for the clinical effect of *Fusarium* infections (3,4).

The *Fusarium* species are a widely spread phytopathogen found in an extensive variety of hosts (5). It causes wilts and root rot disease, which produces secondary metabolites such as T2-toxin, zearalenone and trichothecene, causing huge economic problems through losing crops (6,7). The genus *Fusarium* is seldom able to cause human infections, such as onychomycosis, skin infections or keratitis. The *Fusarium* genus is one of the most heterogeneous fungi and is difficult to classify. Conversely, identification at the species level is required for biological and epidemiological reasons.

Conventional diagnostic techniques for identification of the *Fusarium* species in culture or in infected tissues are based on morphological features. This procedure is time consuming, and it can frequently be difficult to discriminate between similar species. Molecular approaches are more sensitive and quicker. Furthermore, they are employed to the specific detection of the *Fusarium* species. For these reasons, the molecular biological technique has been established in *Fusarium* systematics and the molecular variation at the DNA level has been investigated in numerous studies (8-10). The use of polymerase chain reaction (PCR) with primers targeted to the internal transcribed spacer (ITS) region of the ribosomal DNA (11,12) for identification of the *Fusarium* species has been reported. The ITS region sequences have shown to be highly variable in *Fusarium* genus (13).

PCR-restriction fragment length polymorphism (RFLP) analysis is a useful method in the detection of DNA polymorphism in objective sequences. In the present study, the amplified ITS region of rDNA was digested with 2 restriction endonucleases for genetic variation among *Fusarium* spp. The aim of the study was to identify the phylogenetic associations

Table I. Restriction fragment size of the ITS region of the *Fusarium* species following digestion with *Hae*III and *Sma*I, according to the sequencing of the strains.

Fusarium species	ITS size prior to digestion, bp	*Hae*III, bp	*Sma*I, bp
F. oxysporum	550	347, 88, 86	550
F. verticillidea	550	347, 89, 85	550
F. proliferatum	570	281, 90, 89, 77	570
F. fujikuri	550	281, 91, 77, 61	324, 210
F. solani	570	241, 123, 91, 65	325, 217

ITS, internal transcribed spacer; bp, base pairs.

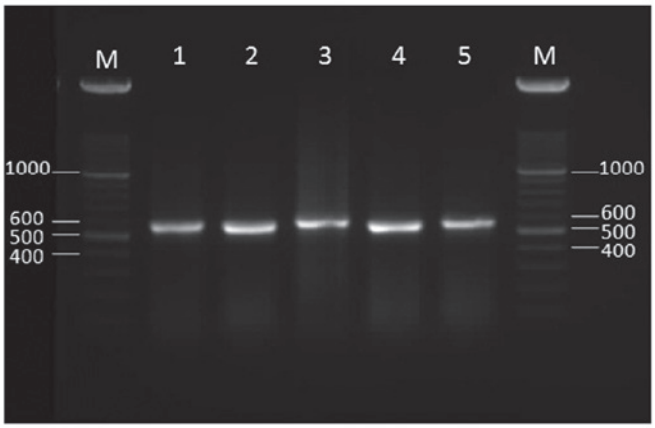

Figure 1. Agarose gel electrophoresis of the internal transcribed spacer region base pair (bp) products of the *Fusarium* species. M, 100 bp ladder; lane 1, *F. oxysporum* PFCC 5115; lane 2, *F. verticillidea* PFCC 15-89; lane 3, *F. proliferatum* PFCC 12-86; lane 4, *F. fujikuri* PFCC 5144; lane 5, *F. solani* PFCC 5284.

