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Molecular phylogeny of *Fusarium* species by AFLP fingerprint

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The high-resolution genotyping method of amplified fragment length polymorphism (AFLP) analysis was used to study the genetic relationships within and between natural populations of five *Fusarium* spp. AFLP templates were prepared by the digestion of *Fusarium* DNA with *Eco*RI and *Mse*I restriction endonucleases and subsequent ligation of corresponding site-specific adapters. An average of 44 loci was assayed simultaneously with each primer pair and DNA markers in the range 100 to 500 bp were considered for analysis. A total of 80 AFLP polymorphic markers were obtained using four primer combinations, with an average of 20 polymorphic markers observed per primer pair. UPGMA analyses indicated 5 distinct clusters at the phenon line of 30% on the genetic similarity scale corresponding to the 5 taxa. The similarity percent of each group oscillated between 87 and 97%. The phenetic dendrogram generated by UPGMA as well as principal coordinate analysis (PCA) grouped all of the *Fusarium* spp. isolates into five major clusters. No clear trend was detected between clustering in the AFLP dendrogram and geographic origin, host genotype of the tested isolates with a few exceptions. The results of the present study provide evidence of the high discriminatory power of AFLP analysis, suggesting the possible applicability of this method to the molecular characterization of *Fusarium*.

Key Words: AFLP, *Fusarium*, molecular phylogeny, selective amplification.

INTRODUCTION

Cotton (*Gossypium barbadense*) is the main agricultural export commodity from Egypt and fungal diseases are among primary constraints to cotton production. *Fusarium* species are frequently isolated from diseased roots of cotton seedlings and often have been reported as pathogens of cotton seedlings (Johnson et al., 1978; Roy and Bourland, 1982; Colyer; 1988). Although the dominant *Fusarium* spp. associated with diseased cotton seedlings vary with geographic location, the species associated with necrotic cotton roots usually include *F. oxysporum, F. solani, F. moniliforme, F. semitectum* (Colyer, 1988; Aly et al., 1996; El-Samawaty, 1999). In recent years numerous molecular phylogeny markers that reveal the genetic diversity of similar organisms have arisen. Random amplified polymorphic DNA (RAPD) analysis is a fast, PCR-based method of genetic typing based on genomic polymorphisms. More recently, amplified fragment length polymorphism (AFLP) analysis 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 analysis, except that only a subset of the fragments are displayed and the number of fragments generated can be controlled by primer extensions. The advantage of AFLP over other techniques is that multiple bands are derived from all over the genome. This prevents over interpretation or misinterpretation due to point mutations or single-locus recombination, which may affect other genotypic characteristics. The main disadvantage of AFLP markers is that alleles are not easily recognized (Majer et al., 1998). PCR has proven to be successful in detecting plant-pathogenic fungi as well as bacteria (Majer et al., 1996; Restrepo et al., 1999). The utility, repeatability, and efficiency of the AFLP technique are leading to broader application of this technique to the analysis of *Fusarium* populations (Abd-Elsalam et al., 2002a,b; Kiprop et al., 2002; Sivaramakrishnan et al., 2002). In this study, we used AFLP with a range of primer

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Abbreviations: AFLP, amplified fragment length polymorphism; GS, genetic similarity; PCA, principal coordinate analysis; UPGMA, unweighted pair group method using arithmetic.

Table 1. Isolates of *Fusarium* spp analyzed in this study.

