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Original Article

Preliminary Identification and Typing of Pathogenic and Toxigenic *Fusarium* Species Using Restriction Digestion of ITS1-5.8S rDNA-ITS2 Region

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Abstract

Background: *Fusarium* species are capable of causing a wide range of crop plants infections as well as uncommon human infections. Many species of the genus produce mycotoxins, which are responsible for acute or chronic diseases in animals and humans. Identification of *Fusaria* to the species level is necessary for biological, epidemiological, pathological, and toxicological purposes. In this study, we undertook a computer-based analysis of ITS1-5.8SrDNA-ITS2 in 192 GenBank sequences from 36 *Fusarium* species to achieve data for establishing a molecular method for specie-specific identification.

Methods: Sequence data and 610 restriction enzymes were analyzed for choosing RFLP profiles, and subsequently designed and validated a PCR-restriction enzyme system for identification and typing of species. DNA extracted from 32 reference strains of 16 species were amplified using ITS1 and ITS4 universal primers followed by sequencing and restriction enzyme digestion of PCR products.

Results: The following 3 restriction enzymes *Tas*I, *Ita*I and *Cfo*I provide the best discriminatory power. Using ITS1 and ITS4 primers a product of approximately 550bp was observed for all *Fusarium* strains, as expected regarding the sequence analyses. After RFLP of the PCR products, some species were definitely identified by the method and some strains had different patterns in same species.

Conclusion: Our profile has potential not only for identification of species, but also for genotyping of strains. On the other hand, some *Fusarium* species were 100% identical in their ITS-5.8SrDNA-ITS2 sequences, therefore differentiation of these species is impossible regarding this target alone. ITS-PCR-RFLP method might be useful for preliminary differentiation and typing of most common *Fusarium* species.

Keywords: Fusarium, *Mycotoxins, PCR-RFLP, ITS*

Introduction

The genus *Fusarium* comprises a large number of species, most of which are soil saprophytic moulds or well-known plant pathogens and food contaminants. *Fusarium* species are capable of infecting a wide range of crop plants including cereals such as maize, wheat, or barley. *Fusarium* contamination is a major agricultural problem since they may reduce crop yield and quality (1, 2). They can rarely cause human infections such as nail infection, keratitis or skin infections in surgical wounds, burns, or deep ulcers. Disseminated fusariosis may occur in immunocompromised patients (3, 4).

Many species of the genus including *F. culmorum, F. graminearum, F. cerealis, F. sporotrichioides, F. poae, F. verticillioides, F. proliferatum, F. nygamai, F. sambucinum, F. acuminatum, F. avenaceum, F. compactum, F. thapsinum, F. pseudograminearum, F. polyphialidicum, F. napiforme, F. oxysporum* and *F. sacchari* produce mycotoxins such as T-2 toxin, deoxynivalenol, zearalenone and fumonisins. The toxins are responsible for acute or chronic diseases in animals and humans. The best example of the diseases is ATA (alimentary toxic aleukia) resulting from ingestion of overwintered cereal grains colonized by the toxigenic *F. sporotrichi-*

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oides and *F. poae*, capable of producing T-2 toxin (5). The high stability of these components during storage and processing, and their occurrence in a wide range of agricultural crop plants lead to this fact that harmful mycotoxins are found in animal feed and human foodstuff (6).

Fusarium is one of the most heterogeneous and difficult to classify fungal genera. On the other hand, identification to the species level becomes necessary for biological, epidemiological and toxicological purposes. Currently, differentiation of the species is based on physiological and morphological characteristics such as the size and shape of the macroconidia, absence or presence of the microconidia, conidiophores and chlamydoconidia, colony morphology and studies based on mycotoxins production profiles and to a lesser degree on host plant association (7). Subtle differences in a single characteristic may delineate species. However, the morphological and physiological characterization of the species is generally time-consuming and only the expert mycologists are able to ensure the correct identification (5). Therefore, in recent years, rapid, sensitive, and reliable methods have received more attention.

DNA-based molecular approaches have been developed for fungal systematic studies and for researches in the fields such as mycotoxicology and plant pathology. The majority of the diagnostic assays are random amplified polymorphic DNA (RAPD) analysis (8), specific diagnostic PCR primers (9), or DNA sequencing (10, 11). Nevertheless, there is still a need for rapid, sensitive, and accurate method for identification and differentiation of common pathogenic and/or toxigenic *Fusarium* species.

In the present investigation, we analyzed ITS1-5. 8SrDNA-ITS2 sequences of the various *Fusarium* species and designed a PCR-restriction enzyme system for preliminary identification and typing of *Fusarium* species and strains. The results of the study can facilitate more studies to exact identification of *Fusarium* isolates.

