PCR - RFLP patterns for the differentiation of the Fusarium species in virtue of ITS rDNA

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Abstract

Background and Purpose: The Fusarium species are among the most important fungi in the medical, veterinary and agricultural fields.

Materials and Methods: In the present study, 172 strains of these fungi have been analyzed. The high molecular weight DNAs were extracted from 23 reference strains as well as from 149 isolated Fusarium species. Using the designed nucleotide primers from rDNA of Fusarium species, PCR analysis was performed for the amplification of ITS regions. Afterwards, the location of the effective endonuclease enzymes has been evaluated within approximately 930 bp of rDNA sequence.

Results: Through the selected enzymes including: HhaI, MspI, TaqI and FagI, the mentioned Fusarium species have been divided into 33 groups. The first three enzymes were able to classify Fusarium species into 23 groups of which 19 groups included one member, one group included two members and three groups included three members of the Fusarium species. This study also revealed the possibility in the identification of F. semitectum, F. solani complex, F. pseudogromineum, F. nitisakadoi, F. coeruleum and F. acuminatum species by one unique enzyme. In addition, our study indicated the ability of the differentiation of F. Compactum from F. equiseti.

Conclusion: As compared to previous studies with more endonuclease enzymes and with limited in identifications, the ITS-RFLP patterns reported here an attempted to evaluate most of the Fusarium species successfully.

Keywords: Fusarium spp., PCR-RFLP, ITS rDNA

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Introduction

The Fusarium species can incite directly the diseases in plants, humans and domesticated animals. The mortality rate for human patients with the systemic Fusarium infections is 70% [1]. In addition, Fusarium spp. produce secondary metabolites associated with plant diseases, as well as with cancer and other growth defects in humans and domestic animals [2-4].

Fusarium is one of the most heterogenous fungal genera in which the classification of species within this genus is very complicated. Currently, the discrimination of Fusarium spp. has been done based on the morphological characteristics such as the shape and the size of the macroconidia, the presence/absence of microconidia and chlamydospores and also the colony morphology. These procedures are time-consuming. They need much effort and an expert staff. Therefore, a rapid and reliable assay for the identification of Fusarium spp. would be beneficial. The polymerase chain reaction (PCR) technique is a sensitive, rapid and a reliable diagnosis method in species identification which will enable us to overcome the poor sporulation of the Fusarium spp. and its identification [5, 6].

Interestingly, the molecular approaches have been developed for Fusarium systematic studies including: Random Amplified Polymorphic DNA (RAPD) analysis, a specific diagnostic PCR primers and DNA sequencing [7-14]. However, the most current methods are
often based on the ribosomal RNA (rRNA) sequences analysis which holds both conserved and variable regions, allowing discrimination at different taxonomic levels [15, 16].

The restriction analysis of PCR-amplified rDNA (rRNA gene) sequences has been shown to be a suitable method for the taxonomic studies in Fusarium spp. [17-19]. A few limited results of PCR-RFLP based on the identification of Fusarium species have been reported so far in Martiella, Elegans, Liseola and Sporotrichiella sections [18, 20-22]. These studies showed that the nucleotide sequences of the ITS regions are useful for identifying Fusarium species. The aim of the current study was to evaluate the utility of PCR-RFLP of the ITS region for discriminating the Fusarium species.

Material and Methods

Fungal isolates

One hundred seventy-two fungal strains including 23 reference Fusarium strains and 149 Fusarium isolates obtained from Iranian cereal grains were included in the present study (Table 1) [23]. All the isolates were identified morphologically according to Nelson et al., subcultured on potato dextrose agar (PDA) medium and incubated at 25°C for 1-2 weeks before storage [24].

DNA isolation

Fungal DNA was extracted according to the standard protocols [25, 26]. Except for a few modifications; the harvested mycelial mass was flash-frozen in liquid nitrogen and proceeded to make a fine powder in a porcelain mortar. The powder was suspended in the DNA extraction buffer including 50mM Tris-HCl (pH 8.0), EDTA (50mM), 3% SDS and 50μl of proteinase-K(20mg/ml). Then the suspension was incubated (65°C for 1h) and the cellular debris removed by centrifugation (15000 g for 30 min). Finally, the DNA pellet was washed with 70% ethanol and re-suspended in distilled water after being air dried.

