Molecular Characterization of *Fusarium oxysporum* isolates from Egypt using IGS Sequencing

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ABSTRACT— ***Twenty isolates of Fusarium oxysporum were collected from different regions of Egypt and characterized using intergenic spacer (IGS) of the ribosomal DNA gene. The identified isolates were compared using sequences analyses on formae speciales level. The length of the amplified IGS region using universal primers CNL12 and CNS1 were about 2.6 kb in all F. oxysporum isolates. For specificity, oligonucleotide primers PNFo and PN22 produced a single DNA fragment of about 1.7 kb for each of the twenty isolates. Sequence cluster analysis using the neighbor-joining method showed that the twenty isolates divided into two clusters, cluster 1 contains isolates from El Fayoum and the remaining isolates on the other cluster. On the whole, there was no clear correlation between geographic origin or plant host and the sequence of IGS region. The results demonstrated the important role for utilization the IGS region as a molecular marker for the rapid and reliable diagnosis, identification and characterization of F. oxysporum pathogen in solanaceous crops in Egypt.***

Keywords— *Fusarium oxysporum*, intergenic spacer region IGS, sequencing, tandem repeats

1. INTRODUCTION

The Solanaceous plants: tomato, pepper and eggplant are the most popular vegetable crops in Egypt. Tomato occupy about 400749.63 hectare, yielding8533803.00 tonnes and eggplant occupy about 42869.00 hectare, yielding 1194115.00 tonnes (2013 FAOSTAT). Many diseases and disorders can affect Solanaceous plants during the growing season. Vascular wilts and root rot diseases of Solanaceous crops caused by fungal pathogens are widespread and very destructive plant diseases, causing economic losses. The survival structures produced by wilt pathogens may remain viable in the soil for more than 20 years, making them a major constraint on agricultural production. *Fusarium oxysporum f. sp. radicis-lycopersici* (FORL) and *F. oxysporum f. sp. lycopersici* (FOL), responsible for crown root rot and a vascular wilt disease, respectively. Formae specials FORL and FOL have strict host specificity. Within FOL, three races have been identified, differentiated by their specific pathogenicity to different cultivars carrying specific resistance genes (Grattidge&O'Brien, 1982). However, no races are known within FORL. Both formae speciales display a considerable genetic diversity. Classically, plant pathogenic fungi were characterized by a series of morphological criteria including cultural characteristics on growth media and diagnostic symptoms on the host along with the presence of the fungus in the affected tissues (Baayen et al. 2000).

Identification of fungi by visual examination of such morphological criteria is very difficult and erroneous. Moreover, these methods have other major limitations such as, reliance on the ability of the fungus to be cultured, time-consuming and laborious nature of identification process and the requirement for extensive taxonomical knowledge, which complicate timely disease management decisions. Molecular approaches that are based on specific DNA sequences in the pathogen genomes could be used for pathogen identification as well as for their phylogenetic studies (Lievens*et. al.*, 2008). Ribosomal DNA (rDNA) regions have been used for taxonomic and phylogenetic studies because sequence data are available and because rDNA regions contain both variable and conserved regions, allowing discrimination at the genus, species, or intraspecific levels. The non-coding regions of rDNA have been used as variable region. The internal transcribed spacers (ITS) of the rDNA can displayed variation within genera and used in the differentiation of species. At the intraspecific level, variability in ITS sequences is generally very low or undetected. The intergenic spacer (IGS) sequence that separates ribosomal repeat unites was variable enough to allow discrimination of closely related fungi. IGS sequences might be good candidates for differentiation of strains at the intraspecific level. (Edel, *et. al.*, 1995)