Materials and Methods

Fungal strains

Thirty-two reference strains of sixteen species of *Fusarium* were used. All standard strains were kindly provided by PROMEC Unit of the Medical Research Council (MRC), South Africa. The species and their reference numbers are listed in Table 1.

DNA extraction

Fungal strains were cultured for 3-5 d on 2% glucose and 1% peptone agar slant at 28° C in stationary conditions. The genomic DNA was extracted and purified from each colony as described previously (12). Briefly, a part of a colony of approximately 10 mm in diameter was collected, suspended in 300 µl lysis buffer [100 mM Tris-HCl, 10 mM EDTA (pH 8), 2% Triton X-100, 1% SDS, 100 mM NaCl) and 300 µl phenol-chlorophorm (1:1)] and vortexed (or shacked by hand) rigorously with 200 µl of glass beads (0.5 mm in diameter), to release DNA. After centrifugation for 5 min at 5000 rpm, the supernatant were mixed with 300 µl chlorophorm, centrifuged again, the supernatant was mixed with equal volume of iso-propanol and 0.1 volume of 3 M sodium acetate (pH 5.2) and centrifuged for 10 min at 10000 rpm. The pellet washed with 70% ethanol, dried and resuspended in 50 µl dd- water and was kept at -20° C as the purified DNA until use.

PCR

The ITS1-5.8SrDNA-ITS2 region of the rDNA was amplified using the forward (ITS1: 5'-TCC GTA GGT GAA CCT GCG G-3') and reverse (ITS4: 5'-TCC TCC GCT TAT TGA TAT GC-3') universal primers (13). Each amplification reaction included 50 µl of premix containing 2.5 U *Taq* DNA polymerase, PCR buffer, 1.5 mM MgCl2 and 200 µM dNTPs (Ampliqon, Denmark), 2 µl (about 10 ng) of template DNA, 1 µl (0.5 µM) of each primers and 46 µl of dd-water in a final volume of 100 µl. Amplification was performed on an Applied Biosystem 2700 thermocycler (Singapore) as follows: 1 cycle of 5 min at 95 °C (primary denaturation), 30 cycles of 45 s at 94 °C (denaturation), 1 min at 56° C (annealing), 1 min at 72 °C (extension) and finally 1 cycle of 7 min at 72 °C. Negative controls (no DNA template) were included for each run to detect the presence of any DNA contamination in reagents and reaction mixtures.

Sequencing

All PCR-amplified products were sequenced using a DNA sequencer (ABI Prism-Perkin-Elmer

310, Genetic Analyzer, Wellesley, MA, via Sinnagen company, Tehran, Iran). Sequencing was performed with forward (ITS1: 5'-TCC GTA GGT GAA CCT GCG G-3') primer. The sequences were analyzed with the Blast (http://www.ncbi.nlm.nih.gov/BLAST/) and DNASIS (Hitachi 2006 Japan) softwares.

Data analyses for choosing restriction enzyme

Sequence data of the 32 *Fusarium* strains sequences in this study together with 192 *Fusarium* isolates available at the GenBank (Table 2) were aligned and restriction patterns were predicted for each of the 610 known restriction enzymes listed in DNASIS software. Restriction fragments were predicted and compared for choosing the best discriminatory enzyme.

Restriction digestion

PCR products for each of the 32 *Fusarium* strains were digested individually with the restriction enzymes. The reactions mixtures were incubated 2-3 h at the optimal temperature (65 °C or 37 °C according to the manufacturer's guidelines) in a total volume of 25 µl containing 1 µl (10 units) of the enzyme, 2.5 µl of related buffer, 10 µl of PCR product and 11.5 µl distilled water. Digested amplification products were subjected to electrophoresis, and the sizes of restriction fragments were determined by comparison with 100-bp ladder standard DNA molecular weight marker (Fermentas, Lithuania).

Electrophoresis

DNA products were electrophoresed on agarose gel in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA) at 100 V for 45-120 min, in a gel composed of 1%, 1.5% and 2% for extracted DNA, PCR products and RFLP products, respectively. All gels were stained with 0.5 µg/ml of ethidium bromide in distilled water for 20 min and then de-stained in distilled water for 10min. The DNA bands were visualized with a UV trans-illuminator and photographed.

Results

Sequence analysis

The theoretic cutting sites and resulting fragment sizes using each of 610 restriction enzymes were analyzed for the one hundred and ninety two ITS1-5.8S rDNA-ITS2 GenBank sequences related to 36 *Fusarium* species, using DNASIS software. Three enzymes the *Tas*I, *Ita*I, and *Cfo*I were found superior as the best for differentiation of the species (Table 2). Using these restriction enzymes the different species and types of *Fusarium* can be classified in several groups. It is interesting that for most *Fusarium* groups the predicted restriction patterns for different enzymes is compatible with each other so that the changes in restriction enzymes do not change the relevant groups.

