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Short Communication: Genetic diversity among *Fusarium* isolates from cereals in Iran assessed using RAPD marker

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Abstract. *Siahpoush S, Darvishnia M. 2019. Short Communication: Genetic diversity among* Fusarium *isolates from cereals in Iran assessed using RAPD marker. Biodiversitas 20: 292-296. Fusarium* species cause important disease on many crops including cereals, and accurate identification and then proper management of this disease will be helpful to reduce economic losses. In this paper, 13 *Fusarium* isolates from root and crown of cereals in western Iran were identified by morphological and molecular methods. Sequencing of translation elongation factor 1-alpha revealed 8 species as follows: *Fusarium acuminatum, F. avenaceum, F. culmorum, F. equiseti, F. proliferatum, F. reticulatum, F. solani, and F. tricinctum.* Species diversity was analyzed by random amplified polymorphic DNA (RAPD). Four primers were used as Rfu9, Rfu10, Rfu23, and Rfu25 which all of them produced distinct and reproducible bands. A dendrogram was developed by UPGMA. Generated polymorphic bands were observed in all 13 different species by 4 primers. Rfu9 by 13 bands and Rfu23 by 10 ones, produced the most and the least bands respectively. Genetic similarity coefficient was between 0.00-0.9. RAPD analysis showed that these isolates were genetically varied and two clusters were formed with *Fusarium* isolates.

Keywords: *Fusarium*, genetic diversity, RAPD, translation elongation factor 1-alpha, UPGMA

INTRODUCTION

Cereals are the main crops in Asia as human and livestock nutrition. Unfortunately, some destructive diseases such as *Fusarium* root rot cause many economic damages to these crops. *Fusarium* head blight is one of the most important diseases all over the world (Yli-Mattila et al. 2009). Latiffah et al. (2007) reported *Fusarium* as one of the main pathogens in plants. Root rot disease is a common problem found in almost all of cereal crops all over the world. Previous studies showed that *Fusarium* species were commonly associated with crown disease. *Fusarium* root, crown, and root rots cause patches of wheat to die prematurely, resulting in areas of whiteheads within a field. Infected plants are typically brown at the base and have poor root development.

Due to limitations of morphological methods to identify *Fusarium* species, molecular markers based on polymerase chain reaction (PCR) are an extended choice (Sabir 2006). Molecular markers especially Random amplified polymorphic DNA (RAPD) (Gupta et al. 2009) and Amplified Fragment Length Polymorphisms (AFLP) (Niessen 2007) are used to differentiate fungal taxa (Steinkellner et al. 2008).

Random Amplified Polymorphic DNA analysis is a fast, PCR-based way of genetic typing based on genomic polymorphisms (Abdel-Satar et al. 2003; Ingle et al. 2009), bacteria and plants (Singh et al. 2011). This technique is used for genetic variability detection (Sabir 2006) and is easy and rapid for evaluation of genetic variation (Niessen 2007; Gupta et al. 2009). Other analysis methods are Amplified Fragment Length Polymorphism (AFLP) and Restriction Fragment Length Polymorphisms (RFLP) based on selective amplification of DNA restriction fragments (Vos et al. 1995; Chulze et al. 2000).

Genetic polymorphism between isolates of *F. solani* was studied by Gupta et al. (2009) in India by RAPD. Smith et al. (2001), Arif et al. (2011), Niessen (2007), Gupta et al. (2009), and Bonde et al. (2013) reported genetic variation among *Fusarium* isolates by RAPD marker.

The aim of this study was determination of genetic diversity of some *Fusarium* species by RAPD-PCR.

MATERIALS AND METHODS

Sample collection

The study was carried out in western Iran, Lorestan fields (Figure 1), during May to October of 2016. The samples were isolated from root and crown of Poaceae species including wheat, barley, corn, and some grass species showing symptoms of root and crown rot.

Figure 1. Study site: Lorestan fields, Iran

Procedures

Fusarium isolation

Nash and Snyder (1962) described a selective medium for *Fusarium* species with a peptone base and pentachloronitrobenzene (PCNB) as a fungal inhibitor. This medium was highly selective for some *Fusarium* species, therefore the isolates were cultured on Nash-Snyder/PCNB and also dichloro-chloramphenicol-peptone agar (DCPA) media, followed by incubation at 28 C° for 7 days (Andrews and Pitt 1986). After about 1week incubation, *Fusarium* colonies emerged from plant materials. A pure culture obtained from a single conidium or hyphal tip of each isolate was inoculated on Potato Dextrose Agar (PDA) (Merck, Germany) for examination of colony color and growth rate at 25 C° (Burgess et al. 1994). For microscopic observations, all isolates were transferred to carnation leaf agar (CLA), synthetic nutrient agar (SNA), KCl plates and sterile distilled water tubes and incubated under 12 h alternating light at 25 ± 2°C for 1-2 weeks (Nelson et al. 1983). All isolates produced typical spores including macro and microconidia and chlamydospores.

