

Full Length Research Paper

Use of AFLPs to differentiate between *Fusarium* species causing root rot disease on durum wheat (*Triticum turgidum* L. var. *durum*)

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Accepted 23 June, 2009

Durum wheat is the main crop in the Mediterranean dryland, where root rot (*Fusarium* spp. and *Helmintosporium sativum*) diseases are common. The aim of this study was to use the AFLP technique to determine variation and genetic relationships between Syrian *Fusarium* isolates; and compare them with some standard *Fusarium* species. To identify the different pathogens causing root rots, AFLP templates were prepared by the digestion of *Fusarium* DNA with *EcoRI* and *MseI* restriction endonucleases and subsequent ligation of corresponding site-specific adapters. The isolates were collected from the main durum wheat growing provinces in Syria (Aleppo, Hassaka and Daraa). A total of 389 AFLP polymorphic bands were obtained using 3 primer combinations. AFLP analyses indicated that the isolate Hassaka31 was the closest to *F. poae*, Hassaka2 to *F. culmorum*. Daraa14, Daraa49 and Tel Hadya16 to *F. equiseti*, and Daraa74 and Daraa62 to *F. graminearum*. Whereas the isolates Tel Hadya10, Tel Hadya19 and Aleppo4 were close to *F. avenaceum*. As for the cluster analysis based on 41 specific traits (morphology and microscopy), it showed that Hassaka31 and Daraa74 were very similar to *F. avenaceum*. Whereas, isolates Aleppo4, Daraa14, Daraa49, Tel Hadya16, Daraa62 and Tel Hadya10 were similar to *F. avenaceum*, Hassaka2 to *F. culmorum* and Tel Hadya19 to *F. equiseti*, *F. graminearum* and *F. poae*. Tel Hadya10 and Daraa62 were the most virulent isolates and they are used in the breeding program for resistance screening.

Key words: DNA, markers, primer, cluster.

INTRODUCTION

Durum wheat is the main agricultural crop in the Mediterranean countries. The fungal diseases are among the primary constraints in the Mediterranean dryland where durum wheat is largely grown, particularly the common root rot (*Fusarium* spp. and *Helmintosporium sativum*). The most obvious symptoms are brown spots or elongated blotches on roots, subcrown, internodes, crowns and lower leaf sheaths. Susceptible cultivars show after heading, white heads without or shriveled kernels (Smiley and Patterson, 1995). *Fusarium* species are isolated from infected roots of wheat plants and often have been reported as pathogens. Although the dominant *Fusarium* spp. associated with infected wheat plants vary with the geo-

graphic locations. The usually found species in the infected wheat plants include often *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. equiseti*, *F. poae* (El-Khalifeh et al., 2006; Mergoum et al., 1997).

Fusarium species are identified using combination of microscopic and colony characters (Ouellet and Seifert, 1993). However, the differences between some species are rather subtle. Specials formae species and races within a species are defined as characters (Miller et al., 1983). Accurate identification of laboratory-reared strains is a prerequisite for field studies of pathogen diversity, spreading and host infection.

In last years, numerous molecular phylogeny markers that reveal the genetic diversity of similar organisms have studied. Among these, the random amplified polymorphic DNA (RAPD) analysis, a PCR-based method of genetic typing has been employed frequently. More recently, amplified fragment length polymorphism (AFLP) analysis

