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GENETIC RELATEDNESS AMONG *Fusarium* SPECIES ISOLATED FROM TAITA TAVETA REGION, KENYA

[RELACION GENETICA ENTRE ESPECIES DE *FUSARIUM* AISLADAS DE LA REGION TAITA TAVETA, KENIA]

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SUMMARY

Genetic relatedness among twenty six *Fusarium* species isolated from soils in six different Land Use Types (LUTs) across a land use gradient was evaluated using Random Amplified Polymorphic DNA (RAPD) assay. The six LUTs were horticulture, maize, napier grass, fallow/shrub land, planted forests as well as indigenous forests. Six primers were used in this assay. Amplification products were examined and presence or absence of each size class of bands was scored as 1 and 0, respectively. The resulting matrix was used to compute Jaccard's similarity coefficients and Unweighted Pair Group Method with Arthmetic mean (UPGMA) cluster analysis using computer package NTSYS-pc. Jaccard's similarity coefficients ranged from 0.257 to 0.583 among the *Fusarium* species studied indicating high genetic diversity. The UPGMA cluster analysis grouped the 26 *Fusarium* species into two main clusters with the first cluster comprising of 17 species with genetic similarity values ranging from 26.2 to 58.3 %. The second cluster comprised of 9 species with genetic similarity values ranging from 29.5 to 56.34 %. *Fusarium avenaceum* and *F. nygamai* depicted the highest genetic similarity of 58.3 %.

Key words: *Fusarium* species; Genetic diversity; RAPD assay; cluster analysis

INTRODUCTION

Fusarium is one of the most ubiquitous, abundant, and important genera of soil microfungi. The genus contains many species of environmental, agricultural and human health importance (Martino *et al*., 1994; Vismer *et al*., 2002). The notoriety of this genus mainly results from pathogenicity towards a wide range of plants. RAPD analysis is a fast, PCR-based method of genetic typing based on genomic polymorphisms. The technique is highly sensitive to nucleotide differences and can assay single nucleotide differences (Williams *et al*., 1990; Welsh and McClelland, 1990). RAPD produces DNA profiles of varying complexity, depending on the primer and template used. Random markers as products of the PCR-RAPD technique (Williams *et al.,* 1990; Hadrys *et al*., 1992) have been developed to differentiate numerous fungi. This technique has been applied widely in the detection and genetic characterization of phytopathogenic fungi (Miller, 1997; Brown, 1998), including race differentiation in several formae speciales of *F. oxysporum* (Bentley *et al*., 1995; Manulis *et al*., 1994; Migheli *et al*., 1998).

RAPDs are already widely used as a diagnostic tool in many laboratories alongside, or as alternative to - Restriction Fragment Length Polymorphism (RFLP) analysis in *Fusarium* studies (Amoah *et al*., 1996). Random PCR approaches are being increasingly used to generate molecular makers, which are useful for taxonomy and for characterizing *Fusarium* populations. RAPD assay have been used extensively to define *Fusarium* populations at species, intraspecific, race, and strain levels (Sabir, 2006). Studies on genetic variations in *Fusarium* populations using RAPD technique have been documented (Nijs *et al*., 1997). Gherbawy *et al*., (2002) used RAPD technique for identification of *Fusarium subglutinans*, *F. proliferatum*, and *F. verticillioides* isolated from maize in Australia. Pasquali *et al*., (2003) characterized isolates of *F. oxysporum* pathogens on *Argyranthemum frutescens* L. using RAPD technique. Abd-Elsalam *et al*., (2003) used RAPD markers to study inter- and intra-specific variation of twelve *Fusarium* species isolated from cotton-growing areas in Egypt.

Many other workers have used RAPD-PCR as a method for analyzing genetic variation within and between *Fusarium* species (Walkers *et al*., 2001;

Khalil *et al*., 2003; Beladid *et al*., 2004). RAPD analysis has been used effectively to distinguish between species of *Fusarium* (Voigt *et al*., 1995; Yli-Mattila *et al*., 1996; Jana *et al*., 2003). RAPD analysis has also successfully delineated groups within *Fusarium* species including *F. avenaceum* (Yli-Mattila *et al*., 1996), *F. graminearum* (Quellet and Seifert, 1993) and *F. verticillioides* (Voigt *et al*., 1995).

This study explored the genetic relationship among *Fusarium* spp. isolated from soils under different land uses in Taita Taveta district, Kenya.