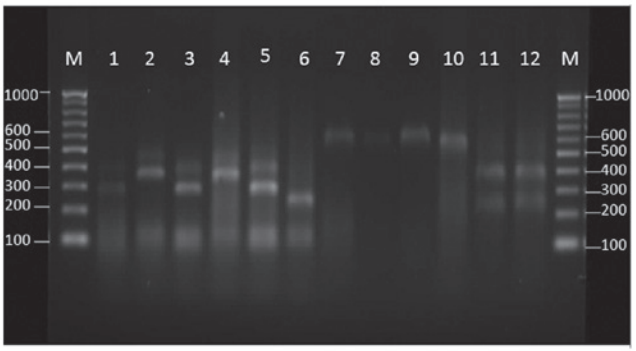

Figure 2. Agarose gel electrophoresis of the internal transcribed spacer region base pair (bp) products of the *Fusarium* species following digestion with *Hae*III (lanes 1-6) and *Sma*I (lanes 7-12). M, 100 bp ladder; lane 1, *F. oxysporum* PFCC 5115; lane 2, *F. verticillidea* PFCC 15-89; lane 3, *F. proliferatum* PFCC 12-86; lane 4, *F. verticillidea* PFCC 53-131; lane 5, *F. fujikuri* PFCC 5144; lane 6, *F. solani* PFCC 5284; lane 7, *F. oxysporum* PFCC 5115; lane 8, *F. verticillidea* PFCC 15-89; lane 9, *F. proliferatum* PFCC 12-86; lane 10, *F. verticillidea* PFCC 53-131; lane 11, *F. fujikuri* PFCC 5144; lane 12, *F. solani* PFCC 5284.

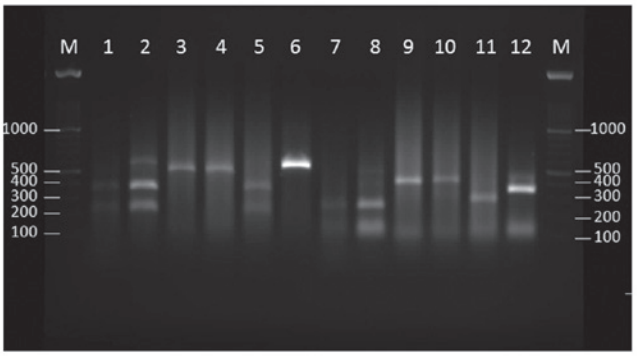

Figure 3. Agarose gel electrophoresis of the internal transcribed spacer region base pair (bp) products of the *Fusarium* species (environmental isolates) following digestion with *Sma*I (lanes 1-6) and *Hae*III (lanes 7-12). M, 100 bp ladder; lane 1, E20; lane 2, E34; lane 3, E35; lane 4, E36; lane 5, E39; lane 6, E42; lane 7, E20; lane 8, E34; lane 9, E35; lane 10, E36; lane 11, E39; lane 12, E42.

and usefulness of the ITS region as a genetic marker within the most clinically important of the *Fusarium* species.

Materials and methods

Microorganisms. A total of 50 strains of *Fusarium* spp. were used in the study including environmental, clinical and reference isolates. The following strains were used as a reference: *F. solani* PFCC 5284, *F. solani* PFCC 5285, *F. oxysporum* PFCC 30067, *F. oxysporu*m PFCC 5115, *F. verticillidea* PFCC 53-131, *F. verticillidea* PFCC 15-89, *F. proliferatum* PFCC 48-125, *F. proliferatum* PFCC 12-86 and *F. fujikuri* PFCC 5144. Enviromental strains were recovered from soil. Two strains were clinical strains, including *F. solani* PFCC 5284.

DNA extraction. In total, 100 ml of YEPD medium in Erlenmeyer flasks was inoculated with 1 ml of thick spore suspension. The flasks were incubated at 200 rpm under agitation at 25˚C for 72 h to obtain mycelium growth. The mycelia were harvested, washed with 0.5 M EDTA and sterile dH_2O and ground into a fine powder with a pestle and mortar using liquid nitrogen.

Approximately 100 mg of the mycelium powder was transferred into a 1.5-ml tube and 400 μl of lysis buffer [100 mM Tris-HCl (pH 8.0), 30 mM EDTA (pH 8.0) and 5% SDS w/v] was added.

Following the incubation of the tubes at 100˚C for 20 min, 150 μl of 3 M acetate potassium was added. The suspension was maintained for 10 min at -20˚C, and centrifuged at 14,000 x g for 10 min in 4˚C. The supernatant was transferred to a 1.5-ml Eppendorf tube (Eppendorf AG, Hamburg, Germany), and subsequently, 250 μl of phenol-chloroform-isoamyl alcohol (25:24:1, v/v) was added and the mixture was briefly vortexed and centrifuged at 14,000 x g for 10 min. Subsequent to transferring the upper aqueous phase to a new 1.5-ml tube, 250 μl chloroform-isoamyl alcohol was added. The tubes were briefly vortexed and centrifuged in 4˚C at 14,000 x g for 10 min. The supernatant was transferred to a new tube, an equal volume of ice-cold 2-propanol was added and the mixture was incubated at -20˚C for 10 min and subsequently centrifuged at 14,000 x g for 10 min. The upper aqueous phase was removed and the pellet was washed with 300 μl of ethanol 70%. The ethanol was removed and the DNA pellet was air-dried and dissolved in 50 μl of dH_2O.