Isolate No.	*Fusarium* spp.	Geographic origin	Previous crops	Sampling date	Host genotype
1	Fusarium oxysporum (Fo)	Dumyat	Alfa-Alfa	August	Giza 45
2	Fusarium oxysporum	Gharbiya	Alfa-Alfa	August	Giza 86
3	Fusarium oxysporum	Minufiya	Alfa-Alfa	August	Giza 89
4	Fusarium oxysporum	Gharbiya	Onion	May	Giza 89
5	Fusarium oxysporum	Sharqiya	Alfa-Alfa	June	Giza 85
6	Fusarium oxysporum	Daqahliya	Faba-Bean	June	Giza 86
7	Fusarium oxysporum	Minufiya	Faba-Bean	April	Giza 89
8	Fusarium oxysporum	Gharbiya	Alfa-Alfa	July	Giza 86
9	Fusarium oxysporum	Minufiya	Alfa-Alfa	July	Giza 89
10	Fusarium oxysporum	Daqahliya	Onion	May	Giza 86
11	Fusarium oxysporum	Fayium	Alfa-Alfa	May	Giza 83
12	Fusarium oxysporum	Beheira	Alfa-Alfa	May	Giza 70
13	Fusarium oxysporum	Beheira	Alfa-Alfa	May	Giza 70
14	Fusarium oxysporum	Beheira	Alfa-Alfa	May	Giza 70
15	Fusarium oxysporum	Beheira	Alfa-Alfa	May	Giza 70
16	Fusarium oxysporum	Beheira	Alfa-Alfa	May	Giza 70
17	Fusarium oxysporum	Beheira	Alfa-Alfa	May	Giza 70
18	Fusarium oxysporum	Beheira	Alfa-Alfa	May	Giza 70
19	Fusarium oxysporum	Beheira	Alfa-Alfa	May	Giza 70
20	Fusarium oxysporum	Beheira	Alfa-Alfa	May	Giza 70
21	Fusarium oxysporum	Beheira	Alfa-Alfa	May	Giza 70
22	Fusarium oxysporum	Minufiya	Alfa-Alfa	June	Giza 89
23	Fusarium oxysporum	Daqahliya	Pisi	May	Giza 86
24	Fusarium oxysporum	Minufiya	Alfa-Alfa	June	Giza 89
25	Fusarium oxysporum	Minufiya	Faba-Bean	July	Giza 89
26	Fusarium oxysporum	Minufiya	Faba-Bean	June	Giza 89
27	Fusarium oxysporum	Fayium	Alfa-Alfa	June	Giza 83
28	Fusarium oxysporum	Fayium	Alfa-Alfa	June	Giza 83
29	Fusarium moniliforme (Fm)	Sharqiya	Alfa-Alfa	August	Giza 85
30	Fusarium moniliforme	Beheira	Alfa-Alfa	July	Giza 70
31	Fusarium moniliforme	Minufiya	Alfa-Alfa	August	Giza 89
32	Fusarium moniliforme	Sharqiya	Alfa-Alfa	August	Giza 85
33	Fusarium moniliforme	Gharbiya	Alfa-Alfa	August	Giza 86
34	Fusarium moniliforme	Gharbiya	Onion	August	Giza 86
35	Fusarium moniliforme	Minufiya	Alfa-Alfa	August	Giza 89
36	Fusarium moniliforme	Minufiya	Faba-Bean	August	Giza 89
37	Fusarium moniliforme	Gharbiya	Alfa-Alfa	August	Giza 86
38	Fusarium solani (Fs)	Daqahliya	Alfa-Alfa	August	Giza 86
39	Fusarium solani	Daqahliya	Pisi	July	Giza 86
40	Fusarium solani	Dumyat	Alfa-Alfa	August	Giza 45
41	Fusarium solani	Dumyat	Faba-Bean	August	Giza 45
42	Fusarium solani	Dumyat	Alfa-Alfa	August	Giza 45
43	Fusarium solani	Beheira	Alfa-Alfa	August	Giza 70
44	Fusarium avenaceum (Fa)	Sharqiya	Alfa-Alfa	June	Giza 89
45	Fusarium avenaceum	Fayium	Alfa-Alfa	July	Giza 83
46	Fusarium chylamdiosporum (Fc)	Beheira	Faba-Bean	June	Giza 70

pairs and found sufficient variation to draw conclusions about the genetic relationships within and between five *Fusarium* species.

MATERIALS AND METHODS

Fungal culture and DNA extraction

Fungal isolates utilized in this study are listed in (Table 1). DNA extractions were conducted as previously described in Liu et al. (2000) from 100 mg fresh fungal mycelium grown in 5 ml potato dextrose broth in 15 ml Falcon tube at 28°C. Cultures were filtered through a double layer of sterile muslin, and the mycelium was washed with sterile distilled water. DNA concentrations were quantified on agarose gels stained with ethidium bromide in comparison with commercially obtained standard.

