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# Characterization of Kenyan Isolates of *Fusarium udum* from Pigeonpea [*Cajanus cajan* (L.) Millsp.] by Cultural Characteristics, Aggressiveness and AFLP Analysis

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With 3 figures

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## Abstract

Isolates of *Fusarium udum* from pigeonpea (*Cajanus cajan*) plants with wilt symptoms were collected from various districts in Kenya and were characterized using cultural characteristics, aggressiveness and amplified fragment length polymorphism (AFLP). The 56 isolates of *F. udum* showed a high level of variability in aerial mycelia growth, pigmentation and radial mycelia growth (colony diameter) on potato dextrose agar. The aggressiveness of 17 isolates of *F. udum* on seven pigeonpea varieties varied and five aggressive groups were observed in the present study. There were no relationships among cultural characteristics and aggressiveness. AFLP analysis of the 56 isolates was tested for genetic variability using seven primer combinations. A total of 326 fragments was generated of which 121 were polymorphic. Ten AFLP groups were identified among the Kenyan isolates and, although they were not genetically distinct, six AFLP subgroups were genetically distinct. AFLP had no relationship with cultural characteristics, aggressiveness and geographical origin of the isolates. This is the first report on the study of genetic variability of *F. udum* using DNA analysis.

## Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is one of the most widely grown food grain legumes in the semi-arid tropics of the world (Nene and Sheila, 1990). Kenya is the world's second largest producer of pigeonpea (van der Maesen, 1983; Nene and Sheila, 1990), where it is the most important pulse crop after common bean (*Phaseolus vulgaris* L.). Fusarium wilt, caused by *Fusarium udum* Butler, is the most important disease of pigeonpea and one of the major causes of low yields (Sen Gupta, 1974; Kannaiyan et al., 1984). The disease has been reported from 15 countries (Nene et al., 1989), but it is apparently more important in India and Eastern Africa. Kiprop (2001) reported wilt incidence of 14.3% in Kenya. The annual loss caused by this disease in Kenya, Malawi and Tanzania was estimated at over US$5 million (Kannaiyan et al., 1984).

*Fusarium udum* isolates from the same site or diverse geographical origins have been shown to exhibit high variability in cultural characteristics (Jeswani et al., 1977; Reddy and Chaudhary, 1985; Gaur and Sharma, 1989) and virulence or pathogenicity on pigeonpea genotypes (Baldev and Amin, 1974; Shit and Sen Gupta, 1978; Gaur and Sharma, 1989; Reddy and Raju, 1993). Studies of genetic diversity using isozyme markers have revealed low variation in *F. udum* isolates (Shit and Sen Gupta, 1980; Okiror, 1986). However, molecular markers such as amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD) have not been used previously to determine the genetic variability of this economically important fungus. The AFLP technique is a recently developed molecular marker that utilizes the reliability of RFLP technique combined with the power of the polymerase chain reaction (PCR) (Lin and Kuo, 1995; Vos et al., 1995). AFLP is a DNA fingerprinting technique that detects genomic restriction fragments and resembles RFLP in this respect, the major difference being that PCR amplification, instead of Southern hybridization is used for detecting fragments. It has been used to detect genetic variation between and within species of fungi (Majer et al., 1996) and to group into haplotype/pathotype isolates of plant pathogenic fungi

 

such as *Colletotrichum lindemuthianum* (Gonzalez et al., 1998) and *Pyrenopeziza brassicae*(Majer et al., 1998). The advantages of AFLP fingerprinting over the other molecular markers (RFLP or RAPD) are that variability is assessed at a larger number of independent loci; AFLP markers are 'neutral' (i.e. they are not subject to natural selection); variation is revealed in any part of the genome; the data are obtained very quickly; and are extremely reproducible (Vos et al., 1995; Janssen et al., 1996; Majer et al., 1996). The main disadvantage of AFLP markers is that alleles are not easily recognized (Majer et al., 1996).

Fusarium wilt is widely distributed in pigeonpea growing districts in Kenya. Twelve isolates of *F. udum* from three districts in Kenya showed high variation in cultural characteristics and virulence on different pigeonpea varieties and low variation in protein profiles (Okiror, 1986). The use of genetic markers such as AFLP analysis to characterize *F. udum* strains could greatly help in the understanding of the variability within this fungus and could help pigeonpea improvement programmes aimed at breeding for wilt resistance. In view of the economic importance of wilt as a constraint to increased yields and the fact that little has been reported on genetic variability of *F. udum*, we aimed to group single-spore isolates from Kenya by cultural characteristics, aggressiveness on different pigeonpea varieties and AFLP analysis and to determine if relationship exists between these characteristics.

## Materials and Methods

### Fungal isolates

Fifty-five single-spore isolates of *F. udum* were obtained from the major pigeonpea growing districts in Kenya during a fusarium wilt survey in 1997 (Table 1). A single-spore isolate of a strain of *F. udum* from Malawi (IMI number 275452) was obtained from the International Mycological Institute, UK. The stem portions of plants showing symptoms of fusarium wilt were collected from a total of 42 pigeonpea farms (sites) in 11 districts. Small pieces (0.5 $cm^2$) of vascular tissue were cut and placed aseptically onto plates containing potato dextrose agar (PDA). The plates were incubated at 25 °C in a 12-h light/dark cycle for 36–48 h and colonies showing growth and morphology typical of *F. udum* (Booth, 1978; Gerlach and Nirenberg, 1982) were transferred onto fresh PDA. The plates were incubated as described above until conidia were produced. Conidial suspension from the cultures was prepared and streaked onto plates with tap water agar and single germinating conidia were transferred to PDA after 24–36 h and maintained as single-spore isolates.