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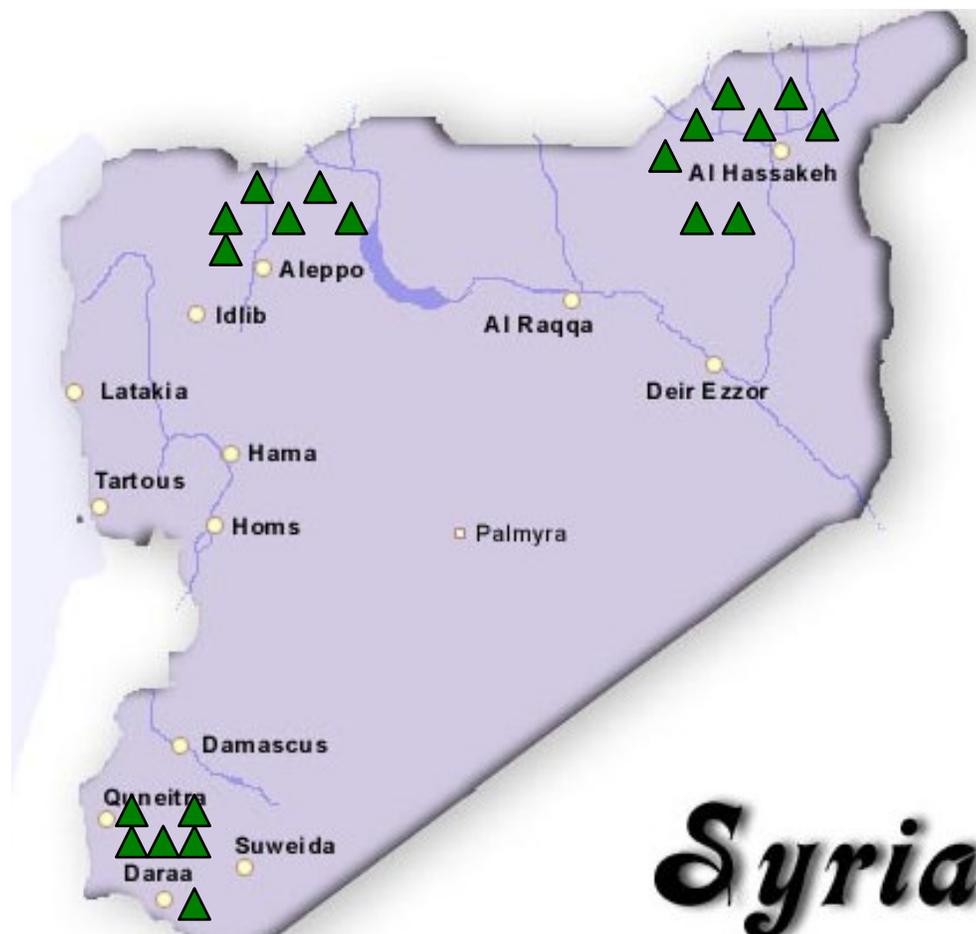


Figure 1. Map of Syria Provinces where *Fusarium* isolates were collected (Hassaka et al., 2003).

has been used for DNA fingerprinting of microorganisms. AFLP analysis is based on selective amplification of DNA restriction fragments (Vos et al., 1995). It is technically similar to restriction fragment length polymorphism (RFLP) analysis, except that the number of fragments generated can be amplified using the PCR technique. The advantage of AFLP over other techniques is that multiple bands are derived from all over the genome. The main disadvantage of AFLP markers is that alleles are not easily recognized (Majer et al., 1998). PCR has proven to be a powerful tool in detecting plant-pathogenic fungi as well as bacteria (Majer et al., 1996; Restrepo et al., 1999). The utility, and efficiency of the AFLP technique are leading to broader application to the analysis of *Fusarium* populations (Abdel-Satar et al., 2003; Sivaramakrishnan et al., 2002, El-Khalifeh, 2006). The aim of this study was to use the AFLP technique to determine the variation and the genetic relationships between Syrian *Fusarium* isolates and compare them with five standard *Fusarium* species (*F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. equiseti*, *F. poae*) obtained by Dr. Mezzalama (CIMMYT, Mexico).

MATERIALS AND METHODS

Fungi material

Hundred-thirty-one *Fusarium* isolates were collected from 3 Syrian provinces in 2003 (Figure 1): 78 isolates from Daraa (South Syria), 22 from Aleppo (North Syria), and 31 from Hassaka (East Syria). The pathogenicity test showed that 58 isolates were pathogenic as follows: 34 in Daraa isolates, 11 in Aleppo, 13 in Hassaka (El-Khalifeh et al., 2006). The pathogenic *Fusarium* isolates were classified into 10 groups according to their cultural and morphological characteristics; from each group one standard isolate was selected for further studies. The cultures were identified and single spore was taken for pure isolate culture using the methods of Nelson et al. (1983).

The Syrian *Fusarium* isolates along the five standard *Fusarium* species (Table 1) were grown on Czapek broth media, in flasks for 2 week at 25°C. For each isolate, the mycelium was harvested by filtration through a double layer of sterile muslin, and the mycelium was washed with sterile distilled water; and ground in liquid nitrogen for later studies.