AFLP analysis

The AFLP procedure was carried out as reported by Vos et al. (1995) with few modifications. In brief, two combinations of restriction endonucleases were used. For the combination *Eco*RI/*Mse*I, genomic DNA (50 ng) was incubated for 2 h at 37°C

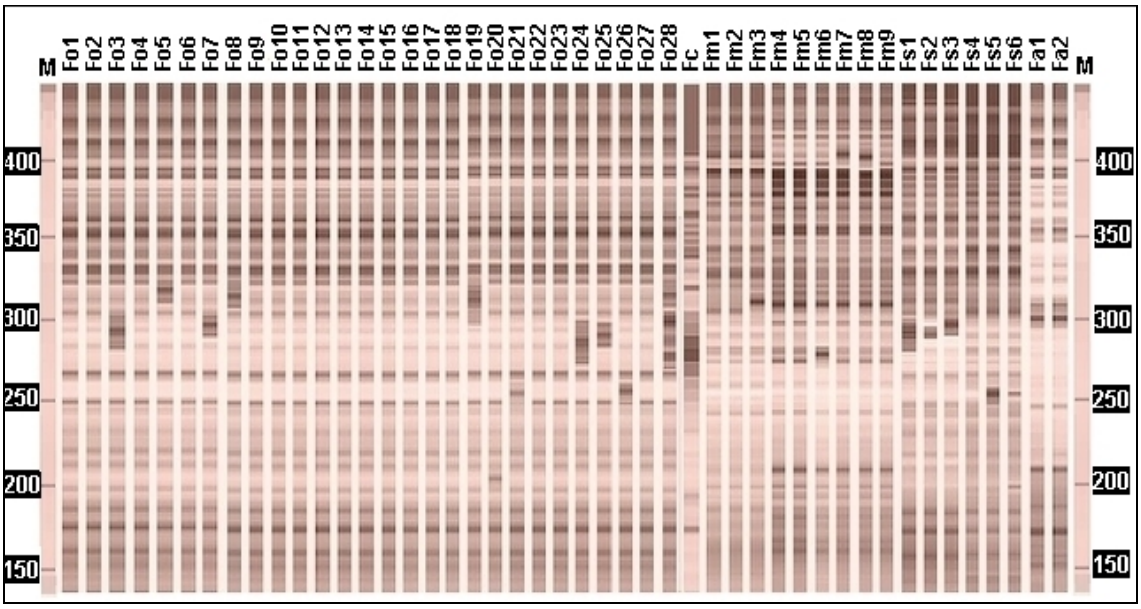

Figure 1. Normalized AFLP band patterns generated from 46 *Fusarium* spp. isolates using primer combinations *Eco*RI+AA/*Mse*I+AG. M is a 50 bp (numbers represent size in bp).

with 2 U of *Mse*I, 5 U of *Eco*RI, 1.2 U of T4 DNA ligase (New England Biolabs, Beverly, Mass.), 50 pmol of *Mse*I adapters and 5 pmol of *Eco*RI adapters. This reaction was done in a volume of 50 µl of restriction-ligation buffer containing 10 mM TRIS-acetate pH 7.5, 10 mM $MgCl_2$, 50 mM potassium acetate, 5 mM dithiothreitol (DTT), 1 mM ATP and 50 ng/µl BSA. For digestion with *Eco*RI/*Mse*I, genomic DNA (50 ng) was digested at 65°C for 1.5 h with 5 U *Mse*I, in a volume of 25 µl of the restriction-ligation buffer described above. The reaction was cooled to 37°C, supplemented with 15 µl of the restriction-ligation buffer containing 5 U of *Eco*RI and incubated at 37°C for an additional 2 h. For adapter ligation, 10 µl of the restriction-ligation buffer, containing 50 pmol of *MseI* adapters, 5 pmol of *Eco*RI adapters, 0.5 mM ATP and 1.2 U of T4 DNA ligase, was added, and the reaction was incubated in 37°C for 3 h. In both cases, a 30-µl aliquot of the adapter-ligated DNA was diluted 1:10 with distilled water to serve as template in the preselective PCR. The remaining 20-µl portion was used to verify that digestion was complete.