### Cultural characteristics

A disc of 6 mm in diameter was cut using a cork borer from 7-day-old single-spore cultures from PDA and transferred onto the centre of fresh PDA plates. Each plate had one culture disc and there were three replications for each of the 56 isolates. The plates were incubated at 25°C in a 12-h light/dark cycle for 8 days. The cultures from these plates were used to determine the nature of aerial mycelium growth, pigments produced on the mycelium and the substrate and radial mycelia growth (colony diameter). The nature of aerial mycelium was determined by visual observation while pigmentation on the mycelium and the substrate determined with the help of a mycological colour chart (Rayner, 1970). The colony diameter of each culture in the plate was measured using a Vernier calliper by taking an average of four radial measurements. The experiments were repeated once.

### Aggressiveness on selected pigeonpea varieties

Based on their geographical origin and cultural characteristics (Table 2), 17 single-spore isolates of *F. udum* were selected for this experiment. Fusarium wilt resistant pigeonpea varieties ICP 8863, ICP 8858, ICP 9174, C-11, ICPL 87105 and ICEAP 00040 and a wilt susceptible variety, KAT 60/8, were obtained from the ICRISAT Centres in Hyderabad (India) and Nairobi (Kenya). Inocula of the isolates were prepared by rinsing the surfaces of 7-day-old cultures on PDA with 100 ml sterile distilled water (SDW). The conidial suspension was filtered through two layers of cheesecloth and its concentrations adjusted to $1.0 \times 10^6$ conidia/ml using a haemocytometer. Seven-day-old pigeonpea seedlings of the seven varieties pregerminated on sterile riverbed sand were uprooted carefully, the roots washed with SDW and trimmed to *c.* 4 cm from the collar region using a sterile scalpel. The seedlings were then dipped into a 500-ml glass beaker containing the inoculum and left for 30 min before transplanting them onto 20 cm diameter plastic pots containing a sterile mixture of red soil (Vertisol) and riverbed sand (3:1 v/v). Five inoculated seedlings were planted per pot and each isolate had three replications. Control seedlings were dipped in SDW. The plants were kept in a glasshouse whose prevailing maximum temperature was 23–30°C with 12 h photoperiod.

Records on percentage of wilted seedlings due to *F. udum* were taken 6 weeks after root-dip inoculation. The disease scale of 0–100% was used where 0–10% wilt indicated resistant plants, 11–20% moderately resistant plants, 21–30% moderately susceptible plants, 31–60% susceptible plants and 61–100% highly susceptible plants (Reddy and Raju, 1997). Some comparisons were made by arbitrary grouping of isolates considered to have similar aggressiveness: those with 21–100% disease incidence were described as more aggressive, while those with 0–20% disease incidence were described as less aggressive. The experiment was performed two times.

### DNA extraction

DNA was extracted from 56 single-spore isolates of *F. udum*. Erlenmeyer flasks (500 ml) containing 200 ml of Czapek Dox (Oxoid) liquid medium (Coddington and Gould, 1992) were inoculated with one agar block of 5 $mm^3$ excised from the margins of 7-day-old colonies on PDA medium. The cultures were placed on a rotary shaker at 120 rpm and incubated in the dark at