Identification of isolates

Fusarium isolates were identified based on general characteristics of the colony (morphology of microconidia, macroconidia, conidiophores, chlamydospore) by using *Fusarium* diagnostic keys (Gerlach and Nirenberg 1982; Nelson 1983; Burgess et al. 1994; Leslie and Summerell 2006). Observations were made using trinocular brightfield microscope (Olympus BX41).

DNA extraction

Molecular identification was performed by examining DNA sequences obtained from translation elongation factor 1-alpha (TEF). Mycelial plugs were transferred from PDA to 50 mL of potato dextrose broth (PDB). Cultures were grown for 5 d at 25 ± 2 °C on a rotary shaker at 100 r.p.m. with 8 h of light every day. Mycelia were collected on to Whatman filter paper using a vacuum pump, rinse and remove excess liquid by placed them between layers of dry filter paper, then were lyophilized at -20 °C.

Genomic DNA was extracted using the established CTAB method (Wu et al. 2001). Freeze-dried mycelium was ground to a fine powder in liquid nitrogen using a pre-cooled pestle and was transferred to microtube. A 1000 µL of extraction buffer (CTAB 1% (w/v), EDTA 10mM pH 8.0, Tris-HCl 100 mM pH 8.0, NaCl 0.7 M, mercaptoethanol 0.2%) was added. After incubation at 65°C for 30 min, and 40 °C for 10 min, DNA was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and precipitated using -20 °C isopropanol (1:1). Precipitated DNA was washed with 70% ethanol, dried and suspended in TE buffer (Tris-hydrochloride buffer –10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). Finally, the DNA was dissolved in 50 µL of pure water and was kept at -20°C (O'Donnell et al. 1998).

Amplification of a partial sequence of TEF

Amplification was performed in a total volume of 25 µL (O'Donnell and Cijelnik 1997). PCR amplification of EF-1α was carried out using a pair of primers EF1(5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and EF2 (5'-GGA(G/A)GTACCAGT(G/C) ATCATGTT-3') primers (O'Donnell et al. 1998) in thermocycler with initial denaturing step of 5 min at 94°C followed by 35 cycles (35 s at 94°C, 55 s at 52°C and 2 min at 72°C) finished by a final extension step at 72°C for 10 min. Electrophoresis of PCR products was performed on 1.5% agarose by gel red for staining. The condition was 100 v for 1 h. Then produced bands were visualized in a UV-transilluminator and photography was carried out by Gel Doc.

RAPD analysis

Thirteen different *Fusarium* isolates were subjected to RAPD-PCR (Table 1). Extracted DNA was amplified by 4 selective primers (Bonde et al. 2013) (Table2). PCR conditions were: an initial denaturation step at 94°C for 2 min, 35 cycles of 94°C for 30 s, 40°C for 60 s, 72°C for 2 min and a final extension at 72°C for 5 min. Total volume of reaction was 25 µL contained 2.5 µL of PCR buffer, 1 µL $MgCl_2$ 50 mM, 0.75 µL of dNTP mixture (200 µmol each), 2 µL of sample DNA (10 ng), 2 µL of primers (10 Pmol/µl), 0.5 µL of Taq polymerase and 16.25 µL sterile distilled water. PCR products were electrophoresed on 1.5% agarose gel including safe stain, then they were observed using Gel Documentation.

Data analysis

Polymorphic RAPD markers were manually scored as binary data with presence as "1" and absence as "0". Cluster analysis was performed employing the (UPGMA) method (Sneath and Sokal 1973) using NTSYSpc version 2.2 (Exeter Software Co. New York).

RESULTS AND DISCUSSION

There were 13 *Fusarium* isolates from cereals in western Iran were identified in this study, using identification keys of Gerlach and Nirenberg (1982), Nelson (1983), Burgess et al. (1994) and Leslie and Summerell (2006) (Table 3).

Table 1. *Fusarium* isolates in RAPD analyses

	Isolate
1	SPF300
2	SPF507
3	SPF516
4	SPF292
5	SPF341
6	SPF441
7	SPF015
8	SPF054
9	SPF548
10	SPF261
11	SPF207
12	SPF501
13	SPF010

Table 2. Primers for RAPD analysis (Bonde et al. 2013)

Primers	Sequences (5'-3')
RFu9	CCTGGGTGCA
RFu10	CCTGGGTGAC
RFu23	CCGGCCATAC
RFu25	CCGGCTGGAA

Table 3. Identified *Fusarium* species by morphological characters

Isolate	Species
SPF300	*Fusarium acuminatum*
SPF507	*F. acuminatum*
SPF516	*F. avenaceum*
SPF292	*F. culmorum*
SPF341	*F. equiseti*
SPF441	*F. proliferatum*
SPF015	*F. proliferatum*
SPF054	*F. proliferatum*
SPF548	*F. proliferatum*
SPF261	*F. reticulatum*
SPF207	*F. solani*
SPF501	*F. solani*
SPF010	*F. tricinctum*

Molecular characterization was used for verification of the species based on the PCR amplified product of EF1 gene. Results of RAPD analysis in a13 isolates showed that all of 4 primers (RFu 9, RFu 10, RFu 23 and RFu 25) produced distinct and reproducible bands (Figure 2). The target amplified fragments were 1-3 kb. Results of UPGMA analysis of the RAPD data separated the species in two main clusters (Figure 3). A total of 85% of the isolates were in the first cluster in which two subclusters were observed. SPF015 and SPF548 showed genetic similarity of GS=70%, isolates SPF341 and SPF054 showed very high genetic similarity of GS=83%. SPF010 constituted one cluster branched from the first main cluster at level of 0.42%. The second cluster consisted of two isolates of *F. solani* at the genetic similarity of GS=54%. The results of the RAPD analysis showed diversity among the *Fusarium* spp.