The preselective PCR contained 5 µl of template, 1 U of AmpliTaq polymerase, 2 µl of 10 AmpliTaq polymerase buffer, 0.25 mM of each of the four dNTPs, 2.5 mM $MgCl_2$ and 25 ng of *Eco*RI and *Mse*I primers with one selective nucleotide (A), in a total volume of 20 µl. The PCR program consisted of thirty cycles of 30 s at 94°C, 1 min at 56°C and 1 min at 72°C, followed by 10 min at 72°C.The selective PCR contained 5 µl of the diluted (1:10) product of the preselective PCR, 0.5 U of AmpliTaq polymerase, 2 µl of 10 AmpliTaq polymerase buffer, dNTPs and $MgCl_2$ as mentioned above, in a total volume of 20 µl. Four primer pairs; *Eco*RI+AG/*Mse*I+AA, *Eco*RI+AA/*Mse*I+AG, *Eco*RI+CC/*Mse*I+AA and *Eco*RI+CC/*Mse*I+CC (MWG-Biotech, Germany) 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 10 cycles, the annealing temperature was reduced by 1°C. The last 25 cycles were carried out at an annealing temperature of 56°C, and the final extension step was carried out at 72°C for 10 min. Each sample was diluted 1:1 with loading buffer, denatured and fractionated on a 5% polyacrylamide sequencing gel in TRIS-borate-EDTA buffer. Gels were run at constant power (55 W), and then stained by a modification of the silver staining method of Creste et al. (2001).

Data analysis

Polymorphic AFLP markers were manually scored as binary data with presence as "1" and absence as "0". Monomorphic markers were not scored. Cluster analysis was performed on the similarity matrix employing the "unweighted pair group method using arithmetic means" (UPGMA) algorithm (Sneath and Sokal, 1973) provided in the computer program NTSYSpc, version 2.1 (Exeter Software Co., New York).

RESULTS

Fast screening of AFLP primers combination

Thirty-two primer combinations were tested on five isolates from different populations. The generated fingerprints were evaluated for overall clearness of the banding pattern and the number of polymorphic markers present was recorded (data not shown). Four primer combinations were chosen for further screening on 46 *Fusarium* spp. isolates. Two primer combinations resulted in not-scoreable fingerprints due to the amplification of too many and/or faint bands. Finally, four primer combinations were chosen for the diversity screening; *Eco*RI+AG/*Mse*I+AA, *Eco*RI+AA/*Mse*I+AG, *Eco*RI+CC/*Mse*I+AA and *Eco*RI+CC/*Mse*I+CC.

Genetic diversity as defined by AFLP fingerprinting

A total of 176 bands were amplified from four primer combinations, of which 80 bands (45%) were polymorphic (Figure 1), with an average of 20 polymorphic bands per primer combinations. The genetic relationship among all AFLP patterns of *Fusarium* spp.