Table 1
Cultural characteristics of *Fusarium udum* isolates

| Isolate | Site[1] | Aerial mycelium growth | Mycelial colour | Substrate colour | Colony diameter (mm) |
|---|---|---|---|---|---|
| MK01 | Makueni 1 | Moderately luxuriant, fibrous | Buff | Buff | 81.3 |
| MK02 | Makueni 2 | Scanty, fibrous | Dark purple | Purple | 81.9 |
| MK03 | Makueni 3 | Moderately luxuriant, fibrous | Buff | Buff | 81.2 |
| MK05 | Makueni 5 | Scanty, fibrous | Buff | Pale luteous | 80.7 |
| MK08 | Makueni 11 | Moderately luxuriant, fluffy | Dark purple | Purple | 77.3 |
| MK10 | Makueni 12 | Luxuriant, fluffy | White | Lilac | 84.2 |
| MS01 | Machakos 13 | Moderately luxuriant, fluffy | Purple | Lilac | 80.8 |
| MS03 | Machakos 14 | Scanty, fibrous | Lilac | Lilac | 80.8 |
| MS04 | Machakos 14 | Scanty, fibrous | Dark purple | Purple | 80.4 |
| MS05 | Machakos 15 | Moderately luxuriant, fluffy | Purple | Purple | 77.3 |
| MS06 | Machakos 16 | Luxuriant, fluffy | White | Lilac | 79.8 |
| MS07 | Machakos 17 | Scanty, fibrous | Dark purple | Dark purple | 77.5 |
| MS08 | Machakos 17 | Moderately luxuriant, fluffy | White | Buff | 79.5 |
| MS09 | Machakos 17 | Moderately luxuriant, fluffy | Dark purple | Mauve | 79.5 |
| MS10 | Machakos 18 | Moderately luxuriant, fibrous | Buff | Buff | 81.4 |
| KT01 | Kitui 19 | Luxuriant, fluffy | White | Buff | 80.2 |
| KT02 | Kitui 19 | Scanty, fluffy | Dark purple | Purple | 78.7 |
| KT03 | Kitui 20 | Scanty, fibrous | Buff | Pale luteous | 82.9 |
| MB01 | Mbeere 24 | Scanty, fibrous | Purple | Purple | 82.6 |
| MB03 | Mbeere 26 | Moderately luxuriant, fibrous | Rose | Lilac | 78.9 |
| MB04 | Mbeere 27 | Scanty, fibrous | Buff | Purple | 78.3 |
| MB05 | Mbeere 27 | Scanty, fibrous | Dark purple | Purple | 72.7 |
| MB07 | Mbeere 28 | Moderately luxuriant, fibrous | Buff | Lilac | 80.9 |
| MR02 | Meru 34 | Moderately luxuriant, fibrous | Buff | Lilac | 82.6 |
| MR03 | Meru 35 | Moderately luxuriant, fibrous | Flax blue | Purple | 83.9 |
| MR04 | Meru 37 | Luxuriant, fluffy | Rose | Purple | 79.5 |
| MR05 | Meru 38 | Luxuriant, fluffy | Purple | Lilac | 83.0 |
| MR06 | Meru 38 | Moderately luxuriant, fibrous | Buff | Purple | 84.3 |
| MR07 | Meru 39 | Moderately luxuriant, fibrous | Flax blue | Dark purple | 84.9 |
| NY01 | Nyambene 40 | Luxuriant, fibrous | White | Luteous | 84.2 |
| NY02 | Nyambene 41 | Luxuriant, fluffy | Buff | Vinaceous buff | 84.3 |
| NY03 | Nyambene 41 | Luxuriant, fluffy | Buff | Pale luteous | 85.7 |
| NY04 | Nyambene 42 | Scanty, fibrous | Buff | Lilac | 82.7 |
| NY07 | Nyambene 44 | Scanty, fibrous | Purple | Purple | 78.4 |
| NY08 | Nyambene 44 | Scanty, fibrous | Purple | Purple | 81.6 |
| TN03 | Tharaka-Nithi 48 | Moderately luxuriant, fluffy | White | Lilac | 81.5 |
| TN04 | Tharaka-Nithi 50 | Scanty, fibrous | Glaucos sky blue | Purple | 85.4 |
| TN05 | Tharaka-Nithi 51 | Luxuriant, fluffy | Purple | Purple | 76.6 |
| TT01 | Taita-Taveta 55 | Moderately luxuriant, fibrous | Luteous | Pale luteous | 78.6 |
| TT02 | Taita-Taveta 56 | Moderately luxuriant, fibrous | Buff | Lilac | 81.5 |
| TT05 | Taita-Taveta 58 | Luxuriant, fluffy | Purple | Luteous | 81.8 |
| TT06 | Taita-Taveta 59 | Scanty, fibrous | Buff | Luteous | 82.5 |
| TT08 | Taita-Taveta 63 | Moderately luxuriant, fluffy | Purple | Purple | 71.4 |
| KR01 | Kirinyaga 75 | Scanty, fibrous | Buff | Pale luteous | 83.5 |
| KR02 | Kirinyaga 76 | Scanty, fibrous | Rose | Pale luteous | 84.6 |
| KR03 | Kirinyaga 76 | Scanty, fibrous | Lilac | Lilac | 81.9 |
| TK02 | Thika 77 | Luxuriant, fluffy | Dark purple | Lilac | 87.7 |
| TK03 | Thika 78 | Luxuriant, fluffy | Dark purple | Purple | 88.5 |
| TK04 | Thika 79 | Luxuriant, fibrous | Purple | Lilac | 85.4 |
| TK05 | Thika 79 | Moderately luxuriant, fluffy | Lilac | Purple | 84.3 |
| TK06 | Thika 79 | Luxuriant, fluffy | Lilac | Lilac | 85.7 |
| NB01 | Nairobi 83 | Luxuriant, fluffy | Mauve | Purple | 83.9 |
| NB02 | Nairobi 83 | Moderately luxuriant, fluffy | White | Pale luteous | 82.6 |
| NB03 | Nairobi 84 | Luxuriant, fluffy | White | Pale luteous | 81.2 |
| NB04 | Nairobi 84 | Moderately luxuriant, fluffy | Rose | Purple | 73.6 |
| MAL01a | Malawi | Luxuriant, fluffy | Purple | Purple | 80.1 |
| Mean | | | | | 81.4 |
| LSD (P = 0.05) | | | | | 1.0 |

[1]All the *Fusarium udum* isolates were from Kenyan districts, except one from Malawi (unknown field site). Figures represent the pigeonpea farm (site) number.