In Arizona and California, based on analysis of the IGS sequence data (Mbofung *et. al.*, 2007) revealed that numerous sequence polymorphisms among *F.oxysporum* forma speciales consisting of insertion, deletion and single nucleotide transitions and substitutions. Repeat sequence analysis revealed several duplicated sub-repeat units that were distributed across much of the region. The consensus sequence GGTGTAGGGTAG was found within most of these repeats. Sequence variation among closely related taxa suggests that it can be used effectively as a diagnostic marker to differentiate pathogenic and nonpathogenic isolates of *F. oxysporum*, including the f. sp. *lactucae.* Based on the heterogeneity of the IGS region, sensitive and specific PCR primers developed for the diagnosis of *F. oxysporum* f. sp. *lactucae*in field soil, lettuce seed, and in planta as an additional tool for the management of Fusarium wilt of lettuce.
O'Donnell*et. al.*,(2009) Identified the sources of the discordance within the IGS rDNA. They suspected the variable numbers of sub-repeats within the IGS rDNA might play a role in the topological discordance. In *F. oxysporum*, the incongruence was attributed to the nucleotide variability localized outside the repeated elements rather than sequence variation within or different numbers of these elements.
The population structures of *Fusarium oxysporum* were previously assessed by a genotypic method based on restriction fragment analysis of polymerase chain reaction-amplified ribosomal intergenic spacer (IGS) DNA (Cai, *et. al.*, 2003; Abo, 2005 and Zombounis *et. al.*, 2007). The isolates were characterized (IGS) DNA typing that represents the combination of patterns obtained with number of the restriction enzymes.
Many researchers have reported that IGS sequence of *Fusarium oxysporum* provides reliable molecular information concerning intraspecific variation and phylogeny of fungal species (Kim & Min 2004; Balogun 2007; Zombounis, *et. al.*, 2007 and Jordão do Amaral, *et. al.*, 2013).
The present study aimed to evaluate the genetic diversity of Egyptian *F. oxysporum* isolates within each of forma speciales FOL and FOLR using sequencing data of the IGS region.

2. MATERIALS AND METHODS

Fungal isolates, culture conditions and DNA extraction:
Twenty isolates of *F. oxysporum* were used in this study (Table 1). All isolates were grown for 10days on PDA plates. Genomic DNA was extracted using a rapid minipreparation procedure (Edel *et al.*, 2001). One ml of lysis buffer (50 mM Tris-HCl (pH 7.5), 50 mM EDTA and 3% SDS) was added to the plate and the mycelium was scraped with a spatula. 500 µl of the buffer mixed with mycelium was recovered in a microtube and mixed using a vortex shaker. The tubes were incubated at 65ºC for 10 min at 4ºC. The supernatants were transferred into new microtubes and the DNA was precipitated by adding 0.5 volume of 3 M sodium acetate and one volume of isopropanol. Microtubes were gently inverted three times and centrifuged at 12 000 *xg* for 15 min at 4ºC. The supernatant was discarded and the pellet was rinsed with 300 µl of ice-cold 70% ethanol. After centrifugation at 12 000*xg* for 5 min at 4ºC, the ethanol was discarded. The DNA pellet was air-dried, dissolved in 100 µl of TE buffer (10 mMTris- HCl (pH 8.0) and 1 mM EDTA) and stored at 4ºC until use.
PCR amplification of IGS region:
The IGS region of the 20 *F.oxysporum* isolates were amplified by the universal primers CNL12 and CNS1. (Appel& Gordon 1996).PCR reactions were performed in a final volume of 50µl containing 25µl (OnePCR- GeneDirx) (Real Bio Gene), 2µl of DNA and 0.4µM of each primer. PCR were conducted in a Minicycler (PTC-150 Minicycle) with an initial denaturation of 2 min at 94ºC followed by 30 cycles of 30 s denaturation at 94ºC, 1 min annealing at 55ºC, 2 min extension at 72ºC and a final extension of 5 min at 72ºC. PCR products were separated by electrophoresis through 1.4% agarose gels, stained with ethidium bromide and visualized under a UV transilluminator.
Partial IGS amplification and direct sequencing:
The IGS region of the rDNA was amplified by PCR with oligonucleotide primers PNFo (5`-CCC GCC TGG CTG CGT CCG ACT C-3`) and PN22 (5`-CAA GCA TAT GAC TAC TGG C-3`) (Edel *et al.*, 1995). Amplifications were performed in a final volume of 50µl containing 25µl (One PCR- GeneDirx), 2µl of DNA and 0.4µM of each primer. PCR reactions were conducted in a Minicycler with an initial denaturation of 2 min at 94ºC followed by 30 cycles of 30 s denaturation at 94ºC, 1 min annealing at 57ºC, 2 min extension at 72ºC and a final extension of 5 min at 72ºC. PCR products were separated by electrophoresis through 1.4% agarose gels, stained with ethidium bromide and visualized under a UV transilluminator. PCR amplification products of all fungal IGS regions were purified using the GenElute PCR Clean-Up Kit-Sigma
Aldrich in order to remove secondary metabolites prior to sequencing.. Sequencing was carried out by the DNA facility in the Laboratory of Sigma Egypt in a Sanger ABI 3730xl sequencer.
Data analysis:
The obtained nucleotide sequences were aligned with other IGS sequences of FOLR(gi|297660795|gb|HM057276.1|) and FOL (gi|297660796|gb|HM057277.1|)from the GenBank (NCBI, National Center Biotecnology Information) using the programs ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and The phylogenetic tree was constructed by Neighbor joining algorithm in the software MEGA v.6 using the Tamura-Nei model .The reliability of results was tested by bootstrap (1000 replications)