Designing Primer

Forward primer ITS5 (5’GGAG TAAAAGT CGTAACAAGG3’) reported by white et al. [27], and newly designed reverse primer as7 (5’CTTCCCTTCAACAATTTC AC3’) from 28S rDNA region was used for the amplification of Fusarium species. Multiple Sequence alignment analysis was performed via MEGA5.1 software.

PCR amplification

Amplification was performed including 2.5 μl of 10X PCR buffer, 0.2 mM of each dNTPs, 0.1 μM of each forward and the reverse primers, template DNA (25ng), and 2.5 U of Taq DNA polymerase. The PCR condition was set up in

| Table 1. The Fusarium reference strains and Iranian isolates used in this study |
|----------------|-----------------|-----------------|----------------|
| Fusarium species | Reference strains | Iranian isolates | Total |
| F. acuminatum | MCR 3231 | - | 1 |
| F. avenaceum | MCR 8381 | 1 | 2 |
| F. babinda | - | 1 | 1 |
| F. campioceras | - | 2 | 2 |
| F. chlamydosporum | - | 1 | 1 |
| F. compactum | MCR 2800 | 4 | 5 |
| F. culmorum | - | 2 | 2 |
| F. dlamin | - | 1 | 1 |
| F. graminearum | MCR 4712, 4927, 6010 | - | 3 |
| F. heterosporum | - | 1 | 1 |
| F. cf.langsethiae | - | 1 | 1 |
| F. nygamai | MCR 8547 | 7 | 8 |
| F. oxysporum | - | 6 | 6 |
| F. poae | MCR 8485, 8486 | - | 2 |
| F. proliferatum | MCR 8549, 8550 | 52 | 54 |
| F. pseudograminearum | MCR 8443 | - | 1 |
| F. pseudonygamai | - | 2 | 2 |
| F. sporotrichioides | VTT D72014, BBA | TCR 8553, 8554 | 20 | 22 |
| F. subglutinans | MCR 8557, 6251 | - | 2 |
| F. thapsinum | - | 4 | 4 |
| F. verticillioides | MCR 8559, 8560, 0826 | 43 | 46 |
| F. xylarioides | - | 1 | 1 |
| Total | 23 | 149 | 172 |
initial denaturation at 94°C for 2 min, 35 cycles (each of 30s at 94°C, 30s at 59°C, 1 min at 72°C), and a final extension at 72 °C for 7 min. Amplified products were visualized by 1% (w/v) agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) stained with ethidium bromide (0.5 μg/ml).

**ITS-RFLP analysis**

The ITS5-A7 sequences of the various Fusarium species obtained from DDBJ/EMBL/GenBank databases were aligned by MEGA3.1 software. The restriction patterns of the PCR products of the species mentioned above were predicted for each of the known restriction enzymes using the Webcutter online software. The predicted restriction fragments were compared for choosing the best discrimination. Finally, the enzymes MspI, HhaI, TaqI and FaqI were selected (Table 2).

Digestion reaction was performed by incubating a 5 μl of PCR product with 2.5 U of enzymes (Fermentas) in a final reaction volume of 15 μl at 37°C for 2h. The restriction fragments were separated by 1.8% agarose gel electrophoresis in TAE buffer for 50 min at 90 V and stained by the ethidium bromide.

**Result**

**Fungal Isolates**

A total of 172 Fusarium isolates including 23 reference strains and 149 Iranian isolates were analyzed. The isolates that belonged to 23 Fusarium species were as follows: F. proliferatum (54), F. verticillioides (46), F. subglutinans (22), F. nygamai (8), F. oxysporum (6), F. compactum (5), F. sporotrichioides (4), F. tricinctum (4), F. graminearum (3), F. poae (2), F. camptoceras (2), F. culmorum (2), F. pseudonygamai (2), F. avenaceum (2), F. thapsinum (2), F. acuminatum (1), F. babinda (1), F. chlamydosporum (1), F. diamini (1), F. heterosporum (1), F. cf.langsethiae (1), F. pseudograminearum (1) and F. xylarioides (1) (Table 1).