PCR

The genomic DNA was successfully amplified with ITS1-ITS4 primers and a product of approximately 550 base pair (bp) was amplified for all *Fusarium* strains, as expected regarding the sequence analyses. Figure 1 shows the agarose gel electrophoresis of the PCR products of standard toxigenic *Fusarium* species.

RFLP

ITS-PCR amplicons of the 32 *Fusarium* strains were digested by three mentioned enzymes separately. Figures 2-4 show the electrophoresis of PCR product of standard strains of *Fusarium* after digestion with the selected restriction enzymes *Tas*I, *Cfo*I and *Ita*I. As it is seen the size of RFLP products are exactly compatible with the size predicted from sequence analysis (Table 2).

Table 1: Reference strains of *Fusarium* species provided by MRC used in the study

***Fusarium* species**	**MRC Number**
F. acuminatum	3231, 8374
F. avenaceum	3227, 8381
F. compactum	2800, 6142
F. graminareum	4712, 4927, 6010
F. napiform	6033
F. nygamai (G. nygamai)	3997, 3546, 8547
F. oxysporum	1380
F. poae	8485, 8486
F. polyphialidicum	3390
F. proliferatum (G. intermedia)	2301, 8549, 8550
F. pseudograminearum	6251, 8443
F. sacchari	1838
F. sporotrichioides	0043, 4333
F. subglutinans	8553, 8554
F. thapsinum (G. thapsina)	8557, 8558
F. verticillioides (G. moniliformis)	0826, 8559, 8560

Table 2: Sequence analysis and digestion patterns of selected restriction enzymes for grouping the *Fusarium* species

Fusarium Species	Types	Size of ITS region	Size of fragments after digestion with			Accession Numbers
			*Tas*I	*Cfo*I	*Ita*I	
F. acuminatum	1	563	305, 250, 8	201, 177, 95, 90	227, 169, 89, 42, 28, 5, 3	AF132802
	2	546	249, 132, 119, 38, 8	251, 200, 95	231, 118,108, 89	U85533
F. acutatum		561	306, 247, 8	293, 175, 93	224, 160, 92, 42, 35, 5, 3	U34573
F. asiaticum		545	247, 171, 119, 8	252, 199, 94	142, 117, 107, 89, 79, 11	AB289552, AB289550, DQ459836, DQ459835, DQ459834, DQ459833
F. avenaceum		561	303, 250, 8	201, 175, 95, 90	227, 167, 89, 42, 28, 5, 3	AB272122, AB272121, AB272120, AJ491296, U26738, U26732
F. graminearum		545	247, 171, 119, 8	252, 199, 94	142, 117, 107, 89, 79, 11	AJ491293, AJ491292
F. poae	1	537	239, 132, 119, 39, 8	285, 252	142, 110, 106, 90, 89	AM262429
	2	535	238, 119, 117, 38, 15, 8	284, 251	231, 110, 105, 89	U85538, AF111058, AF111057
F. proliferatum		558	303, 181, 66, 8	293, 174, 91	224, 159, 90, 42, 35, 5, 3	AJ810449, X94171, U34558, DQ655730, EU314988, EU151490, EU151489, EU151488, EU151487, EU151486
F. sporotrichioides	1	545	248, 132, 119, 38, 8	251, 200, 94	231, 117, 108, 89	AF111055, AF111053, U85541, U38553, U38551, EF464168, EF464167, DQ093674, AY188917, AF414973
	2	563	305, 250, 8	201, 177, 95, 90	227, 169, 89, 42, 28, 5, 3	U38552
F. subglutinans	1	544	247, 157, 117, 15, 8	293, 251	231, 224, 89	AY898264
	2	544	181, 157, 134, 66, 8	293, 251	231, 224, 89	AY898263, AY898251
	3	546	181, 157, 134, 66, 8	294, 252	233, 224, 89	X94167, U34559, U38554
	4	546	181, 157, 134, 66, 8	294, 252	224, 162, 89, 71	U38556
	5	560	305, 181, 66, 8	293, 176, 91	224, 90, 89, 70, 42, 37, 5, 3	U38555
F. verticillioides	1	546	181, 157, 134, 66, 8	293, 253	233, 224, 89	X94166, AY898260 AY898259, AY898258, AY898257, AY898256, AY898255, AY898254, AY898253, U34555
F. nygamai	1	557	302, 181, 66, 8	293, 173, 91	224, 193, 90, 42, 5, 3	AY898252, X94174, U34568
	2	546	181, 157, 134, 66, 8	293, 253	233, 224, 89	U34563
F. thapsinum	1	556	301, 181, 66, 8	293, 172, 91	237, 224, 90, 5	U34560
	2	558	303, 181, 66, 8	293, 174, 91	224, 159, 90, 42, 35, 5, 3	U34574
F. pseudograminearum		542	247, 168, 119, 8	199, 166, 94, 83	142, 117, 107, 89, 87	AJ491295, AJ491294, DQ459871
F. napiforme		546	181, 157, 134, 66, 8	293, 253	233, 224, 89	X94175, U34570, DQ297555, DQ297554, DQ297553, AY303609
F. sacchari	1	546	181, 157, 134, 66, 8	293, 253	233, 224, 89	X94168
	2	545	247, 157, 133, 8	293, 252	232, 224, 89	U34556
F. polyphialidicum	1	547	248, 135, 118, 38, 8	294, 253	233, 225, 89	AJ538042, X94172, U34580
	2	544	232, 223, 89	292, 252	213, 172, 118, 33, 8	AY745991
F. oxysporum	1	555	303, 211, 33, 8	290, 174, 91	415, 90, 42, 5, 3	DQ906171
	2	546	247, 159, 117, 15, 8	293, 253	322, 224	AM262428, AF132799, AF165875, AF069310, U34566
	3	544	247, 157, 117, 15, 8	293, 251	231, 224, 89	AY853769 AY380575X94173
	4	558	303, 247, 8	293, 174, 91	224, 159, 90, 42, 35, 5, 3	AF132800

