

MOLECULAR VARIATION AMONG ISOLATES OF *FUSARIUM* SPECIES

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Abstract

Twelve different isolates of *Fusarium* spp. collected from wheat grown in Syria were analyzed using amplified fragment length polymorphism (AFLP) markers. AFLP analysis allowed differentiation between species on the basis of banding patterns. A total of 654 scorable DNA bands were scored, of which 296 (44.54 %) were polymorphic. UPGMA dendrogram, based on Nei's genetic distances, showed that isolates formed three phylogenetic groups; one of them did not fall into clades correlated to the origin or colour of the isolate, which suggests a regional dispersal of these species. However, isolates belonging to *F. culmorum* and *F. equiseti* collected from north-west of Syria and ICARDA, respectively fell into two separate sub-clusters. The level of genetic variability detected within *Fusarium* isolates by AFLP analysis confirmed that it is a reliable, efficient, and effective marker technology for determining genetic relationships in *Fusarium* spp.

Introduction

Fusarium is one of the most important genera of plant pathogenic fungi with a record of devastating infections in many kinds of economically important plants (Summerell *et al.* 2001). The confusion regarding the taxonomy of *Fusarium* is perhaps best illustrated by the diversity of the proposed taxonomic systems (Nelson 1991). However, species identification by morphological traits is problematic because characteristics like mycelial pigmentation, formation, shape and size of conidia are unstable and highly dependent on composition of media and environmental conditions (Bluhm *et al.* 2002). Moreover, phenotypic variation is abundant but good expertise is required to distinguish between closely related species and to recognize variation within them (Nelson *et al.* 1983, Okuda *et al.* 2013).

Different types of molecular markers have been used to characterize genetic relationships in fungi. Previous studies conducted on worldwide populations of *Fusarium* included PCR methods such as RAPD analysis (Bentley and Dale 1995), DNA amplification fingerprinting (DAF) (Bentley *et al.* 1998 and Gerlach *et al.* 2000), RFLPs (Koenig *et al.* 1997), DNA sequence analysis (Geiser *et al.* 2004) and IRAP marker (Arabi and Jawhar 2010). Although these techniques were able to provide knowledge related to the genetic diversity in *Fusarium*, but they did not always agree in terms of genetic relationships among clonal lineages of this asexually reproducing pathogen.

Amplified fragment length polymorphism (AFLP) markers are the preferred type of molecular markers because of their multiplex power, their efficiency in detecting genetic variability and the robustness of AFLP assays (Vos *et al.* 1995, Okuda *et al.* 2013). The complex DNA fingerprinting patterns produced by the AFLP technique are reproducible and subsets of these data appear to show higher correlations to one another than those observed among many sets of RFLP or RAPD data (Gonzalez *et al.* 1998, Spooner *et al.* 1996).

Fusarium is an important pathogen in Syria and worldwide, but a few studies concerning its genetic variability have been reported. Therefore, our long-term goal was to understand the factors that favour development of the disease. One possible determinate of disease development is the

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genetic variability among isolates of its species. Therefore, the objectives of the current research were (i) to investigate variability within the *Fusarium* spp. using AFLP marker, and (ii) to determine whether or not the observed variability can be used to clarify genetic relationships among *Fusarium* isolates.

Materials and Methods

During preliminary experiments, more than 60 isolates of *Fusarium* spp. were obtained from wheat seeds showing disease symptoms in different locations of Syria, and finally 12 isolates were selected for this study. Seeds were sterilized in 5% sodium hypochlorite for 5 min. After three washings with sterile distilled water, the seeds were transferred onto Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI. USA) with 13 mg/l kanamycin sulphate added after autoclaving and incubated for 10 days, at $23 \pm 1^\circ\text{C}$ in the dark to allow mycelial growth and sporulation. All isolates were identified morphologically according to Nelson *et al.* (1983). The *Fusarium* isolates, their host plants, and geographic origin are listed in Table 1. The cultures were maintained on silica gel at 4°C until needed.

Table 1. *Fusarium* isolates, from wheat seeds, isolate number, location and colony colour.