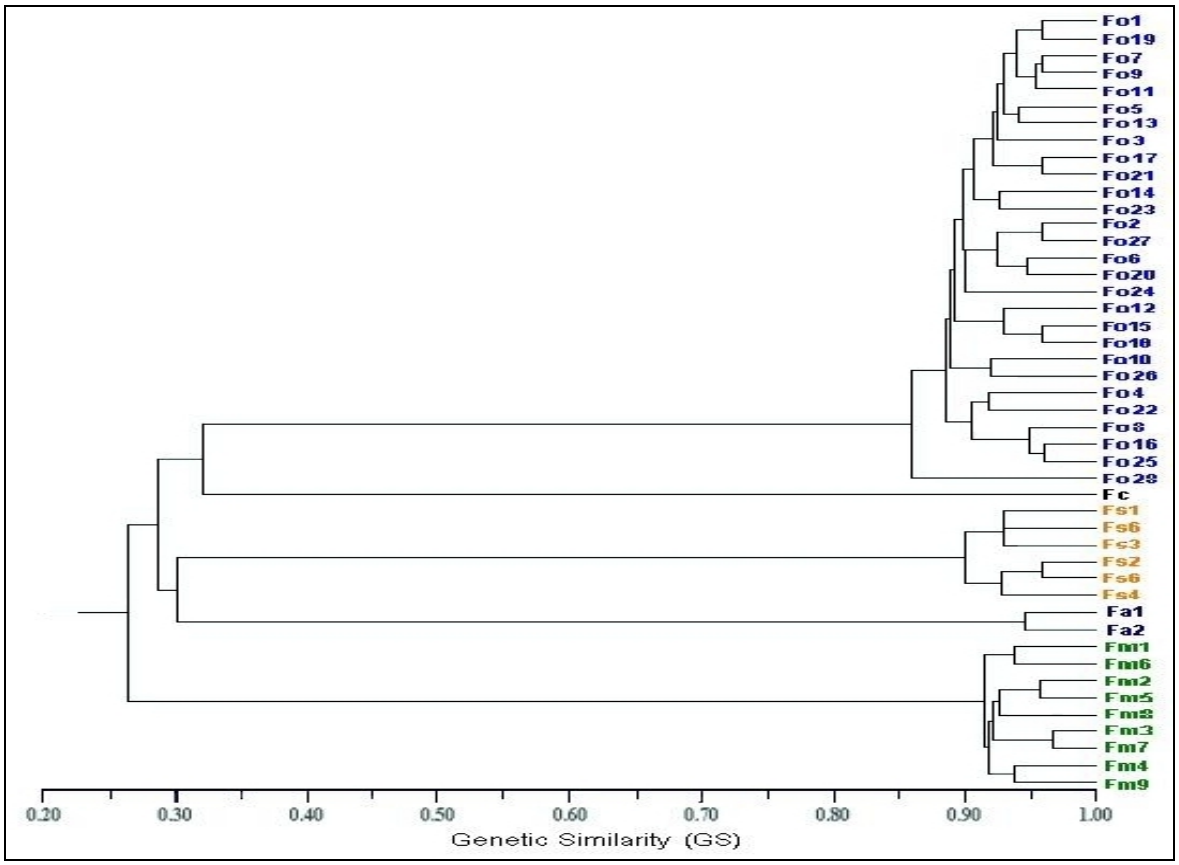

Figure 2. Combined cluster analysis derived from AFLP analysis of 46 *Fusarium* spp. isolates using 4 AFLP primers.

based on the combination of data obtained with the four primers is represented in the dendrogram shown in Figure 2. A total of 80% of the isolates were clustered in the first major cluster with 30% similarity among them. The first major cluster divided into three subclusters, the first subcluster included all *F. oxysporum* with genetic similarity of GS=34%, isolates Fo16 and Fo25 showed very high genetic similarity of GS=99.80%. *F. chlamydosporum* constituted one cluster branched from the second main cluster at level of 33%. The second subcluster consist of the six isolates of *F. solani* at the genetic similarity of GS=90%, isolates Fs2 and Fs5 showed very high genetic relatedness. The third subcluster included all *F. avenaceum* isolates at the genetic similarity of GS=93%. The minor cluster contains all *F. moniliforme* at the genetic similarity of GS=26%, isolates Fm3 and Fm7 showed very high genetic similarity relatedness, although they came from Minufiya governorate and isolated from Giza 89. There was no clear-cut relationship between clustering in the AFLP dendrogram and geographic origin, host genotype of the tested isolates with a few exceptions. The similarity percent of each group oscillated between 87 and 97%. The results of the AFLP analysis showed great genetic diversity among the *Fusarium* spp.

Principal coordinate analysis (PCA)

The PCA is one of the multi-variate approaches of grouping based on the similarity coefficients or variance-covariance values of the component traits of the entities. It is expected to be more informative about differentiation among major groups, while the cluster-analysis provides higher resolution among closely related populations (Liu et al., 2001). In our PCA analysis, more than 70% of the variation in the estimates of genetic similarity was explained by the first three components, indicating the suitability of the AFLP approach for genetic clustering. The isolates of each species and the groups within *Fusarium* spp. were clearly assigned to distinct group. Isolates of *F. moniliforme, F. solani* and *F. oxysporum* are most distinctly separated and were located at the marginal position on the plane defined by X, Y and Z axis respectively. Isolates within the species *F. oxysporum* and *F. moniliforme* were densely aggregated and intra-specific variability was not discernable, *F. solani* isolates formed a more dispersed group. Isolates of *F. avenaceum* and *F. chlamydosporum* were placed between the groups of *F. moniliforme, F. solani* and *F. oxysporum* isolates. Hence, PCA (Figure 3) agreed well with the UPGMA cluster.