Table 2: Countinued…

	5	543	247, 157, 116, 15, 8	293, 250	224, 160, 89, 70	U28161, U28159
F. kyushuense		544	248, 131, 119, 38, 8	250, 199, 95	142, 118, 107, 89, 88	U85547, U85546, U85545, U85544
F. redolens	1	544	247, 157, 117, 15, 8	293, 251	231, 224, 89	AB304483 AB304482 AB304481 AB304480 AB304479 AB304478 AB304477 AB304476 AB304475 AB304474 AB304473 AB304472
	2	559	303, 248, 8	294, 265	225, 197, 95, 42	U34565, X94169
F. cerealis		545	247, 171, 119, 8	252, 199, 94	142, 117, 107, 89, 79, 11	AJ246148, U85534, EU214569, DQ459869, AF006341, AF006340
F. tricinctum	1	562	303, 251, 8	201, 176, 96, 89	228, 168, 88, 42, 28, 5, 3	AF111066 , AF111056
	2	563	267, 250, 38, 8	201, 177, 95, 90	227, 169, 89, 42, 28, 5, 3	AF111054
F. beomiforme		553	298, 247, 8	198, 169, 95, 91	224, 190, 90, 41, 5, 3	X94178, U34582
F. sambucinum	1	534	239, 168, 119, 8	285, 249	229, 111, 105, 89	U34579
	2	562	304, 250, 8	201, 176, 95, 90	227, 168, 89, 42, 28, 5, 3	U38279
	3	545	247, 171, 119, 8	252, 198, 95	142, 118, 106, 89, 79, 11	U38278, U38277
F. dlamini		559	304, 247, 8	293, 175, 91	224, 160, 90, 42, 35, 5, 3	X94177, U34572
F. chlamydosporum	1	550	247, 171, 124, 8	257, 199, 94	142, 117, 107, 94, 79, 11	AB272115
	2	545	248, 132, 124, 38, 8	256, 200, 94	231, 117, 108, 94	AF111059
	3	545	290, 247, 8	252, 198, 95	232, 224, 89	AY213655, EU214561, DQ489296, DQ489289, AJ853773, AY754007
	4	543	289, 246, 8	198, 161, 94, 90	231, 223, 89	AY754001
F. culmorum		545	247, 171, 119, 8	252, 199, 94	142, 117, 107, 89, 79, 11	AB272119, AB272118, AB272117, AB272116, U85535
F. succisae		546	181, 157, 134, 66, 8	293, 253	233, 224, 89	U34561
F. guttiforme		546	181, 157, 134, 66, 8	293, 253	233, 224, 89	U34562
F. pseudonygamai		546	181, 157, 134, 66, 8	293, 253	233, 224, 89	U34563, DQ297563, DQ297562, DQ297561, DQ297560, DQ297559
F. pseudocircinatum		546	181, 157, 134, 66, 8	293, 253	233, 224, 89	U34569
F. inflexum		545	247, 158, 132, 8	293, 252	231, 224, 90	U34577
F. robustum		536	239, 132, 119, 38, 8	285, 251	231, 111, 105, 89	U85539
F. buharicum		566	309, 249, 8	295, 175, 96	226, 195, 95, 42, 5, 3	U34581
F. equiseti	1	556	302, 246, 8	198, 173, 94, 91	223, 193, 90, 42, 5, 3	EU016679, EU016678, EU016677
	2	541	290, 219, 24, 8	252, 198, 91	232, 220, 89	EF611087
	3	546	290, 248, 8	252, 199, 95	232, 225, 89	AB277550, AB272123, EF483926, AY986957, AY147368, AY147367, AY147366, AY147365, AY147364, AY147363, AY147362, AY147361, EU326202, DQ026008, EU030338, EU030331
F. phyllophylum		558	303, 181, 66, 8	293, 174, 91	224, 159, 90, 42, 35, 5, 3	U34574
F. compactum		558	301, 249, 8	200, 170, 95, 93	190, 118, 108, 92, 42, 5, 3	Our study

