The Journal of Animal & Plant Sciences, 26(5): 2016, Page: 1368-1373
ISSN: 1018-7081

DIVERSITY OF INTERNAL TRANSCRIBED SPACER (ITS) REGION OF *FUSARIUM* ISOLATES IN PAKISTAN

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ABSTRACT

The present study evaluate the genetic diversity analysis of the ITS regions of rRNA gene complex of local isolates of *Fusarium* in Pakistan. The genomic DNA of these isolates was amplified using primers ITS1 and ITS4 designed at the end, and start of conserved 18S and 28S region and between ITS1 and ITS4 respectively. By the comparison of RG2, RG3, RG4, RG5, RG6, and RG9 all species have higher genetic diversity while RG2 and RG6 have highest homology with each other that showed that both may be originated from same ancestor. Morphological and molecular data obtained might be useful in determining the taxonomy and diversity of *Fusarium* species. Results obtained reveals inter specific variation among the isolated organisms with limited intra specific variability. It can therefore be inferred based on findings that ITS analysis could provide taxonomic evidence for the isolated organisms but may not be informative enough to score diversity.

Key words: ITS region, Genetic diversity, *Fusarium*, BLAST, CLC BIO, CTAB.

INTRODUCTION

A large group of filamentous fungi species which is widely distributed in soil usually associated with plants comprise of genus *Fusarium*. Most of the *Fusarium* species are relatively abundant members of the soil microbiota and are saprotrophic (Leslie and Summerell, 2006). The well known pathogens of plants, insects and humans are *Fusarium* species Majumbar *et al.* (2008).

Among the different *Fusarium* species, *F. oxysporum* is a specie complex and different host specific individual within this complex are termed as formae specialis and abbreviated as "f.sp." Beckman, (1987). Wilt which is an economically important plant disease with worldwide distribution is caused by *Fusarium oxysporum* which colonizes in vascular tissues and causes wilting of plant Rai *et al*. (2011). Wilt disease is among major yield reducing factors in Pakistan such a wilt epidemic occurred in 1956 and caused more than 75% crop yield losses. In Pakistan, wilt disease causes an estimated annual loss of Rs. 12 million Sattar *et al*. (1953).

Traditionally classification of *Fusarium* isolates was based on morphological characters like presence/absence of chlamydospores, and size and shape of macro and microconidia Leslie *et al*. (2007). *Fusarium* isolates were also classified on the basis of vegetative compatibility groups Puhalla (1985) and host specificity, nevertheless all these parameters were not persistent to develop a consensus scheme.

With the advancement of molecular biology, fungal classification and phylogenetic studies have shifted to DNA sequence base methods Bruns *et al*. (1991). These methods play an important role in *Fusarium* identification Lee *et al*. (2000) and in understanding of genetic diversity of members of genus *Fusarium* Bogale *et al*. (2006). In present study, genetic characterization of local *Fusarium* isolates was done by using ITS sequences of RG complex. There is a significant consensus about the use of the ITS sequences in mold identification as an initial step and as a default region for species identification Balajee *et al*. (2009).

Nuclear rDNA is considered an ideal target for specific PCR primers, as each sequence is variable at the family, genus, or species level. It is a complex of tandem repeats containing genic and nongenic or spacer regions Cullings and Vogler (1998). Component of this gene complex have different domains that evolved at different rates Jorgenson and Cluster (1988). The RG are highly conserved Bruns *et al*. (1991) and have been used in addressing broad phylogenetic hypothesis Lee *et al*. (2000).

The ITS regions are more variable and used to analyze interspecies Vogler and Bruns (1998) and sometimes intra-species relations Bruns *et al*. (1991). In present study, local pathogenic isolates of *Fusarium* were characterized for the first time on genic level. ITS regions of rDNA gene complex were sequenced and compared to determine similarity and diversity of these isolates at genetic level. The study will contribute towards understanding of genetic make-up of local pathogenic *Fusarium* strains and may contribute significantly in crop breeding and disease management.

MATERIALS AND METHODS

Fungal isolates: The different isolates of *Fusarium* sp. were obtained from First Fungal Culture Bank of Pakistan (FCBP) Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan (table 1). All these cultures were preserved at 4°C.