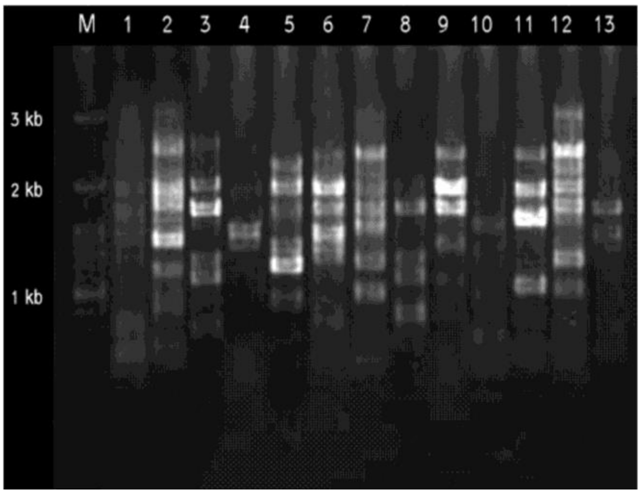

Figure 2. RAPD patterns on 1.5% agarose gel of amplified fragments generated from 13 Fusarium isolates with primer RFu-23. M, DNA marker (1 kb).

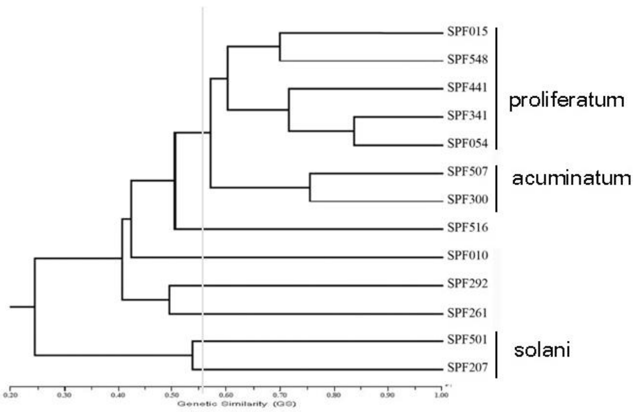

Figure 3. UPGMA dendrogram of 13 isolates of *Fusarium* isolates based on RAPD-PCR

Genetic diversity plays an important role in diseases management programs. Understanding the nature of variation of pathogens is necessary through the use of resistant cultivars. Diversity determination methods are mostly used in various studies and results provide beneficial information for future populations (Thaware et al. 2017).

RAPD analysis for wide range of plants, fungi, and bacteria in many scientific fields is used to uncover genetic variation of closely related taxa. In plant pathology, this method was used to differentiate *Fusarium* species by many researchers. Ingle and Rai (2011) studied *Fusarium* species variation by RAPD. Thaware et al. (2017) used RAPD method to specify variability of some *Fusarium* isolates in India. Some races of *F. oxysporum* f.sp. *vasinfectum* were differentiated by RAPD (Assigbetse et al. 1994). Arici and Koc (2010) estimated genetic variation among isolates of *Fusarium graminearum* and *Fusarium culmorum* from wheat in Turkey by RAPD-PCR. Genetic diversity of *F. oxysporum* f.sp. *lentis* population was determined by some molecular markers including RAPD by Al-Husein et al. (2017) in Syria. *Fusarium* species isolated from eggplant in Turkey were classified on the basis of RAPD by Baysal et al. (2010). Our results showed genetic diversity in *Fusarium* isolates from cereals in Iran. These isolates were identified by morphological features and then by sequencing of partial translation elongation factor 1-alpha. This DNA coding region was used to identify *Fusarium* species. O'Donnel et al. (1998) revealed that unlike RFLP which was insufficient to distinguish species in *Gibberella fujikuroi* complex, RAPD could distinguishes them very well. El-Fadly et al. (2008) identified some *Fusarium* species and diversity between them by TEF sequence and RAPD respectively. The aim of this work was to determine whether our *Fusarium* isolates have genetic diversity. Results showed that amplified DNA bands can use reproducible to differentiate *Fusarium* isolates and this agrees with the results of Balmas et al. (2010), Ingle and Rai (2009), Gupta et al. (2009) and Nagarajan et al. (2004). Gherbawy (1999) in research, used RAPD marker to study genetic diversity among 20 isolates of *Fusarium*. They used data generated from RAPD banding pattern for the UPGMA analysis and found that there were genetic variations in different isolates of *Fusarium* (Abd-Elsalam et al. 2003).

If isolates from these divergent species interbreed, there will be the potential for the production of new genotypes that carry novel combinations of genes for pathogenicity. Therefore, identification of additional isolates is important in the future.

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