Isolate No.	Location	Year of collection	Colony colour
<i>F. culmorum</i>			
5	North-west	2003	Yellow mycelia-red base
6	"	2005	red
7	"	2001	Yellow mycelia-brown base
<i>F. oxysporum</i>			
8	"	2001	White
9	"	2003	"
10	Middle region	2001	"
11	North-west	2003	White mycelia-red base
12	"	2001	red
13	"	2003	red
<i>F. equiseti</i>			
14	ICARDA*	2002	"
15	ICARDA	2003	"
16	ICARDA	2003	White mycelia-red base

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Mycelium was collected and DNA extracted according to standard protocols (Leach *et al.* 1986), resuspended in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). Genomic DNA of a high quality and with concentrations ranging from 100 - 500 ng/ μl was obtained for all *Fusarium* isolates.

The AFLP protocol was carried out as reported by Vos *et al.* (1995). The pre-amplification reaction was performed using E+A and M+C primers (*Eco*RI and *Mse*I primers contain one selective nucleotide at the 3' end to reduce the number of amplified fragments). The sequences of the primer combinations used in this study are presented in Table 2.

Each gel from the AFLP experiments was scored as presence (1) or absence (0) of a specific band for every fungal isolate. Data for all the 19 primer combinations were used to estimate the

genetic distances using the Nei and Li (1979) method. A dendrogram was generated using the Un-weighted pair group of arithmetic means (UPGMA) provided in the computer program Statsoft (2003).

Results and Discussion

The current investigation was aimed at evaluating the potential of the AFLP technique to discriminate among *Fusarium* isolates at the interspecific level. Nineteen of 31 possible primer pairs successfully amplified fragments from the genomic DNA of all studied isolates (Fig. 1). A total of 654 bands were amplified using AFLP assay, of which 296 (44.54%) were polymorphic (Table 2). The number of fragments produced by primer pairs varied from 8 and 28.

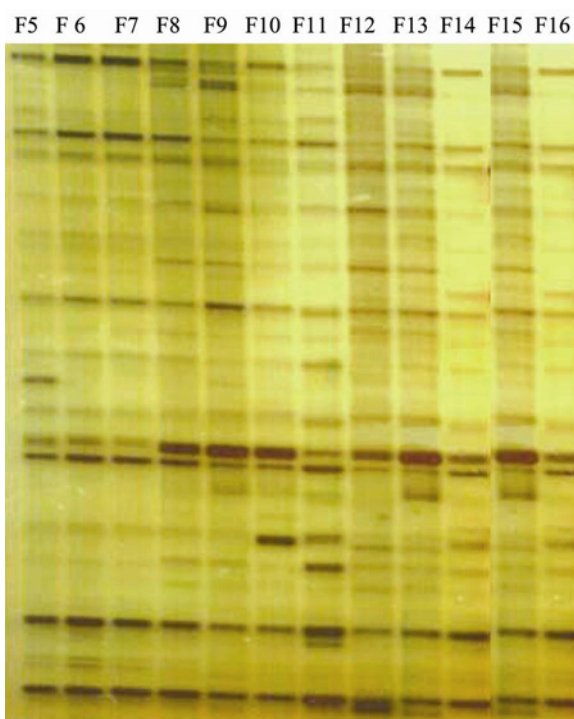


Fig. 1. A portion of a silver stained AFLP gel generated from 12 *Fusarium* spp. isolates using *EcoRI*+*ACA/MseI*+*CTA* primer combination. (Isolates 5, 6 and 7 are *F. culmorum*, 8 to 10 are *F. equiseti* and 11 to 16 are *F. oxysporium*).

The UPGMA dendrogram generated for the AFLP data demonstrated that *Fusarium* spp. isolates clustered into three groups (Fig. 2), the biggest one did not fall into clades correlated to the origin or colour of the isolate, which suggests a regional dispersal of these species. However, isolates belonging to *F. culmorum* and *F. equiseti* collected from north-west of Syria and from ICARDA, respectively fell into two separate sub-clusters. Manicom *et al.* (1987) reported that genetic relationships among *Fusarium* species could be attributed either to a higher evolutionary rate in these species (“fast evolutionary clock”) or to an earlier branching in the Pyrenomycetes.

Although techniques such as RAPD and RFLP were able to provide the scientific community with basic knowledge related to the genetic diversity in *Fusarium*, they did not always agree in terms of genetic relationships among clonal lineages of this asexually reproducing pathogen (Groenewald *et al.* 2005). On the other hand, the advantage of AFLP over these techniques is that multiple bands are derived from all over the genome with a higher reproducibility rate. This prevents over interpretation or misinterpretation due to point mutations or single-locus recombination, which may affect other genotypic characteristics. However, the studied species played a prominent role in plant diseases of diverse host range, therefore, a more comprehensive study of each group could provide useful information on the variation and dispersal of *Fusarium* spp. isolates.