Table 1. Isolates of *Fusarium* with their source of isolation.

Serial No.	Accession No.	Name of fungus	Source of isolation	Isolation date
1	0018	*Fusarium equisetti*	Soil Canal bank Lahore.	20.05.03
2	0031	*Fusarium sp.*	Soil Botanical garden, P.U. Lahore.	12.06.03
3	0237	*Fusarium lateritium*	*Cucumis sativus*, vegetable market.	26.04.04
4	0512	*Fusarium oxysporum*	Soil, *Dalbergia sissoo* , Lahore,	26.08.05
5	0606	*Fusarium oxysporum*	Soil, Lahore.	03.03.06
6	0791	*Fusarium solani*	Potato tuber, Lahore	18.06.07
7	866	*Fusarium oxysporum*	Citrus root, P.U. Lahore.	12.06.08
8	1127	*Fusarium solani*	Ginger, Lahore.	21.02.11
9	1171	*Fusarium anthophylum*	Rhizosphere of Guava, Kasour.	30.01.12
10	1189	*Fusarium semitectum*	Zea maize stored seeds, Lahore.	12.04.12

Revival of *Fusarium* cultures: Different isolates of *Fusarium* were recovered on potato dextrose agar (PDA) and Malt extract agar (MEA) and plates were incubated at 25 ± 2˚C for 3 days. All the auxenic cultures of different isolates of *Fusarium* were identified on the basis of morphological and cultural characters like morphology of colony (colour of the colony, pigmentation, texture of mycelia, etc.), morphology of micro and macroconidia, and chlamydospores (presence or absence of conidia, shape and size of conidia, etc.) on PDA and MEA.

DNA Extraction: Fungal cultures were further purified as a single spore culture on MEA plates by single spore isolation technique Choi *et al*. (1999) and incubated at 25˚C ± 2°C. After 2 weeks fungal colony was removed from the Petri plates by scratching the surface with a sterilized needle and then placed in the (Pre-chilled at -80°C) sterilized mortar. Fungal tissues were ground with liquid nitrogen to form a fine powder with the help of a pestle. Fungal DNA was extracted by using the CTAB method described by Doyle and Doyle (1990) with some modifications.

Polymerase Chain Reaction amplification (PCR): PCR was performed for the amplification of DNA region containing the Internal Transcribed Spacer (ITS) regions (ITS1) and (ITS4) in the *Fusarium* sp (White *et al*., 1990). The PCR conditions were initial denaturation time of 1 minutes at 95˚C temperature following by 35 cycles of denaturation at 95˚C for 1 minute, annealing at 52˚C for 1 minute and extension at 72˚C for 2 minutes. The reaction was finally extended for 10 minutes at 72˚C temperature.

Sequencing and BLAST analysis: The selected purified PCR products were sequence by the Macrogen South Korea with the help of universal primers (ITS1) and (ITS4). The sequences data were assembled with the aid of the Lasergene package of sequences analysis software (DNAStar Inc, Madison, WI, USA). Blastn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to find homology of consensus sequences obtained from multiple sequence runs, with already reported sequences present in nucleotide database. Clustal W (http://www.genome.jp/tools/clustalw), a multiple sequence alignment (MSA) tool was employed to score similarity among different isolates.

Phylogenetic tree: Phylogenetic tree multiple sequence alignments were performed using Muscle software CLC BIO and MEGAALIGN 6. Phylogenetic trees were constructed using Clustal X (neighbour-joining method) displayed, manipulated and printed the phylogenetic tree.

RESULTS AND DISCUSSION

Polymerase Chain Reaction Amplification (PCR): PCR was performed for the amplification of ITS region (ITS1) and (ITS4). PCR Product Purification has done for confirmation of ITS region from the PCR product. The 570 bp product was purified by sigma elute kit.