MATERIAL AND METHODS

Description of the study site

The study was conducted in Ngangao forest, of Taita Taveta district, Coast province in Kenya, and the adjacent farmlands. The area is predominantly under agriculture and has diverse land use cover types (LUT). The six LUTs were natural forest, planted forest, shrub/fallow, napier grass, maize and horticulture. The natural forest consists of a broad diversity of indigenous trees which include; *Strombosia scheffleri, Dicralonepis usambarica, Graibia zimmermanii, Oxyanthus speciosa, Dracaena deremensis, Rauvolvia mannii, Rytiygynia schumanii, Coffea fadenii, Psychotria taitensis, Saintpaulia taitensis,* and *Chassalia discolor*. The rare Eastern Arc type of flora and fauna found in this forest comprises several endemic or globally rare taxa (comprising of 22 plant and 3 animal species) (Newmark, 1998) and the forest is listed among the world biodiversity hotspot areas (Wass, 1995). The natural fallow/shrub consisted of mainly *Croton megalocarpus, Lantana camara, Sporobolus pyramidalis* and *Ficus thoningii*.
In the agricultural areas horticultural crops grown were cabbage (*Brassica oleraceae*), spinach (*Chenopodium spinacia*), tomato (*Solanum lycopersicum*), kale (*Brassica oleraceae* var. *acephala*) and cucumber (*Cucumis sativus*). Maize was mainly intercropped with a variety of crops including beans, potatoes, cowpeas, and bananas. Maize based farming and napier grass (*Pennisetum purpureum*) production were widely distributed in the area for food and fodder. The soils present in Taita hills were composed of high-humic A-horizons overlying pinkish acid sandy loam. The soils were classified as are haplic Acrisols, eutric Cambisols, chromic Luvisols and Regosols. The sandy loams were deep with high infiltration rates, a low pH (3 to 5), low water holding capacity and low in nutrients contents. The soils had high aluminum levels, low calcium levels, resulting in low cation exchange capacity (CEC).

Soil sampling

Soil samples were collected across the land use gradient. Allocation of sample plots within the benchmark site was done in a systematic grid which ensured better coverage of most of the land cover types thus reducing the chance of any stratum being under sampled. These sampling plots were established at fixed intervals along sample strips, which were 200 m apart. Sampling points were geo-referenced using a GPS. Sixty points were selected randomly to represent the six major LUTs, namely; horticulture, maize, fallow/shrub land, napier grass, planted forests (Pines and Cypress) and indigenous forests. Soil augers of 10 cm depth and 6 cm diameter, were used to collect soil samples. At each of the sampling point, twelve soil cores were collected from 0 to 20 cm soil depth level. The twelve sub-samples were thoroughly mixed in sterile containers to constitute a composite sample from which 500 g soil were taken and placed in sterile paper bags and labeled. The auger was sterilized by dipping it in ethanol between sampling points to avoid cross contamination. The 0 to 20 cm surface soil was chosen for mycological considerations since it contains the majority of soil microfungi (Skujins, 1984). Samples were placed cool boxes and taken to the laboratory where they were aseptically air-dried in a laminar flow hood, to prevent microbial activity, for 48 h and then stored at 5 ^{o}C in paper bags until they were processed.

Isolation and identification of *Fusarium* species

Soils were assayed using serial dilution plating (Burgess *et al*., 1988) using 0.1 % Tap Water Agar (TWA) (Brayford, 1993). *Fusarium*-selective PCNB-Peptone Agar (PPA) media were used to recover *Fusarium* isolates from the soil according to the method of Leslie and Summerell (2006). Colonies from PPA media were transferred to Synthetic Nutrient Agar (SNA) media from which very dilute inocula, of 5 to 10 spores per drop of suspension (when viewed at low power magnification), were prepared and spread on 2 % Tap Water Agar plates in order to obtain monosporic cultures. Germlings were then subcultured on different media, that is, SNA, Carnation-Leaf-Agar (CLA) and Potato-Dextrose-Agar (PDA) media plates, for growth and identification. Most of the *Fusarium* species formed sporodochia with robust, uniform macroconidia on the CLA that were used for identification (Leslie and Summerell, 2006). PDA cultures were used to assess pigmentation and gross colony morphology. Cultures grown on SNA were evaluated for microconidia which were more abundant and diverse on this medium, and for chlamydospores, which were common and produced rapidly on this medium.