23–25°C for 7 days. Mycelia were harvested by filtration through two layers of cheesecloth. Samples were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. DNA extraction was performed according to the CTAB (Cetyltrimethylammonium Bromide) method (Doyle and Doyle, 1990). After the digestion of RNA with 10 $\mu$l of 20 $\mu$g/$\mu$l RNase A (Roche Diagnostics Gmbh, Mannheim, Germany), the final DNA pellets were dissolved in 100 $\mu$l TE buffer and stored at 20°C. The DNA was quantified visually by running 3 $\mu$l of each sample on a 1% agarose gel along with undigested $\lambda$ DNA (Roche

Table 2
Aggressiveness[1] (% wilt incidence) of 17 isolates of *Fusarium udum* on seven pigeonpea varieties

| Isolate | Pigeonpea varieties | | | | | | | Mean | LSD ($P < 5\%$) |
|---|---|---|---|---|---|---|---|---|---|
| | KAT 60/8 | ICPL 87105 | ICP 8863 | C-11 | ICP 8858 | ICP 9174 | ICEAP 00040 | | |
| MK02 | 86.7 | 33.3 | 6.7 | 26.7 | 0.0 | 6.7 | 13.3 | 24.8 | 26.5 |
| MK10 | 100.0 | 13.3 | 6.7 | 13.3 | 20.0 | 6.7 | 13.3 | 24.8 | 21.6 |
| MS04 | 93.3 | 53.3 | 0.0 | 13.3 | 0.0 | 6.7 | 26.7 | 27.6 | 17.1 |
| MS10 | 73.3 | 13.3 | 20.0 | 20.0 | 20.0 | 13.3 | 0.0 | 22.8 | 22.9 |
| MB05 | 80.0 | 40.0 | 0.0 | 26.7 | 0.0 | 20.0 | 13.3 | 25.7 | 17.1 |
| KR03 | 60.0 | 13.3 | 13.3 | 20.0 | 6.7 | 0.0 | 0.0 | 16.2 | 22.9 |
| TT02 | 60.0 | 20.0 | 13.3 | 6.7 | 0.0 | 20.0 | 6.7 | 18.1 | 22.9 |
| TT08 | 100.0 | 40.0 | 0.0 | 53.3 | 20.0 | 0.0 | 26.7 | 34.3 | 17.1 |
| MAL01a | 93.3 | 33.3 | 6.7 | 13.3 | 13.3 | 0.0 | 33.3 | 27.6 | 26.5 |
| MR02 | 66.7 | 26.7 | 0.0 | 20.0 | 13.3 | 13.3 | 13.3 | 21.9 | 28.6 |
| MR04 | 66.7 | 26.7 | 13.3 | 13.3 | 13.3 | 13.3 | 6.7 | 21.9 | 24.2 |
| TN05 | 53.3 | 20.0 | 6.7 | 33.3 | 0.0 | 26.7 | 13.3 | 21.9 | 21.6 |
| TK02 | 53.3 | 13.3 | 26.7 | 46.7 | 6.7 | 13.3 | 6.7 | 23.8 | 20.2 |
| NY02 | 86.7 | 13.3 | 6.7 | 26.7 | 6.7 | 13.3 | 20.0 | 24.8 | 22.9 |
| NY07 | 80.0 | 26.7 | 6.7 | 33.3 | 6.7 | 0.0 | 33.3 | 26.7 | 25.3 |
| NB01 | 86.7 | 33.3 | 13.3 | 40.0 | 6.7 | 0.0. | 13.3 | 27.6 | 17.1 |
| NB03 | 86.7 | 20.0 | 6.7 | 33.3 | 6.7 | 26.7 | 26.7 | 29.5 | 26.5 |
| Mean | 78.0 | 25.9 | 8.6 | 25.9 | 8.2 | 10.6 | 15.7 | 24.7 | 5.1 |
| LSD ($P < 5\%$) | 22.3 | 26.7 | 18.0 | 23.2 | 19.7 | 16.8 | 22.3 | 7.9 | |

[1]Aggressiveness: less aggressive = 0–20% wilt incidence and more aggressive = 21–100% wilt incidence using a disease scale of 0–100% (Reddy and Raju, 1997).

Diagnostics) of a known concentration (20 ng/$\mu$l) and making comparisons of their relative fluorescence in the presence of UV light and 0.1 $\mu$l/ml ethidium bromide solution.

**AFLP analysis**

AFLP assays were performed with the AFLP Analysis System II of GibcoBRL (Life Technologies, 1995) following the manufacturer's instructions and as described by Lin and Kuo (1995) and Vos et al. (1995). Genomic DNA was digested by restriction endonucleases, ligated to *Eco*R I and *Mse* I adapters and amplified by PCR, using primers that contain the common sequences of the adapters and *Eco*R I + 2 and *Mse* I + 3 nucleotides as selective sequences. Primary template DNA was prepared in a one-step restriction-ligation reaction and the preamplification reaction was performed in a GeneAmp PCR system 9600 (Perkin-Elmer, Cetus, CT, USA) using the pre-amp mix provided in the kit at a temperature profile of 94°C for 30 s, 56°C for 60 s and 72°C for 60 s, as described in the instruction manual. The *Eco*R I + 2 primers used in the selective AFLP amplification was radioactively labelled with [$\gamma$-$^{32}$P]ATP (Amersham International plc, Buckinghamshire, UK) by using T4 kinase. Selective amplification was performed with 5 $\mu$l of the diluted preamplified fragments, $^{32}$P-labelled *Eco*R I + 2 primers and unlabelled *Mse* I + 3 primers (contains dNTPs) (Table 3) and *Taq* DNA polymerase (Roche Diagnostics). The PCR amplification temperature profile was one cycle at 94°C for 30 s, 56°C for 30 s and 72°C for 60 s and then the annealing temperature was lowered during each cycle by 0.7°C for 12 cycles to give a touch down phase of 13 cycles. The remainder of the amplification was 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s.