The indigenous *Fusarium sp*. (FCBP Acc. No. 0031) isolated from botanical garden's soil, PU, Lahore showed highest homology of 90% with the USA isolate of *Fusarium sp*. while the *F. lateritium* (FCBP Acc. No. 0237) isolated from *Cucumis sativus*, vegetable market showed highest homology of 94% with the Japan isolate of *F. lateritium*. The *F. oxysporum* (FCBP Acc. No. 0512) isolated from Soil, *Dalbergia sissoo* , Lahore showed highest homology of 97% with the Japan isolate of *F. oxysporum* while the *F. oxysporum* (FCBP Acc. No. 0606) isolated from soil, Lahore showed highest homology of 97% with the Mexico isolate of *F. oxysporum*. The *F. solani* (FCBP Acc. No. 0791) isolated from Potato tuber, Lahore showed highest homology of

90% with the USA isolate of *F. solani* while the *F. anthophylum* (FCBP Acc. No. 1171) isolated from Rhizosphere of Guava, Kasour showed highest homology of 95% with the China isolate of *F. anthophylum*.

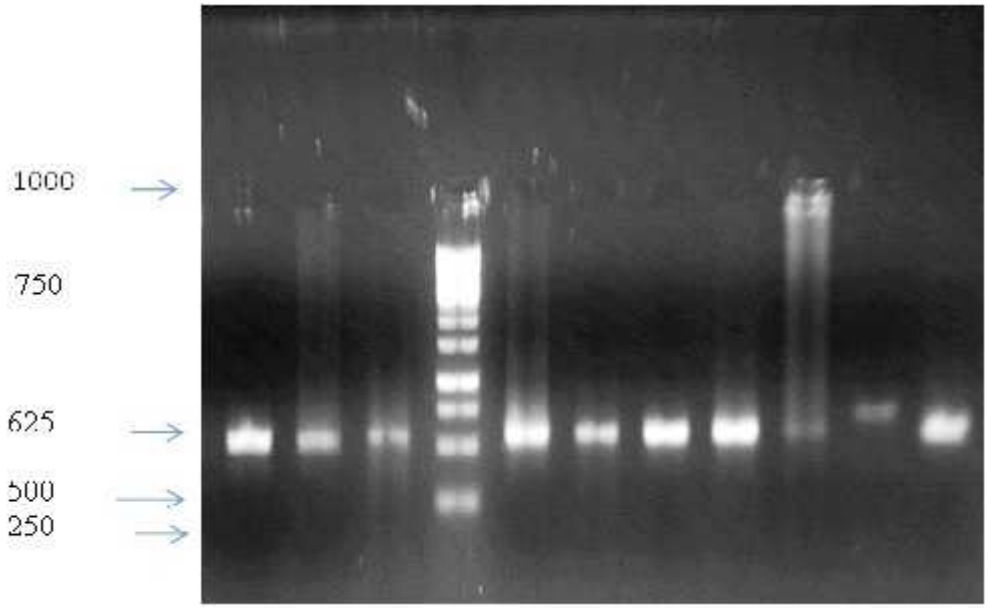

Figure 1. Amplification of ITS region of different *Fusarium* species. [Lane 1- *F. equiseti* (Acc. No.0018) ,Lane 2- *Fusarium sp.* (Acc. No.0031), Lane 3- *F. lateritium* (Acc. No.0237), Lane 4-DNA marker, Lane 5- *F. oxysporum* (Acc. No.512) , Lane 6- *F. solani* (Acc. No.791), Lane 7 -*F. solani* (Acc. No.1127) , Lane 8- *F. oxysporum*(Acc. No.606), Lane 9- *F. oxysporum* (Acc. No.866), Lane 10 - *F. anthophylum* (Acc. No.1171), Lane 11- *F.semitectum* (Acc. No.1189)].

Table 2. NCBI Nucleotides highest homology with sequenced FCBP Nucleotides.