**Molecular characterization of the Fusarium Isolates**

The size of amplified PCR products was estimated to be 930 bp while using ITS5 and

**Table 2. Size of rDNA ITS gene PCR products from a number of Fusarium species in accordance to the GenBank / EMBL data library**

| Species                  | Size of PCR product (bp) | EMBL accession no. |
|--------------------------|--------------------------|---------------------|
| F. chlamydosporum        | 895                      | EU715615            |
| F. sambucinum            | 913                      |AY188921             |
| F. poae                  | 915                      |AY188915             |
| F. venenatum             | 920                      |AY188922             |
| F. pseudograminearum     | 924                      |DQ459871             |
| F. sporotrichioides      | 925                      |AY188917             |
| F. proliferatum          | 925                      |GQ167230             |
| F. oxysporum             | 926                      |DQ535184             |
| F. semitectum            | 926                      |AY633745             |
| F. subglutinans          | 926                      |GQ167235             |
| F. culmorum              | 927                      |DQ459870             |
| F. crookwellense         | 927                      |DQ459869             |
| F. austroamericanum      | 927                      |DQ459837             |
| F. boothii               | 927                      |DQ459848             |
| F. brasilicum            | 927                      |DQ459860             |
| F. cortaderiae           | 927                      |DQ459859             |
| F. lunulosporum          | 927                      |DQ459868             |
| F. meridionale           | 927                      |DQ459842             |
| F. mesosamericanum       | 927                      |DQ459844             |
| F. graminearum           | 927                      |DQ459830             |
| F. verticillioides       | 927                      |GQ168840             |
| F. asiaticum             | 928                      |DQ459835             |
| F. equiseti              | 928                      |EU595566             |
| F.armeniacum             | 930                      |GQ505462             |
| F.latertitium            | 939                      |AY188920             |
| F. tricinctum            | 943                      |AY188923             |

As 7 primer pair. The actual size of PCR products of rDNA region in some species of Fusarium is shown in Table 3 in comparison with the existing data in the NCBI. The lowest and the highest number of the fragments were belonged to the digestion of F. chlamydosporum and F. tricinctum PCR products, respectively. The patterns and the estimated sizes of the restriction fragments that generated four restriction enzymes (HhaI, MspI, TaqI and FaqI) (Table 3) revealed that HhaI and FaqI enzymes showed 7 patterns (A to G), TaqI enzyme, 6 patterns (A to F) and the highest number of patterns (14 different patterns) that were obtained via MspI enzyme. The selected enzymes divided the Fusarium species in 33 groups (Table 3, 4). In the present study, by using three restrictive enzymes including HhaI, MspI, TaqI, the 23 different groups were identified (Table 4). 19 out of them
Table 3. Band Patterns (A to N) and their estimated restriction fragment sizes (base pairs) obtained from rDNA ITS digestions

| Band Pattern | Enzyme | Hha I (Clo I) | Msp I | Taq I | FaqI |
|--------------|--------|---------------|-------|-------|------|
| A            | E      | 530, 315      | 825, 100 | 490, 240 | 700, 220 |
| B            | E      | 530, 200, 120 | 710, 120 | 430, 235 | 580, 220, 135 |
| C            | E      | 450, 315      | 570, 330 | 340, 235 | 430, 300, 220 |
| D            | E      | 450,200,120   | 525, 330 | 290, 235 | 410, 240, 220 |
| E            | E      | 370, 315, 175 | 500, 445 | 230, 235, 145 | 320, 250, 220, 135 |
| F            | E      | 370, 200, 170, 120 | 500, 380 | 235, 210, 125 | 300,275, 220, 135 |
| G            | E      | >320, 250     | 500, 335, 140 | - | >385, 185 |
| H            | E      | -             | 445, 375, 100 | - | - |
| I            | E      | -             | 445, 120 | - | - |
| J            | E      | -             | 430, 375 | - | - |
| K            | E      | -             | 430, 300, 120 | - | - |
| L            | E      | -             | 430, 250, 120 | - | - |
| M            | E      | -             | 380, 200 | - | - |
| N            | E      | -             | 300, 160, 120 | - | - |

1. Considering the short registered sequences recorded in GenBank / EMBL database, cannot completely cut location and length of components identified.