PCR amplification. The primer sets (ITS1, 5'-TCCGTA GGTGAACCTGCGG-3') and (ITS4, 5'-TCCTCCGCTTAT TGATATGC-3') were used to amplify an ~600-base pair (bp) DNA fragment of the ITS region. PCR reactions were prepared to a final volume of 50 μl, containing reaction buffer, 2.2 mM $MgCl_2$, 200 μM of each dNTP, 2.5 unit of Taq DNA polymerase (CinnaGen, Karaj, Iran), a 30-ng DNA template and 50 pmol of each primer.

An initial denaturation step for 5 min at 95˚C was followed by 30 cycles of denaturation at 94˚C for 40 sec, annealing at 58˚C for 40 sec and extension at 72˚C for 40 sec, with a final extension at 72˚C for 5 min. The PCR product was run on a 1% agarose gel in Tris-base, acetic acid and EDTA (TAE) buffer, and stained with ethidium bromide. The PCR amplification of the ITS region resulted in ~595-bp of fragment.

RFLP analysis. Two restriction enzymes were analyzed. *Hae*III and *Sma*I had restriction sites in the ITS region of the *Fusarium* species. The reaction mixture for each enzyme was carried out in a total volume of 20 μl containing 10 units of the enzyme, 2 μl of the related buffer, 5 μl of the PCR product and Ultrapure water to create the 20-μl volume. Digested PCR products were subjected to electrophoresis on a 1.5% agarose gel in TAE buffer, and stained with ethidium bromide.

Results

PCR amplification of the ITS regions. PCR amplification of the ITS region with primers ITS1 and ITS4 resulted in an ~550-bp band (Fig. 1). The fragment was obtained from all 50 *Fusarium* strains. The ITS-region products were sequenced from several isolates, including the reference strains. A Basic Local Alignment Search Tool search showed that the ITS PCR products from 5 medically significant *Fusarium* reference strains, which were *F. solani*, *F. oxysporum*, *F. verticillidea*, *F. proliferatum* and *F. fujikuri*, exhibited a 99% homology with the associated sequences in the GenBank.

Restriction patterns for the Fusarium strains. Different restriction patterns were obtained following digestion with the *Hae*III and *Sma*I enzymes for the reference *Fusarium* strains, including *F. solani*, *F. oxysporum*, *F. verticillidea*, *F. proliferatum* and *F. fujikuri* (Table I and Fig. 2).

The restriction patterns of 6 environmental *Fusarium* strains with *Hae*III and *Sma*I are shown in Fig. 3. Strain E42 was recognized as *F. oxysporum*, based on the sequencing and restriction enzymes pattern.

Discussion

The ITS region was confirmed in the present study to be particularly suitable for the purpose of providing target genes for molecular identification of the *Fusarium* species. Variation in the nucleotide composition of the ITS region was successfully employed for recognition among the species (11-13). A variety of targets have been employed for DNA-based identification and dicrimination of pathogenic *Fusarium* species. DNA diversity in intergenic spacer (14) or ITS regions (13), β-tubulin, calmodulin, elongation factor 1α (15) and mycotoxins biosynthetic genes (16) as target genes for identification of *Fusarium* species have been tested with the PCR method. Several studies have shown that ITS1 and ITS2 are useful targets for detection of certain *Fusarium* species (13,17). The study by O'Donnell (18) reported an unexpected level of divergence for ITS sequences within the *F. sambucinum* species. There are certain advantages for the ITS region being a good target for detection reasons. The ITS region is comparatively conserved within numerous fungal species. It is present as multiple copies in the genome of fungi, and yields adequate taxonomic resolution for the majority of fungi (19). Furthermore, there are a large number of sequences from this locus in the GenBank, which enable the comparison of the obtained sequences. Therefore, nucleotide sequence heterogeneity in this region could be employed for classification of the majority of pathogenic fungi (20).

In the present study, the ITS1 and ITS4 primers were used to amplify the 5.8S rDNA gene. RFLP using *Hae*III and *Sma*I restriction enzymes provided a genus-specific assay for the rapid identification of medically significant *Fusarium* genus. According to the RFLP result, five species of *Fusarium*, which were *F. solani*, *F. oxysporum*, *F. verticillidea*, *F. proliferatum* and *F. fujikuri*, were divided into five RFLP types when used with the *Hae*III and *Sma*I enzymes.

In conclusion, it appears that the present PCR-RFLP method produces a restriction profile for the differentiation of the most medically significant *Fusarium* species.

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