Serial No.	FCBP Accession No.	Gene Bank No.	Code	Sequence	Similarity	Country
1	0031	LN864516	RG2	Kc808235	90	USA
2	0237	LN864517	RG3	KF607063	94	Japan
3	0512	LN864518	RG4	KF6070-1	97	Japan
4	0606	LN864519	RG5	KF607063	97	Mexico
5	0791	LN864520	RG6	Kc808235	90	USA
6	1171	LN864521	RG9	KJ540092	95	China

In this study, genetic diversity among 10 *Fusarium* isolates was evaluated through sequence analysis of the ITS region. The ITS region proved to be a useful tool for assessing genetic variability within this pathogen. Because the ITS regions have important biological meaning in RG processing. The ITS sequences of *Fusarium* sp. were deposited in GenBank under different accession No. Different *Fusarium* species that were taken from (FCBP) show considerable levels of genetic variation. Intraspecific genetic divergence based on the percentage of homology. So sequence variations found in all these species that were much higher in *Fusarium* species. The high intraspecific genetic variation in *Fusarium species* is mainly due to the existence of deep divergent clades as revealed by the phylogenetic analysis.

Genetic distance was different among all *Fusarium* species and genetic identity ranged from the highest value of divergence and minimum value of homology. RG2 shows 87% homology with jq690080 and this nucleotide sequence was taken from China. RG2 also showed 97 % homology with RG6 that was isolated from Pakistan with 90% homology. These results shows that RG2 and RG6 may be belong to the same ancestors but with the passage of time both are different genetically and both has the high level of genetic diversity with the NCBI nucleotide and diversity in genetics may be due to environmental conditions.

Table 3. NCBI Nucleotides with highest homology with FCBP nucleotides.

Samples	NCBI nucleotides and percentage of homology			
RG2	1	2	3	4
	RG6	Jq690080	Kc808235	Kc8082-1
	90%	87.7%	90%	87.7%
RG3	DQ297555	KF607063	KJ540092	KF6070-1
	91%	94%	91%	94%
RG4	KJ540092	KC341959	KF607063	KF6070-1
	85%	92%	97%	97%
RG5	KJ540092	JX914477	KC341959	KF607063
	85%	81%	91%	97%
RG6	RG2	Jq690080	Kc808235	Kc8082-1
	90%	87.7%	90%	87.7%
RG9	KF6070-1	KJ540092	KC341959	JX960431
	90%	95%	84%	82%