Table 2. Percent polymorphism, band numbers and polymorphic bands produced by 19 primer combination used in the study.

No.	Primer combination	Polymorphism (%)	Total No. of bands	Polymorphic bands
1	E-ACT × M-CTG	51.1	47	24
2	E-AAG × M-CTA	62.2	45	28
3	E-ACG × M-CAC	40.5	37	15
4	E-ACA × M-CTA	47.1	51	24
5	E-ACA × M-CAT	36.6	41	15
6	E-AAC × M-CAC	45.2	31	14
7	E-AAC × M-CAA	42.9	28	12
8	E-ACC × M-CTG	50.0	28	14
9	E-ACC × M-CAA	50.0	32	16
10	E-AAG × M-CTA	55.6	36	20
11	E-ACG × M-CAT	32.0	25	8
12	E-ACG-M-CAA	30.8	26	8
13	E-ACC × M-CAT	36.1	36	13
14	E-ACG × M-CTA	43.3	30	13
15	E-ACG × M-CAT	60.0	30	18
16	E-ACG × M-CAT	24.2	33	8
17	E-ACG × M-CAA	47.2	36	17
18	E-AAG × M-CAT	55.9	34	19
19	E-ACA × M-CTG	35.7	28	10
Average		44.54	654	296.00

Different colony colours were observed among isolates after 10 days of growth on PDA media, but these were not correlated with colony origin within the country (Table 1). Burgess *et al.* (1989) attributed the morphological changes to the effects of different environmental conditions and considered constant environmental conditions necessary for taxonomic studies in *Fusarium*. The observed variation among *Fusarium* isolates in this study is in line with the results of Windels (1991) and Kiprof *et al.* (2002). However, the patterns generated from AFLP in this study demonstrated variability among *Fusarium* species that may have arisen through point mutations, gene flow and/or recombination.

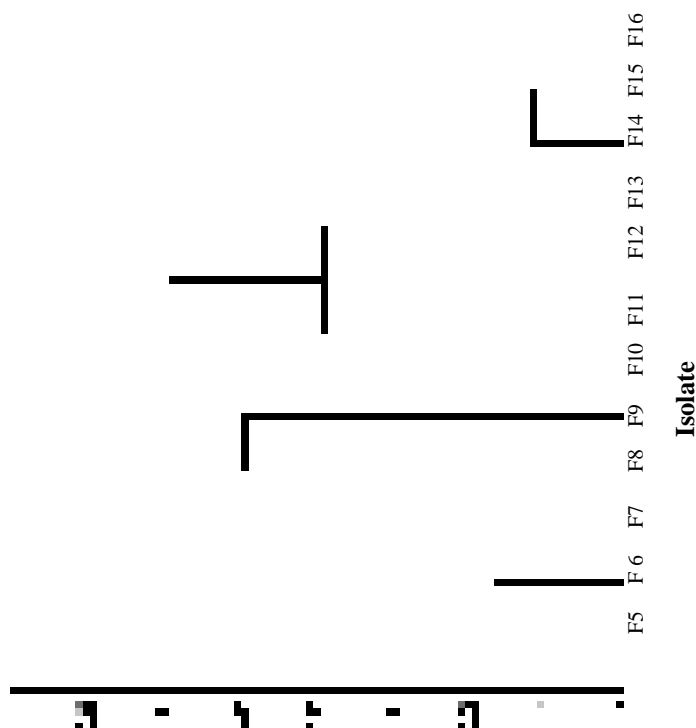


Fig. 2. UPGMA dendrogram showing genetic relationships among 12 isolates of *Fusarium* spp. The dendrogram was constructed using AFLP marker and was based on the genetic distances calculated according to Nei and Li (1979).

In this study, AFLP exhibited a high level of efficiency in detecting DNA polymorphism among *Fusarium* spp. isolates. The phylogeographic distribution of AFLP diversity suggests that there has been a previous dispersal of *Fusarium* spp. isolates throughout the interior of Syria. Moreover, this study could provide crucial information needed for future selection of isolates in order to develop wheat resistance against these species.

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