DNA extraction

100 mg from the end product was extracted with 1 ml of extraction

Table 1. Syrian *Fusarium* isolates and standard *Fusarium* species.

S/N	Sources of isolates (provinces)	Name of isolate
1	(ICARDA Station), Aleppo, North Syria	Tel Hadya10
2	(ICARDA Station), Aleppo, North Syria	Tel Hadya16
3	(ICARDA Station), Aleppo, North Syria	Tel Hadya19
4	Aleppo, North Syria	Aleppo4
5	Daraa, South Syria	Daraa49
6	Daraa, South Syria	Daraa14
7	Daraa, South Syria	Daraa74
8	Daraa, South Syria	Daraa62
9	Hassaka, East Syria	Hassaka2
10	Hassaka, East Syria	Hassaka31
Standard <i>Fusarium</i> species used		
1	CIMMYT-Mexico	<i>F. graminearum</i>
2	"	<i>F. avenaceum</i>
3	"	<i>F. equiseti</i>
4	"	<i>F. culmorum</i>
5	"	<i>F. poae</i>

buffer (1 M Tris-HCl pH 7.5, 5 M NaCl, 0.5 M EDTA, CTAB) and 1 ml (Chloroform+Iso-Amyl-Alcohol 24:1). After centrifugation (30 min at 4000 rpm), the DNA in the upper aqueous phase was precipitated by additional of 1 ml of iso-propanol. After centrifugation (6 min at 10000 rpm), the DNA pellets were washed with 2 ml 75% ethanol. After centrifugation (5 min at 10000 rpm), the DNA pellets were dissolved in 100 µl of TE (1 M tris pH 8, 0.5M EDTA pH 8) and incubated at 37°C for 30 min with RNAase (0.3 µl/100 µl). Thereafter of sodium acetate (1 µl/10 µl) and 1 ml pure ethanol (-20°C) were added. After centrifugation (5 min at 10000 rpm) the DNA pellets were dissolved in 100 µl TE. DNA concentration was quantified on spectrophotometer machine.

AFLP analysis

The AFLP procedure was carried out as reported by Vos et al. (1995) with modifications made by the Marker Assisted Durum Selection Lab. at ICARDA.

Restriction digestion: The 0.65 µl of DNA (80 ng) was incubated for 4 h at 37°C with 1 µl of 5× reaction buffer, 0.4 µl of EcoRI/MseI enzymes mix, 2.95 µl of AFLP-grade water. The product was incubated for 15 min at 70°C for inactive the enzyme.

Ligation of adapters: 2.4 µl of EcoRI/MseI adapters, 0.1 µl of T4 DNA ligase were added to digest DNA and incubated at 20°C or room temperature for 2 h. After that was made dilution 1:5 with TE buffer and stored at -20°C.

Preamplification reaction: The preselective PCR contained 0.5 µl of diluted ligated DNA, 4 µl of pre-amp primer mix with 0.5 µl of 10× PCR buffer, 0.38 µl of Taq polymerase, in a total volume of 5 µl. The PCR program consisted of 20 cycles of 30 s at 95°C, 1min at 56°C, 1 min at 72°C.

Selective amplification: The selective PCR contained 1.3 µl of the diluted (1:10) product of the preamplification PCR, 2.8 µl of ddH₂O, 0.5 µl of 10× PCR buffer AFLP, 1.17 µl of each primer pairs, 0.038 µl of Taq polymerase. Three primer pairs: EcoI+CAC/MseI+AAG, EcoI+CTT/MseI+AGG and EcoI+CTC/MseI+AGG were used for the

selective amplification. The first amplification cycle was carried out for 30 s at 94°C, 30 s at 65°C and 1 min at 72°C. in each of the following 13 cycles, The annealing temperature was reduced by 0.7°C. The last 23 cycles were carried out for 30 s at 94°C, 30 s at 56°C and 1 min at 72°C. Each sample was diluted 1:1 with loading buffer, denatured and fractionated on a polyacrylamide sequencing gel in Tris-borate-EDTA buffer. Gels were run at constant power (70 W), and then stained by method of Nachit et al. (2001).