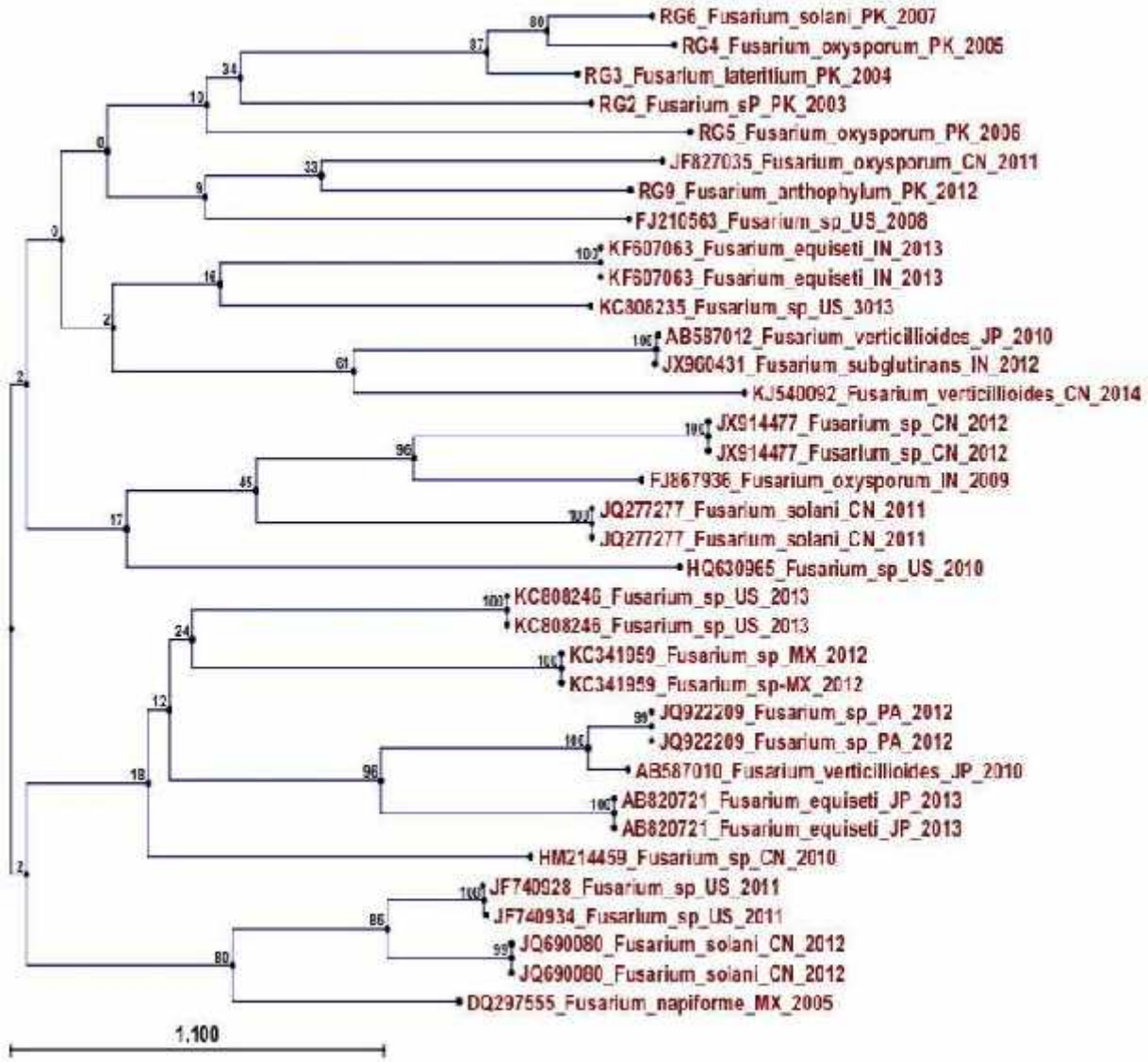

Figure 2. Phylogenetic tree by CLC BIO: Neighbour joining tree was from by CLC BIO by aligned muscle in CLC BIO with 1000 boosteps in which minimum JC+G and BIC was used.

RG3 isolated from soil in Lahore Pakistan shows highest homology with these NCBI nucleotide sequences KF607063 that isolated from India, and KF6070-1 that was isolated from Pakistan with the percentage of homology 94.% By analysis of phylogenetic tree it may be concluded that RG3 and RG9 both belongs to same species. RG4 isolated from soil in Lahore Pakistan shows highest homology with these sequences. These NCBI nucleotide sequences KF6070-1 that was isolated from Japan and KF607063 isolated from Mexico with the percentage of homology 97%. In phylogenetic tree RG4 appeared with the high level of genetic diversity that showed RG4 has the same genera but different spp because it has least homology. RG5 isolated from soil in Lahore Pakistan shows highest homology with nucleotide sequences KC341959 that was isolated from Panama and KF607063 isolated from Mexico with the percentage of homology 97%. RG5 showed the high level of genetic diversity in phylogenetic tree but less than RG4.

RG9 isolated from soil in Lahore Pakistan shows highest homology with nucleotide sequences KF6070-1 that was isolated from Japan and KJ540092 isolated from china with the percentage of homology above 90%. By the comparison of RG2, RG3, RG4, RG5, RG6, and RG9 all species have highest genetic diversity only RG2 and RG6 have highest homology with each other that showed that both are may be originated from same ancestor. All these species not only present in Pakistan but these may be present most of the countries with high level of genetic diversity. But all these results require more molecular techniques for correct identification at specie level. In this study ITS region was used to estimate the intra and inter-species genetic relationships *of Fusarium species* from different countries. Finally, a large portion of variation in *Fusarium* species may have resulted from an isolate by environment interaction, which poses a major constraint to the reliability Pariaud *et al*. (2009).

In this study it was concluded that dependence on environmental factors, such as temperature and soil moisture affected the *Fusarium species*. By the comparisons of all isolates it was concluded that they were not similar with each other at species level but show similarity at genus level. So it has been concluded that *Fusarium* species may have changed their genetic material with passage of time for their survival. It can be concluded that morphologically similar strains of the same fungal species may show genetic variations so by this study species genotypes were identified which add knowledge about *Fusarium* species genotypes in Pakistan and this work helps to understand the genetic mechanisms for the efficient breeding programs to breed the resistant cultivars of different crops.

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