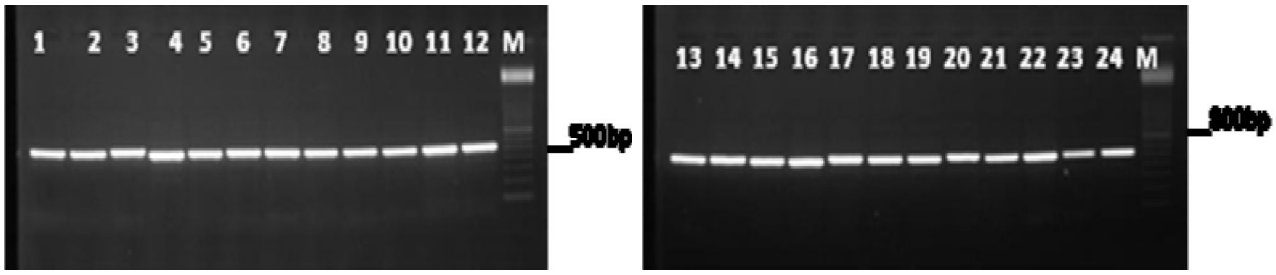

Fig. 1: Agarose gel electrophoresis of ITS PCR products of different *Fusarium* species: Lanes 1 to 24: *F. polyphialidicum* (MRC 3390), *F. pseudograminearum* (MRC 8443), *F. acuminatum* (MRC 3231), *F. poae* (MRC 8486), *F. subglutinans* (MRC 8554), *F. thapsinum* (MRC 8557), *F. avenaceum* (MRC 8381), *F. proliferatum* (MRC 8549), *F. compactum* (MRC 2800), *F. graminearum* (MRC 4712), *F. sporotrichioides* (MRC 0043), *F. sporotrichioides* (MRC 4333), *Fusarium no. 23*, *F. proliferatum* (MRC 8549), *F. verticillioides* (MRC 8559), *F. poae* (MRC 8485), *F. proliferatum* (MRC 8550), *F. sacchari* (MRC 1838), *F. verticillioides* (MRC 0826), *F. acuminatum* (MRC 8374), *F. napiforme* (MRC 6033), *F. pseudograminearum* (MRC 6251), *F. graminearum* (MRC 4927) and *F. graminearum* (MRC 6010) respectively. Lanes M: 100 bp molecular size marker.