Data analysis

Polymorphic AFLP markers were visually scored as binary data with presence as "1" and absence as "0". Monomorphic markers were discarded. Clusters analyses were performed on SYSTAT 7.0 program, using Ward and Gamma method.

RESULTS

A total of 389 polymorphic bands with good quality reading were amplified using three primer combinations (Figure 2), including 133 bands per EcoI+CAC/MseI+AAG, 172 per EcoI+CTC/MseI+AGG, and 84 per EcoI+CTT/MseI+AGG. The genetic relationship among all AFLP patterns of *Fusarium* spp. based on the combination of data obtained with the three primers is represented in the dendrogram shown in Figure 3. The *Fusarium* isolates were clustered in two major clusters with diversity distance (DD) at 2.5. The two major clusters were divided into two subclusters, the first subcluster (1.1) included *F. poae* and *F. culmorum* with two Syrian isolates from (Hassaka31 and Hassaka2) at DD=0.7, *F. poae* and Hassaka31 isolate were very similar (DD=0.1); the Hassaka2 isolate was similar to *F. culmorum* (DD=0.3).

The second subcluster (1.2) consist of *F. equiseti* and

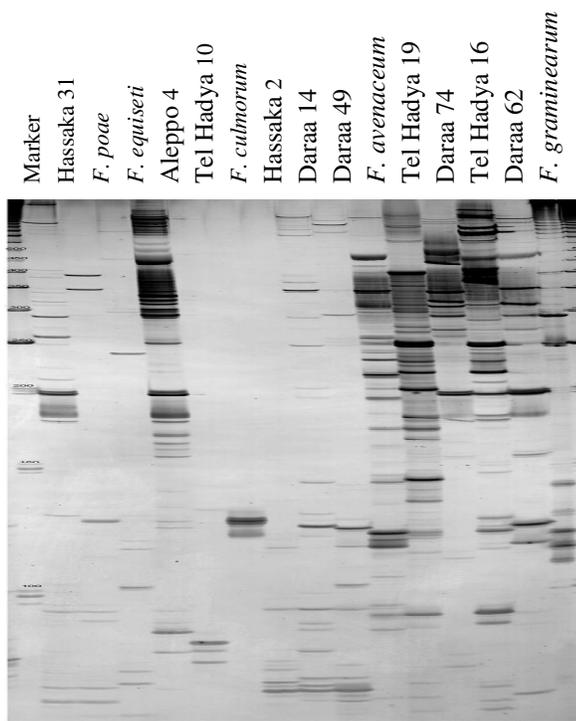
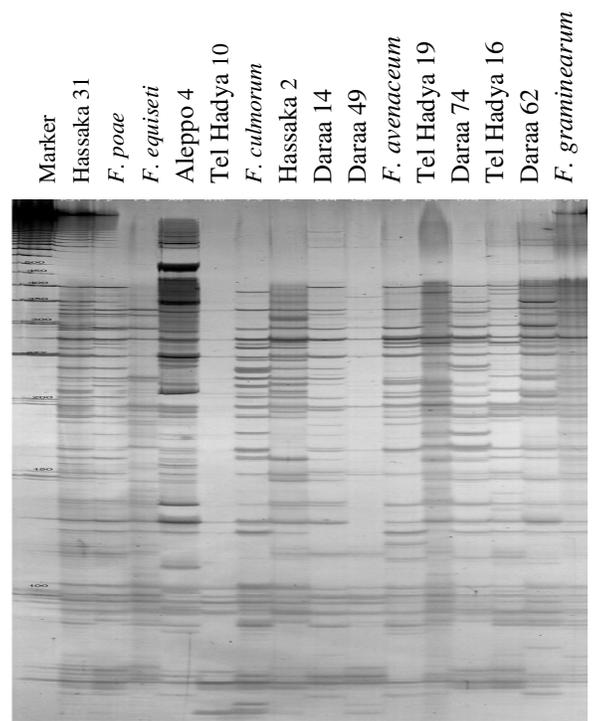
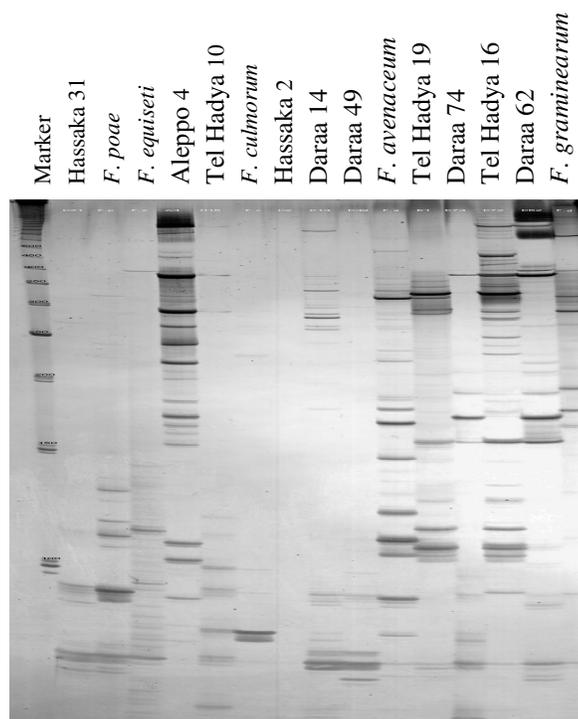
E-CAC/M-AAG**E-CTC/M-AGG****E-CTC/M-AGG**