Table 4. Band Patterns (A to N) revealed by restriction analysis of PCR-amplified ITS rDNA region among Fusarium isolates

| Isolate | Enzyme | Hha I | Msp I | Taq I | FaqI | Type |
|---------|--------|-------|-------|-------|------|------|
| F. dimorum | A      | E     | B     | D     | AFBBD |
| F. nivalis (Microdochium nivalis) | A      | E     | B     | B     | ABBD |
| F. semitectum (F. incarnatum) | B      | A     | F     | E     | BAPE |
| F. solani complex | E      | H     | A     | C     | EAC |
| F. pseudogroenianum | D      | A     | C     | B     | DAB |
| F. australamericanum | B      | A     | D     | B     | BABD |
| F. saccchari | A      | I     | C     | E     | ACE |
| F. nisikadoi | C      | A     | C     | B     | CACB |
| F. brevicatenuatum | A      | L     | C     | E     | ACE |
| F. acutatum, F. concinicum, F. redolens | E      | N     | B     | B     | ENBB |
| F. venenatum | A      | A     | D     | A     | AADA |
| F. subulatum | B      | L     | B     | F     | BLBF |
| F. mangelera | A      | L     | B     | B     | ALBB |
| F. concolor | F      | C     | C     | B     | FCCB |
| F. coeruleum | G      | E     | G     | G     | GBGG |
| F. beomiform | F      | N     | B     | B     | FNB |
| F. ambrosiod | E      | H     | D     | C     | BDHC |
| F. tricinctum, F. avenaceum, F. heterosporum | F      | C     | B     | B     | PBPP |
| F. verticillioides, F. polyphialidicum, F. daminis | E      | K     | B     | B     | EBKB |
| F. sporotrichioides, F. langsetiae | B      | A     | C     | F     | BACF |
| F. chlamydosporum, F. camptocera | B      | A     | C     | E     | BACE |
| F. equiseti | F      | B     | B     | E     | FBB |
| F. compactum | F      | H     | B     | B     | FBB |
| F. acuminatum | F      | D     | B     | B     | FBB |
| F. kyushuense, F. crookswelliae, F. culmorum, F. graminearum, F. flocciferum, F. lunducomporum, F. bonei, F. meridionalis, F. mesoamericanum, F. asiaticum, F. brassicicum, F. cortaderiae | B      | A     | C     | B     | BACB |
| F. poae | A      | A     | C     | E     | AACE |
| F. sambucinum, F. tumidum | A      | A     | C     | A     | ACA |
| F. robustum | A      | A     | C     | B     | AACB |
| F. lateritium, F. thapsinum, F. proliferatum, F. udim, F. globosum | E      | L     | B     | B     | ELBB |
| F. nygmaei | E      | L     | B     | A     | ELBA |
| F. buharicum | E      | L     | B     | F     | ELBF |
| F. polyphilidicum | A      | B     | C     | F     | ABCF |

1. Fusarium graminearum complex, 2. Gibberella fujikuroi complex, 3. Fusarium oxysporum complex. *including: F. solani, F. virguliforme, F. tucumaniae, F. phaseoli, F. brasiliensis
** For identifying and differentiating from other species using underlined patterns is not required.
that contain one species equally, one of them includes two species and three of them contain three species.

This study showed that using one specific enzyme could distinguish *F. nivale* (*Microdochium nivale*), *F. semitectum* (*F. incarnatum*), *F. solani* complex, *F. pseudo-raminearum*, *F. nisikadoi*, *F. coeruleum* and *F. acuminatum* species (Table 4). As well by two enzymes such as; *Hha*I and *Msp*I, *F. dimerum*, *F. sublunatum*, *F. beomiform*, *F. equiseti* and *F. compactum* could be differentiated (Table 4). As well by two enzymes such as; *Msp*I and *Taq*I, *F. sacchari*, *F. brevicatenulatum*, *F. concolor* and *F. ambrosium* could be distinguished. The restriction pattern of PCR –

amplified rDNA of the *Fusarium* strains digested with *Msp*I, *Hha*I and *Faq*I enzymes is shown in Figures 1-3. It shows that the bands generated corresponded to the predicted sizes.