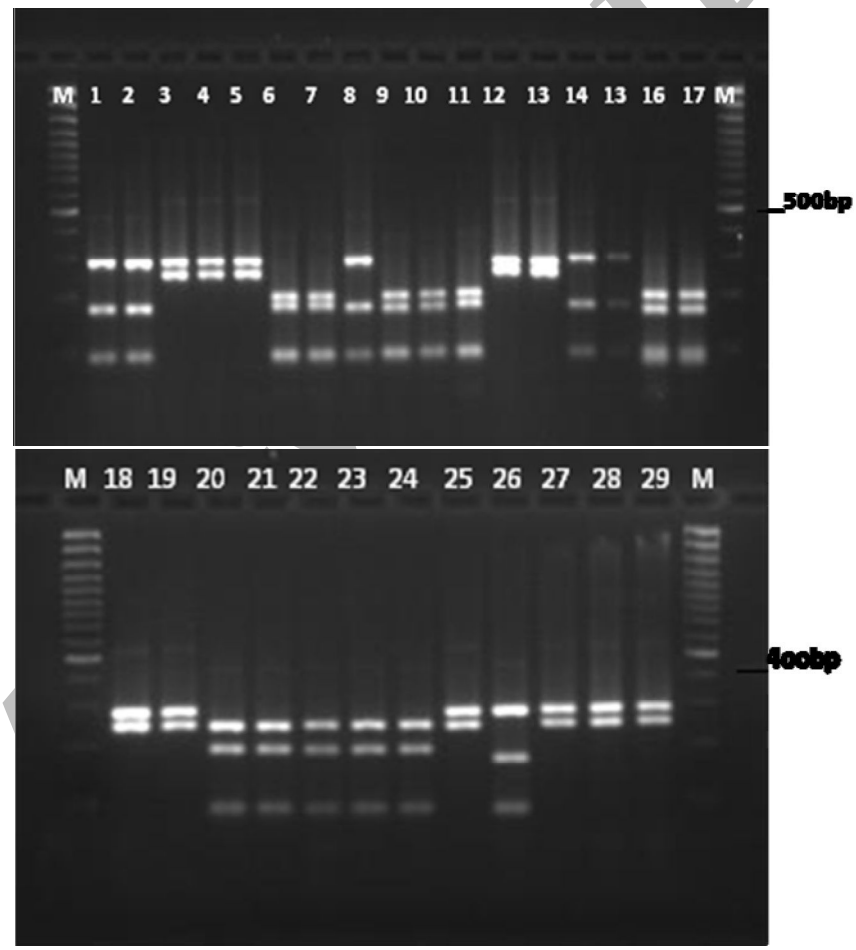

Fig. 2: Agarose gel electrophoresis of ITS PCR products of *Fusarium* species after restriction digestion with *Cfo*I. Lanes 1 and 2: *F. proliferatum* (MRC 8549 and MRC 8550), 3, 4 and 5: *F. verticillioides* (MRC 0826, MRC 8559 and MRC 8560), 6 and 7: *F. acuminatum* (MRC 8374 and MRC 3231), 8: *F. thapsinum* (MRC 8557), 9 and 10: *F. compactum* (MRC 2800 and MRC 6142), 11: *F. avenaceum* (MRC 8381), 12 and 13: *F. poae* (MRC 8485 and MRC 8486), 14 and 15: *F. nygamai* (MRC 8547 and MRC 8546) 16 and 17: *F. pseudograminearum* (MRC 6251 and MRC 8443), 18 and 19: *F. subglutinans* (MRC 8553 and MRC 8554), 20 and 21: *F. sporotrichioides* (MRC 0043 and MRC 4333), 22, 23 and 24: *F. graminearum* (MRC 4927, MRC 4712 and MRC 6010), 25: MRC 1838, (*F. sacchari*), 26: MRC 1380 (*F. oxysporum*), 27: MRC 6033 (*F. napiforme*), 28: MRC 3390 (*F. polyphialidicum*) and 29: MRC 0023. Lanes M 100 bp molecular size marker.