After completion of the cycle programme, an equal volume (20 $\mu$l) of formamide dye [98% (w/v) formamide, 10 mM EDTA, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol] was added to each reaction mixture. Prior to gel loading and electrophoresis, mixtures were heated for 3 min at 95°C and then rapidly cooled on ice to prevent nucleic acid secondary structures from reannealing. The amplified fragments were analysed on 6% denaturing polyacrylamide sequencing gels. Autoradiographs were obtained by exposing Kodak Biomax MR film (Kodak Scientific Imaging Film, Eastman Kodak Co., NY, USA) to the dried gel in an exposure cassette for 15–17 h at room temperature.

**Data analyses**

The data on colony diameter on PDA medium and fusarium wilt incidence on pigeonpea varieties of *F. udum* isolates were analysed by ANOVA procedure using the SAS system computer package release 6.12 (SAS Institute Inc., Cary, NC, USA). Seventeen isolates of *F. udum* were classified based on aggressiveness with five pigeonpea varieties (ICP 8863, ICP 9174, ICEAP 00040, ICPL 87105 and C-11) by a distance dendrogram that was produced using the average method of the simple matching distance measure from SYSTAT version 8.0 (SPSS Inc., Chicago, IL, USA). Bands observed by AFLP analysis were assigned a number in relation to their migration within the gel. The band with the highest molecular weight was assigned number 1 and so on in ascending order until the band of the lowest molecular weight was assigned. For each individual, the presence or absence of each band was determined and designated 1, present or 0, absent in order to obtain binary banding data. Binary banding data from seven primer combinations were

Table 3
Primers, amplified bands and polymorphic bands obtained from 56 isolates of *Fusarium udum* using AFLP analysis

| Primer combination (*Eco*R I/*Mse* I) | Amplified bands | Polymorphic bands | Polymorphism (%) |
|---|---|---|---|
| TA/CAT | 30 | 20 | 67 |
| AA/CAG | 72 | 28 | 39 |
| AG/CAA | 46 | 17 | 37 |
| AG/CAG | 45 | 11 | 24 |
| AC/CAG | 55 | 15 | 27 |
| TG/CAC | 50 | 22 | 44 |
| AG/CAC | 28 | 8 | 29 |
| Total | 326 | 121 | 37 |

combined in order to determine the relationships among the *F. udum* isolates by evaluating their genetic distances. The proportion of shared bands between each pair of isolates was determined as $Dx = 2Nxy/(Nx + Ny)$, where $Nxy$ is the number of bands shared between a pair of isolates and $Nx$ and $Ny$ are the number of bands in isolate $x$ and $y$, respectively (Nei and Li, 1979). The matrix of pairwise $(1 - Dx)$ values, that is, the proportion of bands that are not shared, was generated with RFLPfrag (X. Vekemans, ULB, Belgium). The phylogenetic relationship among the isolates was constructed by the UPGMA method using procedure NEIGHBOR from the PHYLIP program (Felsenstein, University of Washington, Washington, DC, USA). One thousand sets of bootstraps were performed by sampling, with replacement, individual bands from the original data set, computing a new matrix $(1 - Dx)$ for each bootstrap, constructing trees as described above and summarizing the results using the procedure CONSENSE from PHYLIP. The phylogenetic tree was drawn using the TREEVIEW program.

## Results

### Cultural characteristics

The *F. udum* isolates exhibited high variability in cultural characteristics on PDA (Table 1). The growth of aerial mycelium was luxuriant, moderately luxuriant and scanty with 30.4, 37.5 and 32.1% isolates, respectively. Nine and eight different colours were observed on the mycelium and substrate, respectively. However, the dominant colour was the purple group (lilac, purple, dark purple) on both the mycelium and the substrate with 42.9 and 67.9% isolates, respectively. The yellow group (buff, vinaceous buff, pale luteous, luteous) appeared to be the second dominant pigment. White colour on the mycelium was observed on 14.3% isolates while pink (rose, mauve) and light blue (flax blue, glaucous sky blue) were the least produced pigments on both the mycelium and the substrate. There were significant differences ($P < 0.05$) among the isolates in radial mycelium growth (colony diameter). The minimum and maximum radial mycelium growths were 71.4 and 88.5 mm in diameter on TT08 and TK03 isolates, respectively, with a mean of 81.4 mm. For comparative purposes, the radial mycelium growth of the isolates was grouped into fast growth (≥84.0 mm diameter), moderate growth (78.0–83.9 mm diameter) and slow growth (<78.0 mm diameter). The isolates with fast, moderate and slow growths were 23.2, 64.3 and 12.5%, respectively. Isolates that had a similar character, for example luxuriant mycelium growth, varied in other cultural characteristics such as pigmentation on mycelium and substrate or radial mycelium growth.