All the pure isolates subcultured on PDA, CLA and SNA were incubated for ten to twenty days at 25 ^{o}C under fluorescent lamps (Sylvania cool white tubes) with a 12 hour photoperiod. *Fusaria* were identified to the species level where morphological characters were used as the basis of identification (Nelson *et al*., 1983). Identification was made according to Nelson *et al*., (1983), Burgess *et al.,* (1988), Brayford (1993) and Leslie and Summerell (2006). After identification, the single spore cultures were stored in agar slants of SNA in screw capped bottles at 4 ^{o}C and in sterilized soil in screw cap bottles.

Molecular Analysis

In order to obtain DNA from each of the identified *Fusarium* species, single-spore *Fusarium* species were grown for six days at 24 ^{o}C in 50 ml of Potato Dextrose Broth (PDB) (Difco) on a rotary shaker at 150 rpm. The mycelia were harvested from the cultures by pouring the liquid media containing fungal growth through a non-gauze milk-filter. The culture filtrate was allowed to drain briefly for 30 minutes and the filtered mycelia were washed twice with sterile distilled water, while still in the filter funnel. The washed mycelia samples were removed from the funnel with a clean sterile spatula placed on a pad of clean paper towels then and blotted dry. The mycelia samples were then freeze-dried overnight, before storing them at -20 ^{o}C.

Frozen mycelia samples of each of the identified *Fusarium* spp. were ground with mortar and pestle in liquid nitrogen. DNA from the ground mycelia were extracted according to the method described by Khalil *et al.,* (2003). To a 1.5 ml Eppendorf tube containing 500 µl of lysis buffer (400 mM Tris HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1 % Sodium Dodecyl Sulphate) a small lump of mycelia was added by using a sterile toothpick, with which the lump of mycelia was disrupted. The tube was then left at 24 ^{o}C for 10 minutes. After adding 150 µl of Potassium, the tubes were vortexed briefly and centrifuged at 13,000 X g for 1 minute. The supernatant was transferred to another 1.5 ml Eppendorf tube and centrifuged again as described above. After transferring the supernatant to a new 1.5 ml Eppendorf tube, an equal volume of isopropyl alcohol was added and the tube mixed briefly by inversion. Then the tube was centrifuged at 12,000 X g for 2 minutes and the supernatant discarded.

The resultant DNA pellet was washed in 300 µl of 70 % ethanol. The pellet was centrifuged at 10,000 X g for 1 minute and the supernatant discarded. The DNA pellet was then air-dried by inverting the tube on a tissue paper before it was dissolved in 100 µl of TE buffer. Fifteen microlitres of each sample were added to 735 µl TE and mixed well. OD_{260} and OD_{280} was read to determine the purity of the DNA using Beckmann DU-65 Spectrophotometer. The concentration of each DNA sample was adjusted with TE to 50 ng/ µl and stored at -20 ^{o}C. This procedure was followed to extract DNA from all the 26 *Fusarium* species identified.

RAPD Assessment and Primer screening

Ten different 10-mer primers (Invitrogen Corporation, San Diego, CA, USA) were screened for amplification of template DNA. The RAPD technique described by Williams *et al.*, (1990) was used as starting point for setting up a standard protocol suitable for *Fusarium* spp. Primarily, concentration of template DNA, $MgCl_2$, *Taq* polymerase and the thermal cycling profile were varied independently to define reaction conditions which generate reproducible and scorable

RAPD profiles. RAPD–PCR procedures were carried out as described by Khalil *et al*., (2003). The amplification reactions were performed in 25 µl volumes in thin-walled PCR tubes after optimization. PCR reactions were performed in 25 µl volumes in a PTC-100 (Programmable Thermal Controller), programmed for an initial cycle of 2 minutes at 94 ^{o}C, 30 seconds at 31 ^{o}C and 2 minutes at 68 ^{o}C, followed by 35 cycles of 30 seconds at 94 ^{o}C, 30 seconds at 31 ^{o}C and 2 minutes at 68 ^{o}C. There was a final extension step of 5 minutes at 68 ^{0}C followed by a cooling to 4 ^{o}C until samples were recovered.