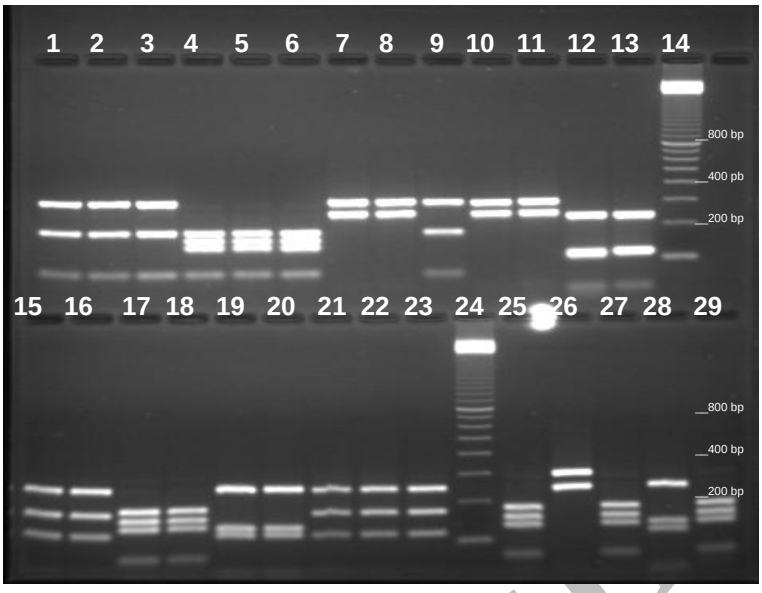

Fig. 3: Agarose gel electrophoresis of ITS PCR products of *Fusarium* species after restriction digestion with *Tas*I. Lanes1, 2 and 3: *F. proliferatum* (MRC 8549 and MRC8550), 4, 5 and 6: *F. verticillioides* (MRC 0826, MRC 8559 and MRC 8560), 7 and 8: *F. acuminatum* (MRC 8374 and MRC 3231), 9: *F. thapsinum* (MRC 8557), 10: *F. compactum* (MRC 2800), 11: *F. avenaceum* (MRC 8381), 12 and 13: *F. poae* (MRC 8485 and MRC 8486), 15 and 16: *F. pseudograminearum* (MRC 6251 and MRC 8443), 17 and 18 *F. subglutinans* (MRC 8553 and MRC 8554), 19 and 20: *F. sporotrichioides* (MRC 0043 and MRC 4333), 21, 22 and 23: *F. graminearum* (MRC 4927, MRC 4712 and MRC 6010), 25: , MRC 1838 (*F. sacchari*), 26: MRC 1380 (*F. oxysporum*), 27: MRC 6033 (*F. napiforme*), 28: MRC 3390 (*F. polyphialidicum*) and 29: MRC 0023, Lanes 14 and 24 are 100 bp molecular size marker.

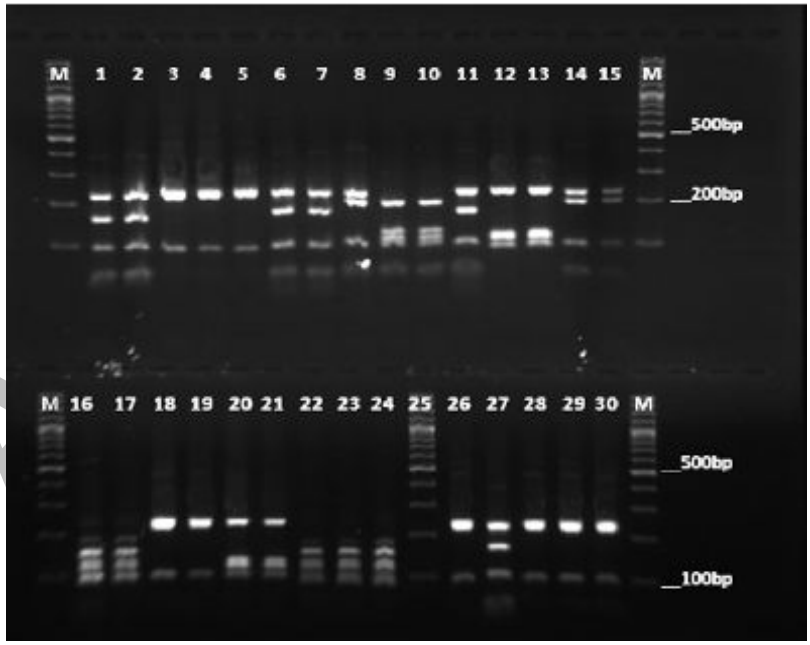

Fig. 4: Agarose gel electrophoresis of ITS PCR products of *Fusarium* species after restriction digestion with *Ita*I. Lanes 1 and 2: *F. proliferatum* (MRC 8549 and MRC 8550), 3, 4 and 5: *F. verticillioides* (MRC 0826, MRC 8559 and MRC 8560), 6 and 7: *F. acuminatum* (MRC 8374 and MRC 3231), 8: *F. thapsinum* (MRC 8557), 9 and 10: *F. compactum* (MRC 2800 and MRC 6142), 11: *F. avenaceum* (MRC 8381), 12 and 13: *F. poae* (MRC 8485 and MRC 8486), 14 and 15: *F. nygamai* (MRC 8547, MRC 8546) 16 and 17: *F. pseudograminearum* (MRC 6251 and MRC 8443), 18 and 19: *F. subglutinans* (MRC 8553 and MRC 8554), 20 and 21: *F. sporotrichioides* (MRC 0043 and MRC 4333), 22, 23 and 24: *F. graminearum* (MRC 4927, MRC 4712 and MRC 6010), 26: MRC1838, (*F. sacchari*), 27: MRC 1380 (*F. oxysporum*), 28: MRC 6033 (*F. napiforme*), 29: MRC 3390 (*F. polyphialidicum*) and 30: MRC 0023. Lanes M and 25: 100 bp molecular size marker.

Discussion

Identification of *Fusarium* species is ideally carried from growth on carnation leaf agar, an effective medium for macroconidium production, however, this medium is not readily available to the non-specialist. Recently many investigations have been focused on molecular approaches for identification and speciation of moulds including *Fusarium* species. Various targets have been used for DNA-based identification and differentiation of pathogenic *Fusarium* species. The use of DNA diversity in the ribosomal regions such as intergenic spacer (IGS) (14) or internal transcribed spacer (ITS) regions (15, 16), elongation factor 1α (*EF-1α*) (17), β-tubulin (β-*TUB*), calmodulin (*CAM*) (18), 28S rRNA gene (19), RNA polymerase II second largest subunit (*RPB2*), and mycotoxins biosynthetic genes, as targets to identify the species have been examined using PCR amplification of the DNA. Following the amplification, the methods such as restriction fragment length polymorphism (RFLP) analysis (5, 14, 20), DNA probe hybridization or DNA sequencing analysis (10, 11, 19) eliminate the need for several cultures prior to identification.