3. RESULTS

The samples of diseased tomato, pepper and eggplant plants showing the symptoms of wilt or root rot were collected from several localities of the agricultural production region of Egypt during 2012-2013 growing season. Isolate (10) obtained from Assiut university Mycological center (AUMC) Faculty of science and isolate (11) was recovered from tomato seeds cultivar Alfarah F, Hybrid imported from China. Microscopic observation showed that 19 isolates originating from Egypt originally and one from China identified as *Fusarium oxysporum.* Identification of the isolated fungi was verified according to cultural and microscopical characters described by Booth (1971) in Department of Plant Pathology, Faculty of Agriculture, Alexandria University, and Plant Pathology institute, Agriculture Research Center, Cairo. (Table1).

Table 1: Original host, collection sites, and time of isolation of 20 *Fusarium oxysporum* isolates used in this study.

Isolates	Original host	Cultivar	Location	Year
1	Tomato root		El-Behira	2012
2	Tomato root		El-Behira	2012
3	Tomato root		El-Behira	2012
4	Tomato root		El-Fayoum	2012
5	Tomato root	Superhagin	Haron village El-nubaria El-Behira	2013
6	Tomato root		El banger village 5 El-Behira	2013
7	Tomato root		El banger village 15 El-Behira	2013
8	Tomato root		El-Behira	2013
9	Tomato root		Assiut	2013
10	Tomato root		Assiut(AUMC)	2013
11	Tomato seeds	Alfarah F, Hybrid	China	2013
12	Pepper root		Slah El-abd El noubariaEl-Behira	2012
13	Pepper root	Kaha	Qalyubia	2012
14	Pepper root	Baldy	El banger village 4 El-Behira	2013
15	Pepper root		El banger village 5 El-Behira	2013
16	Pepper root		El banger village 15 El-Behira	2013
17	Eggplant root		Alexandria	2013
18	Eggplant root	Aros	El banger village 4 El-Behira	2013
19	Eggplant root		El banger village 5 El-Behira	2013
20	Potato root		El-Behira	2012

Molecular identification:

The universal primers CNL12 and CNS1were used to amplify a single double stranded product of approximately 2.6 kbp, representing to the IGS region in twenty isolates of *F. oxysporum*. Figure (1).

For specificity of *F. oxysporum* forma specials, oligonucleotide primers PNFo and PN22 successfully amplified a single DNA fragment of about 1.7 kbp for each of the 20 isolates Figure (2). The nucleotide sequences produced were aligned with other partial IGS region sequences of genes of *F.oxysporum f.sp. lycopersici* (FOL) HM057277.1, *F. oxysporum. f.sp. radicis-lycopersici* (FORL) HM057276.1 and *Fusarium* sp. GU737460.1obtained from the GenBank (NCBI, National Center Biotecnology Information) using the program ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). All tested isolates were identified as *F. oxysporum*, with homology range from 92% to 99%.