The aerial mycelium growth, pigmentation on mycelium and substrate and radial mycelium growth of *F. udum* isolates generally varied within and between regions (districts). Isolates from the same site showed similar or different cultural characteristics. For example, isolates with similar characters were MS03 and MS04, NY02 and NY03, and NY07 and NY08 while those with some different characters were MS07, MS08 and MS09, MB04 and MB05, MR05 and MR06, KR02 and KR03, TK04, TK05 and TK06, NB01 and NB02, and NB03 and NB04. Isolates obtained from the same pigeonpea plant that showed differences in one or more cultural characters were MS08 and MS09, and TK04 and TK05.

### Aggressiveness on different pigeonpea varieties

The aggressiveness of 17 isolates of *F. udum* to seven pigeonpea varieties differed significantly ($P < 0.05$) (Table 2). The wilt incidences varied from 0 to 100% with a mean of 24.7%. All the isolates were more aggressive to the susceptible variety KAT 60/8 and less aggressive to variety ICP 8858. Isolates showed higher variation of reaction in varieties KAT 60/8, C-11, ICPL 87105 and ICEAP 00040 while lower variation was observed in varieties ICP 8858, ICP 8863 and ICP 9174. Isolate TT08 induced the highest mean wilt incidence of 34.3% wilt while inoculation of isolates KR03 and TT02 gave the lowest mean wilt incidence of 16.2 and 18.1% wilt, respectively. Isolates TT08, NY07 and NB03 caused reaction of susceptibility (≥21% wilt) in four pigeonpea varieties whereas isolates MK10, MS10, KR03 and TT02 caused reaction of susceptibility only on variety KAT 60/8.

The 17 isolates of *F. udum* were classified into five aggressive groups based on the simple matching distance using the wilt incidence of five pigeonpea varieties (Fig. 1, Table 2). Groups I, II, III, IV and V comprised of 2, 1, 4, 6 and 4 isolates, respectively. Group IV isolates appeared less aggressive while isolates in the other four groups were more aggressive. Cultural characteristics of *F. udum* appeared to be independent of aggressiveness. No relationship between aggressiveness and geographical origin of the isolates was observed.

### AFLP analysis

A total of 326 bands were amplified from seven primer combinations, of which 121 bands (37%) were polymorphic (Table 3, Fig. 2), with an average of 17 polymorphic bands per primer combination. Polymorphism was highest among isolates with primer combination

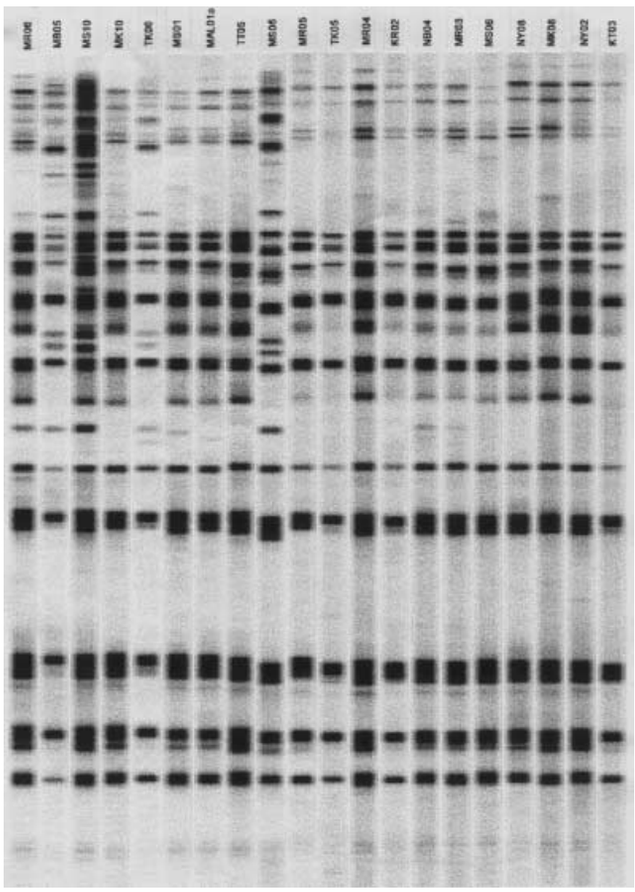

Fig. 1 Dendrogram of *Fusarium udum* isolates constructed from aggressiveness data based on average method using simple matching

*Eco*R I (E) + TA/*Mse* I (M) + CAT, followed by E + TG/M + CAC, E + AA/M + CAG and E + AG/M + CAA. The lowest polymorphism was observed among isolates with primer combinations E + AG/M + CAC, E + AC/M + CAG and E + AG/M + CAG.

Analysis of the binary banding data from all the seven primer combinations showed that isolates of *F. udum* from Kenya could be grouped into ten AFLP groups and included groups I to VIII plus isolates TT06 and NB01 as separate groups (Fig. 3). However, all the ten AFLP groups were not significantly (confidence interval of <50% at their forks) distinct. Six AFLP subgroups were genetically distinct (confidence interval of ≥50%), and includes isolates MR06, TK06, MR02, MB05, MS10 and MS05, and isolates MK01 and TK03 in AFLP group V; isolates MS03 and MS08, isolates NY07 and MB03, and isolates MB04 and KT03 in AFLP group I; and isolates NB03, MB01 and KT02 in AFLP group VIII.