Figure 2. Normalized AFLP band patterns generated from 10 Syrian *Fusarium* virulent isolates and 5 standard *Fusarium* spp. using 3 primer combinations *Eco*RI+3/*Mse*I+3. Marker (each 50 bp).

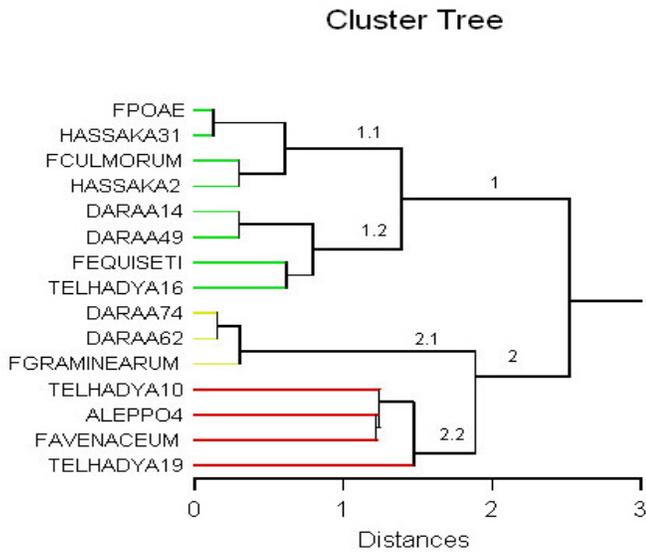


Figure 3. Combined cluster analysis derived from AFLP analysis of 10 Syrian *Fusarium* virulent isolates and 5 standard *Fusarium* spp. using 3 AFLP primers with a total number of polymorphic bands 389. FPOAE: *Fusa-rium poae*; FEQUISETI: *Fusarium equiseti*; FGRAMI-NEARUM: *Fusarium graminearum*; FAVENACEUM: *Fusarium avenaceum*.

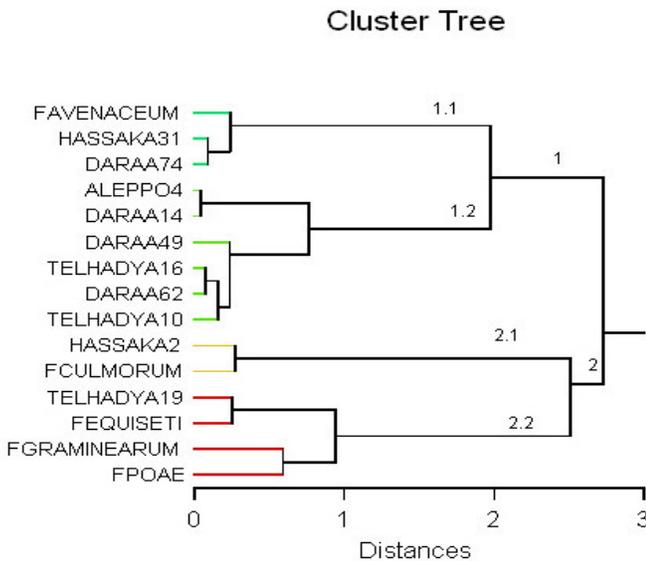


Figure 4. Combined cluster analysis derived from morphological and cultural description analysis of 10 Syrian *Fusarium* virulent isolates and 5 standard *Fusa-rium* spp. FPOAE: *Fusarium poae*; FEQUISETI: *Fusa-rium equiseti*; FGRAMINEARUM: *Fusarium graminea-rum*; FAVENACEUM: *Fusarium avenaceum*.