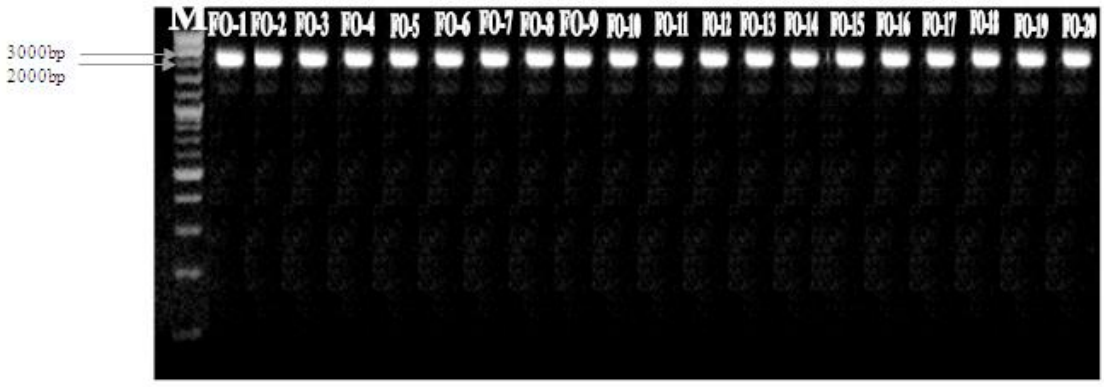

Figure 1. Polymerase chain reaction (PCR) amplification corresponding to the intergenic spacer (IGS) regions of 20 fungal isolates using primers CNL12/CNS1 visualized on EtBr-stained agarose gel (1.4 %)

Figure 2. Polymerase chain reaction (PCR) amplification corresponding to the intergenic spacer (IGS) regions of 20 fungal isolates using primers PNFo/PN22 visualized on EtBr-stained agarose gel (1.4%).

Based on differentiations were observed among alignment of obtained sequences of amplified IGS region. The phylogenitic tree Figure (3), showed that all tested isolated divided into 2 main clusters, cluster I includes just one isolate FO-4 from (tomato El-Fayoum). Cluster II divided into 5sub-cluster, sub group I contains isolates FO-5 from (tomato El-Behira) and FO-13 from (pepper-Qalyubia). Sub-cluster II contains isolate FO-10 from (tomato-Assiut (AUMC) and FO-12 from (pepper, El-Behira) beside one group included each of isolates FO-3 and FO-7 from (tomato, El-Behira). Sub cluster III included isolate FO-19 from (eggplant, El-Behira) and one group branches to sub group contains isolates FO-17 and FO-18 from eggplant (Alexandria and El-Behira, respectively) beside another branch included isolate FO-8 (tomato, El-Behira). Sub cluster IV divided to one isolate FO-20 (potato El-Behira) ,one group branches to one branch include isolate FO-2 (tomato El-Behira) beside one sub group included each of isolate FO-6 (tomato El-Behira) and isolate FO-16 (pepper El-Behira). Finally, sub cluster V included one isolate FO-14 (pepper El-Behira) and one group consisted of the remaining isolates. Results demonstrated that no clear correlation was found between geographic origin or genus of plant and sequence of IGS region, Tested isolates have been deposited in the GenBank (NCBI, National Center Biotecnology Information) under accession numbers from KP898390 to KP898409 as FOL (FO-2, 5, 7, 8, 14, 16, 17, 18, 19 and FOLR (FO-1, 6, 9, 12, 13,15, 20). (Table 2)

Analysis of partial IGS sequence revealed several tandem repeats. The consensus sequence GGTGTAGGGTAGG was found within most of repeats. These motifs repeated four times in each of the isolates FO-2, 5, 7, 13&19. In isolates FO-3 and FO-11 repeated one time while in isolate FO-10 repeated two times, in the remaining isolates repeated three times except isolate FO-4 there is no repeat. Two other repeated sequences were found included GTGAGTCGATTTTTTTGTTT and TGAATTTTGCGGA, as described in table (3).