AFLP groups and subgroups of *F. udum* had no relationship with cultural characteristics and aggressiveness. The isolates in each aggressive group were spread among various AFLP groups (Figs 1 and 3). No relationship between AFLP and geographical origin of the isolates was observed.

## Discussion

Single spore isolates of *F. udum* varied greatly in cultural characteristics and 56 isolates were classified into three groups by aerial mycelium growth and into three groups by radial mycelium growth (colony diameter). The isolates produced nine pigments on the mycelium and eight pigments in the substrate with a higher variation in the former. Okiror (1986) also found variation among 12 isolates of *F. udum* obtained from Machakos, Makueni and Thika districts (Kenya) in mycelia growth, pigmentation and colony diameter on PDA incubated at room temperature for 10 days. Variations in cultural characteristics on isolates of *F. udum* have also been reported elsewhere (Shit and Sen Gupta, 1978; Gupta et al., 1988 ; Gaur and Sharma, 1989). Gerlach and Nirenberg (1982) reported that the colonies of *F. udum* are fast growing, reaching 7.8–8.2 cm diameter in 8 days at 25°C on PDA, which correlates with our results.

No relationship was found among cultural characteristics of *F. udum*. Thus, the cultural characteristics of *F. udum* were not associated with a particular region or district, although isolates from Thika district produced the purple group of pigments and with fast radial mycelium growth. These phenotypic characteristics were variable even with isolates from the same site or the same plant. Single spore isolates from single strains of *F. udum* have been found to vary among themselves with regard to growth pattern, segmentation and capacity to secrete metabolic products (Upadhyay and Rai, 1992).

The differential reactions of seven pigeonpea varieties to 17 isolates of *F. udum* confirmed the variability in aggressiveness of the isolates. Gaur and Sharma (1989) have also made this observation using 18 pigeonpea varieties against seven isolates of *F. udum* from India and Okiror (1986) using six pigeonpea genotypes against 12 isolates of *F. udum* from Kenya. Similar findings using pigeonpea varieties against *F. udum* isolates have been reported elsewhere (Baldev and Amin, 1974; Shit and Sen Gupta, 1978; Gupta et al., 1988). Five aggressive groups exist in *F. udum* among the Kenyan isolates. The aggressiveness of *F. udum* isolates appeared to be independent of cultural characteristics, as reported also by Okiror (1986) and Gaur and Sharma (1989). However, Shit and Sen Gupta (1978) found that isolates of *F. udum* producing luxuriant mycelia growth were weakly to moderately pathogenic (or aggressive).

We have demonstrated that AFLP markers are useful in the study of genetic variation of *F. udum* isolates. Using seven primer combinations with *Eco*R I (E) + 2 and *Mse* I (M) + 3 selective nucleotides at the 3′-end of the primers on 56 isolates, a total of 326 bands were amplified with 121 polymorphic bands. However, the number of amplified bands and polymorphism obtained from individual primer combinations varied, with primers having more Ts and As showing higher polymorphism and generally more