three Syrian isolates at DD=0.8, the Syrian isolates: Daraa14 and Daraa49 showed very high genetic relatedness between them; whereas Tel Hadya16 was very similar to *F. equiseti* (DD=0.6). The first subcluster in second major cluster (2.1) includes *F. graminearum* and

two Syrian isolates (Daraa74 and Daraa62) at DD=0.4. The second subcluster (2.2) includes *F. avenaceum* and three Syrian isolates (Tel Hadya10, Tel Hadya19, and Aleppo4) DD= 1.5. Tel Hadya10 and Aleppo4 were very similar to *F. avenaceum* (DD= 1.3).

Further, we have also analyzed the morphology and microscopy characterization of the tested isolates using 41 specific traits (Growth speed of isolates on artificial media, spores traits, culture color, etc). The isolates were clustered in two major clusters (Figure 4). The two major clusters divided into two subclusters. The first subcluster (1.1) consist of *F. avenaceum*, and Two Syrian isolates (Hassaka31 and Daraa74) at DD=0.3.

The second subcluster (1.2) consist six Syrian isolates at DD=0.8. Where Tel Hadya16 and Daraa62 were very similar (DD=0.1). The first subcluster (2.1) included *F. culmorum* and Hassaka2 isolate at DD= 0.2. The second subcluster (2.2). Which consisted of *F. equiseti*, *F. poae*, *F. graminearum* and the Tel Hadya19 isolate at DD= 0.9.

The results obtained showed that there was no-clear-cut relationship between clustering in the AFLP dendrogram and morphology/microscopy description clustering of the tested isolates with a few exceptions.

This study showed that five *Fusarium* species (*F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. equiseti*, *F. poae*) were found spread in Syria; *F. graminearum* in Daraa province, *F. culmorum* and *F. poae* in Hassaka province, *F. avenaceum* in Aleppo province, and *F. equiseti* in Daraa and Aleppo provinces. The most virulent *Fusarium* species were *F. graminearum* and *F. avenaceum*.

The virulent isolates Tel Hadya10 (close to *F. avenaceum*) and Daraa62 (close to *F. graminearum*) are used in our breeding program for the disease resistance.

DISCUSSION

The variation and genetic relationships between Syrian *Fusarium* isolates were compared with five standard *Fusarium* species (*F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. equiseti*, *F. poae*) using AFLP technique that have broad taxonomic applicability. This technique has been also used effectively in a variety of taxa including fungi by Majer et al. (1998) and Janssen et al. (1996). A total of 389 good polymorphic bands were amplified using three primer combinations with *EcoR1* (E)+3 and *MseI* (M)+3 at the 3'-end of the primers. This study has enabled us to identify *Fusarium* isolates which taxa had been difficult to determine based on morphological criteria. Janssen et al. (1996) have showed that the choice of the restriction enzymes and the length and composition of selective nucleotide will determine the complexity of the final AFLP fingerprint. The present findings are consistent with that of Majer et al. (1996) who also used 3 nucleotides in analyzing AFLP of pathogenic isolates of *Cladosporium fulvum*. The primer selectivity is related to genome size. Good selectivity is found with primers

having three selective nucleotides (El-Khalifeh, 2006). However, with addition of the fourth nucleotide, selectivity is lost as indicated by Vos et al. (1995).

Statistical analysis of AFLP data enabled the classification of *Fusarium* isolates into 2 AFLP groups, although these groups were not morphologically distinct. There was no correlation between AFLP and geographic origin of the isolates and morphology description; our results are in harmony with those obtained by Abdel-Satar et al. (2003) and Sivaramakrishnan et al. (2002). The results of this study demonstrate clearly that the use of the AFLP is a powerful, simple and rapid technique in studying the identification and the genetic relationships between *Fusarium* species.

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