Table 2: Fusarium isolates used in this study, results of the *F. oxysporum* specific PCR assay using primers PNFo/PN22 with original and optimized amplification conditions, the total size of IGS partial sequences and accession numbers in Genbank.

Isolates name with assigned accession number	**Identification**	**Total length**	**Accession number of the most matched isolate in the genbank**	***E*-value**
FO-1 KP898390	*F. oxysporumf. sp. radicis-lycopersici*	1109	HM057276.1	0.0
FO-2 KP898391	*F. oxysporum f. sp. lycopersici*	1054	HM057277.1	0.0
FO-3 KP898392	*F. oxysporum*	1092	GU737459.1	0.0
FO-4 KP898393	*F. oxysporum*	823	GU737459.1	3e-82 1e-81
FO-5 KP898394	*F. oxysporum f. sp. lycopersici*	1092	HM057277.1	0.0
FO-6 KP898395	*F. oxysporumf. sp. radicis-lycopersici*	1070	HM057276.1	0.0
FO-7 KP898396	*F. oxysporum f. sp. lycopersici*	1113	HM057277.1	0.0
FO-8 KP898397	*F. oxysporum f. sp. lycopersici*	1013	HM057277.1	0.0
FO-9 KP898398	*F. oxysporumf. sp. radicis-lycopersici*	1091	HM057276.1	0.0
FO-10 KP898399	*F. oxysporum*	1101	GU737459.1	0.0
FO-11 KP898400	*F. oxysporum*	1089	GU737459.1	0.0
FO-12 KP898401	*F. oxysporumf. sp. radicis-lycopersici*	1102	HM057276.1	0.0
FO-13 KP898402	*F. oxysporumf. sp. radicis-lycopersici*	935	HM057276.1	0.0
FO-14 KP898403	*F. oxysporum f. sp. lycopersici*	1004	HM057277.1	0.0
FO-15 KP898404	*F. oxysporumf. sp. radicis-lycopersici*	1100	HM057276.1	0.0
FO-16 KP898405	*F. oxysporum f. sp. lycopersici*	1045	HM057277.1	0.0
FO-17 KP898406	*F. oxysporum f. sp. lycopersici*	1024	HM057277.1	0.0
FO-18 KP898407	*F. oxysporum f. sp. lycopersici*	1070	HM057277.1	0.0
FO-19 KP898408	*F. oxysporum f. sp. lycopersici*	1111	HM057277.1	0.0
FO-20 KP898409	*F. oxysporumf. sp. radicis-lycopersici*	926	HM057276.1	0.0