NCBI information showed that the *Fusarium* section of *Sporotrichiella* including *F. poae*, *F. tricinctum*, *F. chlamydosporum* and *F. sporotrichoides* / *F. langsethiae* could be distinguished using the candidate enzymes (Table 4). But practically, it was seen that only *F. poae* and *F. tricinctum* could be discriminated. In order to discriminate *F. chlamydosporum* from *F. sporotrichoides* / *F. langsethiae*, *Mbo*I enzyme is required (Figures 4, 6). *F. compactum* from *F. equiseti* discrimination is also possible with *Hha*I, *Msp*I & *Taq*I enzymes (Figure 5).

**Discussion**

The restriction fragment analysis of the PCR-amplified region of rDNA from 172 isolates belonging to the 23 *Fusarium* species...
was used. By using ITS1 and ITS4 primers, this could distinguish a limited number of Fusarium species in the PCR-RFLP method [20, 21, 28]. The ITS1-ITS4 or ITS4-ITS5 or ITS4-ITS5 primer pairs often amplify a 550 ± 50bp fragment from the ITS element [27] which is not lengthy enough for the PCR-RFLP technique especially in the case of Fusarium genus. Therefore, the reverse primer for 28S rRNA gene sequence was designed to create a larger fragment and be seen in agarose gel clearly. Besides, more patterns have been generated that can be discriminative for more species of the Fusarium genus.

By virtue of these findings, we conclude identifying 33 groups of Fusarium species and at least 22 species of them are possible through four endonuclease enzymes while the previous studies reported that via the seven enzymes can discriminate 12 Fusarium species (Table 3) [20, 28].

HhaI and MspI are able to discriminate F. equiseti and F. makes a distinction on conidia morphology solely [2].

There are some isolates in which their new band patterns were not observed in our pattern list (Table 3). The RFLP pattern of morphology method in F. babinda was FLBE and also the RFLP pattern of three isolates of F. Subglutinans was diagnosed as -M-C. Therefore, it seems that it is possible to identify the species that are not listed in Table 4 using the proposed enzymes.

The similar patterns were observed for the several species which were distinguishable by the tease mount method. For example, the rDNA restriction pattern for F. tricinctum, F. avenaceum and F. heterosporum is FCBB or -CB- can be discriminated by tease mount method. F. tricinctum produces abundant microconidia that are napiform, oval, pyriform and citriform. F. avenaceum produces long and straight macroconidia but microconidia are produced sparsely by some isolates. Moreover, F. heterosporum has medium length macroconidia and no microconidia. The second example is BACE pattern that can approve the existence of F. camptoceras using the tease mount method. According to NCBI GenBank, the FaqI cleavage pattern in F. sporotrichioides should be F (Table 3), but pattern E was observed practically (Figure 5) indicating the weakness of ITS gene in the F. sporotrichioides identification.

In this study, all isolates and the reference strains of F. verticillioides had -B-E pattern whilst the GenBank information showed other patterns. The accession numbers including EU364843, EU364845 and EU364846 showed -K- B pattern while EU714404 the -D- E pattern. Consequently, it can be deduced that ITS gene in some species such as F. verticillioides induces interspecies’ differences although it is possible that some records have been registered mistakenly.

**Conclusion**

In conclusion, it could be concluded that using four endonucleases, namely, HhaI, MspI, TaqI and FaqI at least 22 species of Fusarium can be differentiated. For the
identification of the unknown *Fusarium* isolates, it is recommended to use the three enzymes, initially and *MspI*, *HhaI* and *TaqI*, sequentially.

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**Authors’ contributions**

R.K. designed, applied all tests and wrote manuscript, S.R. and MH.Y. managed the research and were supported financially, N.S. was scientific consultation, A.G. provided most of the reference *Fusarium* strains, F.N. applied some of the tests, V.P. edited the final manuscript.

**Conflicts of interest**

The authors have not supplied their declaration of conflict of interest.

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No financial interests related to the material of this manuscript have been declared.

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