Several studies have demonstrated that ITS1 and ITS2 are useful targets for identification of some species complexes of *Fusarium* (21, 22). There are some disadvantages for using the ITS region as a target including insufficient variability to distinguish the various species in the *Fusarium* species complexes and probable problems with reliability of the ITS sequences deposited in the reference databases (23). On the other hand, available data demonstrate that sequences of ITS region and domains D1 and D2 of the 28S ribosomal DNA (rDNA) are too conserved to resolve important fusaria at the species level (21, 22). Moreover, use of the ITS within the *Gibberella fujikuroi* species complex and *F. oxysporum* species complex (11) and β-tubulin within the *F. incarnatum-equiseti* species complex and *F. solani* species complex could be confusing due to paralogous or duplicated divergent alleles (24). Nevertheless, several advantage for ITS, make the region to be still a good target for identification purposes. The region is relatively conserved within many species, is present as multiple copies in the fungal genome, and yields sufficient taxonomic resolution for most fungi (25). Furthermore, the GenBank contains a large number of sequences from this locus, enabling a ready comparison of the sequence from an unknown species (25). For these reasons nucleotide sequence heterogeneity within this region can be used to phylogenetically classify the majority of pathogenic fungi (26).

In the present study, a new PCR–restriction enzyme profile for rapid and low price differentiation of *Fusarium* species based on the sequence variation in the ITS-5.8S rDNA-ITS2 was developed based upon the analysis of published ITS sequences and the cutting sites of more than 600 restriction enzymes. The method was next validated using 32 *Fusarium* strains. The following three enzymes of *Tas*I, *Cfo*I and *Ita*I provided the highest discriminatory power. Using three enzymes, the 36 different *Fusarium* species were divided into some types. For the following species *F. acuminatum, F. avenaceum, F. proliferatum, F. pseudograminearum, F. napiforme* and *F. culmorum* no intra-species variation was observed, while for *F. poae, F. sporotrichioides, F. subglutinans, F. verticillioides, F. sacchari* and *F. oxysporum,* individual isolates within each species had different RFLP pattern illustrating a potential of this method for genotyping of these species.

The following species could be reliably identified to the species level using our method: *F. acutatum, F. oxysporum* type 2, *F .redolens, F. dlamini, F. acuiseti, F. asiaticum, F. pseudograminearum, F. cerealis, F. subglutinans* types 3 and 4, *F. nygamai* type 2, *F. sacchari, F. verticillioides* type 2, *F. nygamai* type1, *F. thapsinum* type 1, *F. polyphialidicum, F. kyushuense, F. poae* type 1, *F. oxysporum* type 2 and 5, *F. acuiseti* and *F. chlamydosporum* types 3 and 4. However, a minority of the species (*F. subglutinans* type 2, *F. verticillioides* type 1, *F. napiforme* and *F. sacchari)* has identical ITS-5.8S rDNA-ITS2 sequences and thus inclusion of another DNA target or morphological criteria is necessary for correct identification.

Although previous studies have investigated the use of PCR based techniques for the diagnosis of *Fusarium* isolates, the methods have been

hampered by including only a limited number of *Fusarium* species in their validation. Furthermore, our study is sequence-based and we analyzed considerable number of ITS sequences and tested as many as 610 (nearly all known) restriction enzyme for understanding and applying the best digestion profile for differentiation or typing the species in the best way. As it is seen in Figures 2-4, the electrophoretic patterns achieved by PCR-restriction digestion of the PCR products of the tested standard strains were completely comparable and coordinated with computerized data (Table 2) and this findings result in to trust to the method. It is noteworthy that although there are different RFLP types for some *Fusarium* species, as it is shown in Figures 2-4 the different strains (with different collection number) of same species tested in our study, have identical RFLP patterns.

In conclusion, it seems that the PCR-RFLP method reported in the study, generates a sufficiently detailed restriction profile for preliminary differentiation and typing of most common *Fusarium* species and can be developed for recognition of more species. This method has now been implemented in our laboratory for the testing of toxicogenic *Fusarium* from different food sources in Iran.

Ethical Considerations

All ethical issues including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc have been completely observed by the author.

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