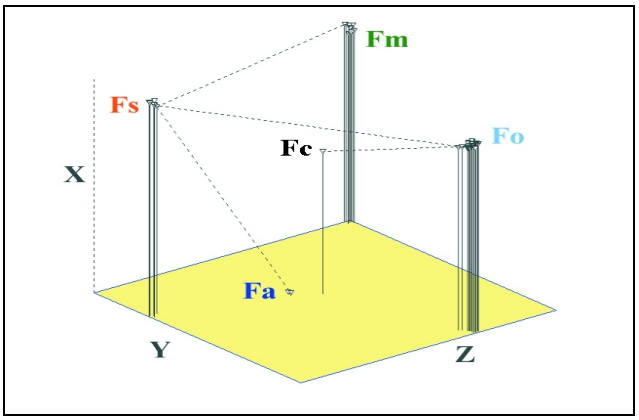

Figure 3. Three-dimensional display generated by NTSYS of principal coordinate analysis (PCA) of five *Fusarium* spp. based on the combination of data obtained with the four AFLP primers. X, Y and Z-axes are accounted 70% of the variation observed.

DISCUSSION

For a wide range of taxa, including plants, fungi and bacteria, AFLP markers have been used to uncover cryptic genetic variation of strains, or closely related species, that have been impossible to resolve with morphological or other molecular systematic characters. Therefore, AFLP have broad taxonomic applicability and have been used effectively in a variety of taxa including bacteria (Huys, 1996) and fungi (Majer et al., 1998). In view of the results of the present study, complex AFLP patterns were obtained using four different primer pairs and genomic similarity analyses derived from qualitative data enabled us to identify 46 isolates of five *Fusarium* spp. whose taxa had been uncertain based on morphological criteria. We have demonstrated that AFLP markers are useful in the study of genetic variation of *Fusarium* isolates. Using four primer combinations with *Eco*R1 (E) + 2 and *Mse*I (M) + 2 at the 3`-end of the primers on 46 isolates, a total of 176 bands were amplified with 80 polymorphic bands. 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 finding is consistent with the work of Majer et al. (1996) in the AFLP analysis of pathogenic isolates of *Cladosporium fulvum* where they used E + 2 and M + 2 nucleotides. Gonzalez et al. (1998) also used two instead of three selective nucleotides (E + 2 and M + 2) in order to generate adequate number of fragments for AFLP analysis of *C. lindemuthianum* isolates. Primer selectivity is good for primers with one or two selective nucleotides in simple genomes such as fungi, bacteria and some plants, although selectivity is still acceptable with primers having three selective nucleotides, but it is lost with addition of the fourth nucleotide (Vos et al., 1995).

Statistical analysis of AFLP data enabled the classification of *Fusarium* isolates from Egypt into 5 AFLP groups, although these groups were not genetically distinct. There was no correlation between AFLP and geographic origin of the isolates, our result in harmony with these obtained by Abd-Elsalam et al. (2002a) and Kiprop et al. (2002). The similarity matrices generated by each of four primer pairs were highly correlated and were combined to determine the genetic relationships among the *Fusarium* species and isolates. Genetic similarity was detected among and within *Fusarium* spp. More than 30% similarity was found between 46 isolates of five *Fusarium* spp, and there was more than 85% similarity between *F. solani* and *F. moniliforme*.

AFLP analysis will be useful in the identification of genetic diversity and analysis of population structure within complex genera such as Fusaria. Leissner et al. (1997) has employed AFLP fingerprinting methodology to study 18 different *Fusarium graminearum* strains. Fifteen of the 18 strains showed a high degree of similarity in banding patterns. The banding patterns of the remaining three strains completely differed from the *F. graminearum* pattern found. Of these strains, one was revealed as *F. cerealis* by comparison of its pattern against an AFLP database of different *Fusarium* species. O'Neill et al. (1998) also found that there was less than 70% similarity between *F. udum* and *F. oxysporum* formae species pathogenic to coca, cowpea, and tomato. In our PCA analysis, more than 70% of the variation in the estimates of genetic similarity was explained by the first three components, indicating the suitability of the AFLP approach for genetic clustering. Use of the AFLP fingerprinting method resulted in a high degree of discrimination and identification of *Fusarium* spp. isolates and was found to be useful and practical.

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REFRENCES

Abd-Elsalam KA, Khalil MS, Aly AA, Asran-Amal A (2002a). Genetic diversity among *Fusarium oxysporum* f. sp. *vasinfectum* isolates revealed by UP-PCR and AFLP markers. Phytopathol. Mediterr. 41:1-7.