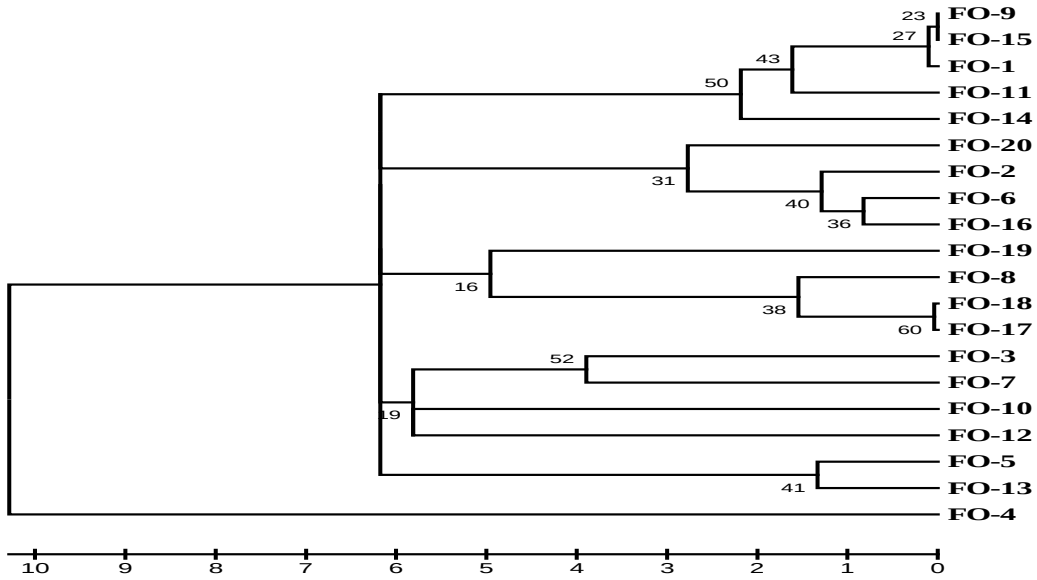

Figure3.Phylogram based on IGS (rDNA) region of the 20 isolates of *F. oxysporum*. The IGS region was amplified with PNFo primer. The phylogram was generated by the neighbor-joining method using software MEGA4. The numbers on the branches indicate the bootstrap value support.

Table 3: The frequency of the three detected sequence motifs in the 20 *F. oxysporum* isolates

Isolate	**Motif1** GGTGTAGGGTAGG	**Motif2** GTGAGTCGATTTTTTTGTTT	**Motif 3** TGAATTTTGCGGA
FO-1	**3**	**1**	**1**
FO-2	**4**	**1**	
FO-3	**1**	**1**	**1**
FO-4	**0**	**0**	**0**
FO-5	**4**	**1**	**1**
FO-6	**3**	**0**	**1**
FO-7	**4**	**1**	**1**
FO-8	**3**	**0**	**0**
FO-9	**3**	**1**	**1**
FO-10	**2**	**1**	**0**
FO-11	**1**	**0**	**1**
FO-12	**3**	**1**	**1**
FO-13	**4**	**0**	**0**
FO-14	**3**	**0**	**0**
FO-15	**3**	**1**	**1**
FO-16	**3**	**0**	**1**
FO-17	**3**	**1**	**0**
FO-18	**3**	**1**	**1**
FO-19	**4**	**1**	**1**
FO-20	**3**	**0**	**0**

4. DISCUSSION

The main purpose of this study was to evaluate sequencing of IGS as a rapid and reliable molecular marker to detect and diagnose the pathogen, where it is essential for undertaking appropriate and timely disease management measures.
It has been reported that the length and sequence heterogeneities in higher plants and animals mostly occur in IGS region. Whereas, data for different fungal species indicate that intraspecific differentiation among the IGS is normal, but the structure of fungal IGS is not known for many species.(Kim,*et. al.*, 2001)
At the first, we used the universal primers CNL12 and CNS1 to amplify the IGS region of all *F.oxysporum* isolates (Appel&Gorden 1996; Cai, *et. al.*, 2003; Kim,*et. al.*, 2001;Srinivasan,*et. al.*, 2010 and Salvador*et. al.*, 2013). For partial IGS amplification, several researches (Edel, *et. al.*, 1997, 2001 & 2011; Lori, *et. al.*, 2004; Abo, 2005 and Erlangung des Grades.2010) have reported primers PNFo and PN22 to amplify ion a single DNA fragment of about 1.7 kb. for the identification of *F. oxysporum* to the forma speciales level.
Sequences of unidentified organisms can be easily characterized and morphological identification can be confirmed by using identity matches resulting from a BLAST search. Complementation of alignments with organism sequences from data bases can significantly increase the information content of the phylogenetic analysis. (Erlangung des Grades.2010). A BLAST search of our partial IGS sequences isolates showed similarity from 92 to 99 % and Cluster analysis of IGS region sequences do not reflect any resemblance and have not been correlated with their geographical origin.
The repeats elements of the IGS sequences were similar with the findings of each of (Mbofung *et. al.*, 2007;O'Donnell *et. al.*, 2009 and Salvador *et. al.*, 2013). O'Donnell *et. al.*, 2009 suspected that variable numbers of sub-repeats within the IGS rDNA might play a role in the topological discordance. These elements have been reported in a diverse range of fungi in which they have contributed to IGS length heterogeneity even within the same isolate.
Here, we have suggested that sequence polymorphism in the IGS region is considered a suitable tool for the development of PCR-based diagnostic such as ISSR (Inter simple Sequence Repeat), because of its sensitivity and specificity.

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