Tubes containing all reaction components except DNA were used as negative controls. Amplicons were analyzed by electrophoresis in a 2 % Agarose gel with 1 X TBE buffer (0.09 M Tris-borate, 0.002 M EDTA, 1 M EDTA [Ph 8]), stained with Ethidium Bromide at 100 volts, constant voltage, for 2 hr. RAPD reactions were performed twice for 4 replicates of each species. The gels were slide onto a UV transilluminator and photographed with a Polaroid apparatus. A 50-bp ladder (Promega, Germany) was used as a molecular maker in determining the sizes of the amplification products. All experiments were done using the same reagents and high ddH_2O to ensure repeatability and consistent results. Aliquots of the working stocks were prepared for ach experiment and stored frozen (- 20 ^{o} C). All manipulations were carried out with dedicated DNA-free pipettes in a sterile hood to minimize risk of contamination.

In order to identify primers that generate informative arrays of PCR Products, five *Fusarium* spp. were selected from entire panel of species. Ten oligonucleotides, each 10 nucleotides long, with a G/C content of 60 to 70 % were tested. The choice of selected primers was based on the number of bands as

well as the quantity of different and reproducible patterns yielded. A combined binary matrix was generated from the molecular data with the six selected RAPD primers. Genetic similarities among *Fusarium* spp. were calculated by scoring presence or absence of each size class as 1 or 0, respectively. The resulting matrix was used to compute Jaccard's similarity coefficients (Sneath and Sokal, 1973) and UPGMA (Unweighted Pair Group Method with Arithmetic means) cluster analysis using computer package NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) version 1.80 (Rohlf, 1993).

RESULTS

Isolation of *Fusarium* species

A total of 1865 isolates classified into 26 *Fusarium* spp. were recovered (Table 1). The most frequently isolated species was *F. oxysporum* (37.9 %) followed by *F. solani* (10%). The two accounted for 47.9 % of all *Fusarium* isolates recovered and were present in six LUTs.

Table 2. Frequency of isolation of *Fusarium* spp. from different Land Use Types.

Fusarium spp.	Overall Proportion (%)
F.oxysporum	37.9
F.solani	10.0
F.sporotrichioides	7. 2
F.graminearum	5. 9
F.avenaceum	5. 7
F.poae	4. 8
F.chlamydosporum	4. 5
F.acuminatum	4. 0
F.xylarioides	3. 2
F.verticillioides	2. 5
F.polyphialidicum	2. 0
F.dlamini	2. 0
F.denticulatum	1. 8
F.nygamai	1. 3
F.lateritium	1. 2
F.torulosum	1. 0
F.phyllophilum	0. 9
F.semitectum	0. 7
F.decemcellulare	0. 7
F.nelsonii	0. 6
F.scirpi	0. 5
F.redolens	0. 4
F.heterosporum	0. 4
F.sp.	0. 3
F.beomiforme	0. 3
F.compactum	0. 2

RAPD Assessment and identification of informative primers

Template DNA concentrations from one to 100 ng had no significant effect on changing the RAPD pattern. However concentrations above 100 ng reduced the number of amplified fragments and with 1 ng of template DN, no products were formed. This finding was verified with various primers and with template DNA of different species. Furthermore, varying the annealing temperature from 28 to 38 ^{o} C had no obvious influence on the RAPD patterns. In contrast, only a narrow range of $MgCl_2$ concentration (2 to 4 Mm) resulted in constantly reproducible patterns. The established protocol (Table 2) was then utilized to generate informative RAPD patterns.

Out of the 10 primers six were selected since they produced bands highly reproducible and consistently well amplified (Table 3).

Table 2. The protocol used for PCR-reactions.

Component	Stock solution	Volume	Final concentration
ddH_2O		14. 8 µl	
dNTP mix	10 mM	2.0 µl	0. 8 mM
Taq Buffer	10 X	2. 5 µl	1 X
$MgCl_2$	25 mM	2. 5 µl	2. 5 mM
Taq Polymerase	5 U/ µl	0. 2 µl	1 U
Primer	10 µM	0. 5 µl	0.2 µM
DNA	50 ng/µl	2. 5 µl	5 ng

Table 3. Sequences of oligonucleotide primers used for RAPD analysis.

Primer Code	5'-Sequence-3'	(G +C) %
FS01/06	GTGACGTAGG	60
FS02/06	TGCCGAGCTG	70
FS04/06	AATCGGGCTG	60
FS05/06	AGGGGTCTTG	60
FS06/06	GGTCCCTGAC	70
FS08/06	CAGGCCTTCA	60

Genetic relationship among *Fusarium* spp.