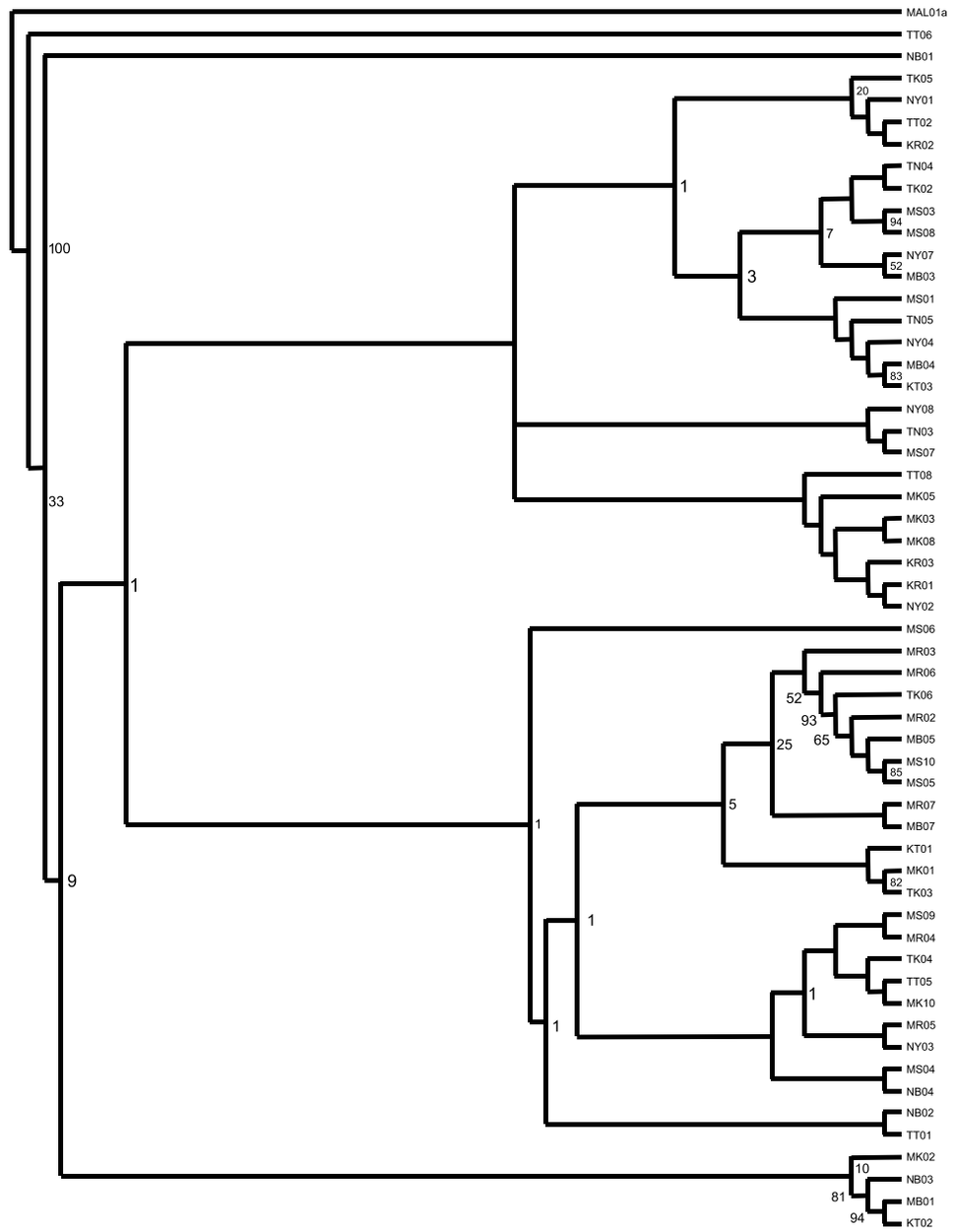


Fig. 2 Typical banding pattern of AFLP fingerprint obtained by AFLP analysis using primer combination *Eco*R I + AG/*Mse* I + CAA

bands amplified than primers having more Gs and Cs. An exception was observed with primer combination E + TA/M + CAT which showed the highest polymorphism (67%) but with the second lowest number of amplified bands (30 bands). Janssen et al. (1996) have showed that the choice of the restriction enzymes and the length and composition of the selective nucleotide will determine the complexity of the final AFLP fingerprint. The present findings are consistent with the work of Majer et al. (1996) in the AFLP analysis of pathogenic isolates of *Cladosporium fulvum* and *P. brassicae*, although they used E + 2 and M + 2 nucleotides. Gonzalez et al. (1998) also used two instead of three selective nucleotides (E + 2/M + 2) in order to generate adequate number of bands 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 the addition of the fourth nucleotide (Vos et al., 1995).

Statistical analysis of AFLP data enabled the classification of *F. udum* isolates from Kenya into 10 AFLP groups, although these groups were not genetically distinct. Six AFLP subgroups were, however, genetically distinct. There was no correlation between AFLP and geographical origin of the isolates. *F. udum* is a deuteromycete and therefore natural populations of the pathogen may consist of clonal lineages produced by asexual reproduction. Using AFLP analysis, Gonzalez et al. (1998) have classified *C. lindemuthianum* isolates from Mexico analysis into two major groups according to the type of common bean cultivar or

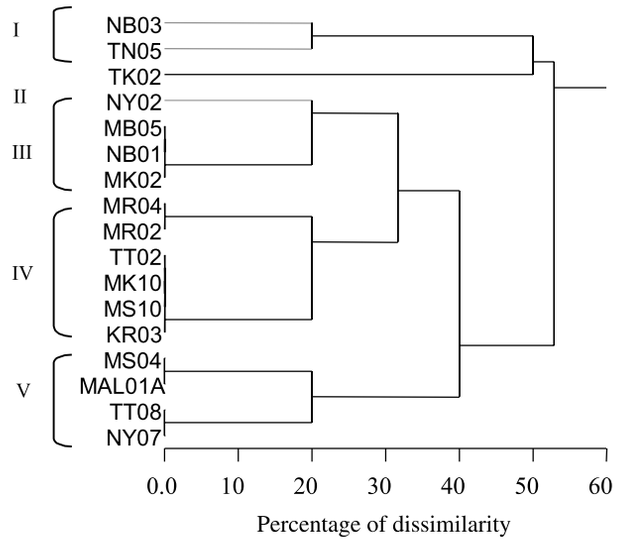


Fig. 3 Dendrogram showing genetic relationships of 56 isolates of *Fusarium udum*. The most parsimonious trees and the strict consensus tree (rooted) were produced by PHYLIP program. The numbers on the branches represent the confidence intervals generated by bootstrapping with 1000 replications

system of cultivation from which they originated and into smaller subgroups generally associated with the geographical location from which they were obtained. Koenig et al. (1997) identified 10 clonal lineages of *F. oxysporum* f.sp. *cubense* using RFLP analysis and the two largest lineages had pantropical distribution, while the minor lineages were found only in limited geographical regions. AFLP appeared independent of cultural and virulence traits. Okiror (1986) found no relationship between protein profiles of 12 isolates of *F. udum* from three districts in Kenya and their virulence.

The genome analysis of *F. udum* isolates from Kenya by AFLP has provided evidence that this pathogenic fungus varies genetically. This variability should be taken into consideration in pigeonpea improvement programmes aimed at breeding for wilt disease resistance. In order to determine the extent of genetic variation of this economically important fungus and relationships with cultural and pathogenic traits, more isolates from other countries and/or geographical origins should be assayed using AFLP analysis or other DNA based molecular techniques.

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