Abd-Elsalam KA, Schnieder F, Verreet JA (2002b). Population analysis of *Fusarium* spp. Phytomedizin 3:18-19.

Aly, AA, Hussein EM, Mostafa MA, Ismail AI (1996). Distribution, identification, and pathogenicity of *Fusarium* spp. isolated from some Egyptian cottons. Menofiya J. Agric. Res. 4: 819-836.

Colyer PD (1988). Frequency and pathogenicity of *Fusarium* spp. associated with seeding diseases of cotton in Louisiana. Plant. Dis. 72: 400-402.

Creste S, Tulmann-Neto A, Figueira A, (2001). Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. Plant Mol. Biol. Reptr. 19: 1–8.

El-Samawaty MA (1999). Studies on cotton root rot disease. MS.c. Thesis. Assiut Univ., Egypt.

Gonzalez M, Rodriguez MEZ, Jacabo JL, Hernandez F, Acosta J, Martinez O, Simpson J (1998). Characterization of Mexican isolates of *Colletotrichum lindemuthianum* by using differential cultivars and molecular markers. Phytopathology 88: 292–299.

Huys G, Coopman R, Janssen P, Kersters K (1996). High resolution genotypic analysis of the genus *Aeromonas* by AFLP fingerprinting. Int. J. Syst. Bacteriol. 46:572-580.

Janssen P, Coopman R, Huys G, Swings J, Bleeker H, Vos P, Zabeau M, Kersters K (1996). Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. Microbiology 142: 1881–1893.

Johnson LF, Baird DD, Chambers AY, Shamiyeh NB (1978). Fungi associated with postemergence seedling disease of cotton in the soils. Phytopathology 68: 917-920.

Kiprop EK, Baudoin JP, Mwang'ombe AW, Kimani PM, Mergeai G, Maquet A (2002). Characterization of Kenyan isolates of *Fusarium udum* from Pigeonpea [*Cajanus cajan* (L.) Millsp.] by cultural characteristics, aggressiveness and AFLP analysis J. Phytopathol. 150: 517-527.

Leissner CEW, Niessen ML Vogel RF (1997). Use of the AFLP technique for the identification and discrimination of *Fusarium graminearum*. Cereal Res. Comm. 25: 555-556.

Liu D, Coloe S, Baird R, Pedersen J (2000). Rapid mini-preparation of fungal DNA for PCR. J. Clinic. Microbiol. 38: 471.

Liu F, Sun GL, Salomon B, Bothmer von R (2001). Distribution of allozymic alleles and genetic diversity in the American Barley Core Collection. Theor. Appl. Genet. 102:606-615.

Majer D, Mithen R, Lewis BG, Vos P, Oliver RP (1996). The use of AFLP fingerprinting for the detection of genetic variation in fungi. Mycol. Res. 100: 1107–1111.

Majer D, Lewis BG, Mithen R, (1998). Genetic variation among field isolates of *Pyrenopeziza brassicae*. Plant Pathol. 47:22-28.

O'neill NR, Bailey BA, Van-Berkum PB (1998). AFLP, a novel PCR-based DNA analysis technique, reveals level of genomic variation within species of *Colletotrichum, Fusarium* and *Dendryphion*.7th International Congress of Plant Pathology, held in Edinburgh, Scotland from 9-16 August 1998.

Restrepo S, Duque M, Tohme J, Verdier V (1999). AFLP fingerprinting: an efficient technique for detecting genetic variation of *Xanthomonas axonopodis* pv. *Manihotis*. Microbiology 145: 107–114.

Roy, KW, Bourland FM (1982). Epidemiological and relationships in cotton seedling disease in Mississippi. Phytopathology 72:868-872.

Sivaramakrishnan S, Kannan S, Singh SD (2002). Genetic variability of *Fusarium* wilt pathogen isolates of chickpea (*Cicer arietinum* L.) assessed by molecular markers. Mycopathologia 155: 171-178.

Sneath, P, and Sokal R (1973). Numerical Taxonomy. Freeman, San Fransisco.

Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995). AFLP: a new technique for DNA fingerprinting. Nucl. Acids Res. 23: 4407-4414.