The six primers generated 702 scorable bands. The size of the DNA fragments ranged from 230 to 2,950 bp and the number of markers generated by each primer ranged from 83 for primer FS06/06 to 146 for primer FS04/06. Figure 1 indicates the banding pattern produced by primer FS 08/06. Jaccard's similarity coefficients ranged from 0.257 to 0.583 among the *Fusarium* spp. indicating a high genetic diversity

(Figure 2). The UPGMA cluster analysis grouped *Fusarium* species into two main clusters with 25.7 % genetic similarity. The first main cluster comprised of seventeen species with genetic similarity ranging from 26.2 – 58.3 %. The species in this cluster are; *F. oxysporum, F. beomiforme, F. semitectum, F. scirpi, F. avenaceum, F. nygamai, F. poae, F. redolens, F. verticillioides, F. graminearum, F. heterosporum, F. chlamydosporum, F. sporotrichioides, F. decemcellulare, F. compactum, F. sp.,* and *F. torulosum.* The cluster was regrouped into two sub clusters with the first sub cluster comprised of twelve species with genetic similarity values ranging from 32.83 to 58.3 %. The second sub cluster having five species with genetic similarity values ranging from 38.53 to 45.89 %. *Fusarium avenaceum* and *F. nygamai* depicted the highest genetic similarity of 58.3 %, followed by *F. verticillioides* and *F. graminearum* with a genetic similarity value of 57.3 %.

The second major cluster comprised of nine species namely; *F. acuminatum, F. dlamini, F. nelsonii, F. xylarioides, F. denticulatum, F. lateritium F. polyphialidicum, F. solani* and *F. phyllophilum* and with genetic similarity values ranging from 29.5 to 56.34 %. This cluster was further separated into two sub clusters at 26.2 % genetic similarity. The first sub cluster comprised of six species with genetic similarity range of 31.52 to 56.34 %. The second sub cluster consisted of three species with a genetic similarity value ranging from 30.67 to 34.13 %.

The dendrogram showed that there was low correlation between some the clusters of *Fusarium* species and LUT. For example, species present in all the LUTs (*F. polyphialidicum, F. solani* and *F. phyllophilum*) were placed in same sub cluster (Figure 2). On the other hand, *F. sporotrichioides, F. decemcellulare, F. compactum, F. torulosum* and *F.* sp. isolated from all the LUTs except soils under napier grass were clustered in the same region. The dendrogram show low correlations between maize and horticulture LUTs.

DISCUSSION

Occurrence of *Fusarium* spp. present in the soil was influenced by land use type. These findings were consistent with those of Wakelin *et al*., (2008) who demonstrated the effect of plant type on soil *Fusarium*. Cultivated soils which are highly disturbed, such as those under horticulture and maize based LUTs recorded the highest fungi abundance. The effect of the crops was a major factor which influenced *Fusarium* abundance in the soils. The low occurrence of *Fusarium* species in indigenous forest soils could be associated with suppression by the saprophytic fungi present or that the fungus alternatively lack susceptible host in this ecosystems.

This study has also showed that the method of Khalil *et al.,* (2003) was adequately efficient for use in extraction of DNA from *Fusarium* spp. The results demonstrate that 2 % Agarose gel gave a satisfactory resolution of PCR amplification products. RAPDs were already widely used as diagnostic tools in many laboratories along side or as an alternative to RFLP analysis determining genetic variability among *Fusarium* species (Walkers *et al*., 2001; Jana *et al*., 2002; Khalil *et al*., 2003; Beladid *et al*., 2004). RAPD markers had been used to study inter- and intraspecific variation of 12 species isolated from cotton growing areas in Egypt by Abd-Elsalam *et al*., (2003). Similar analysis has also been used to identify *Fusarium* spp. in Kenya (Macdonald and Chapman, 1997).

Diverse gene pool in the genus with high genetic variability existed among the recovered *Fusarium* species as revealed by RAPD markers. A low genetic similarity value for the species under study was observed (GS= 25.7 %). Clustering analysis separated the species into distinct RAPD classes. The results agree with Abd-Elsalam *et al*., (2003) who did not find a clear relationship between RAPD profiles of *Fusarium* species across the LUTs in Egypt. Sabir, (2006) used RAPD analysis on isolates of *F. sambucinum* did not find any correlation between RAPD patterns and geographical location of where the isolates were made. Similarly, Pasquali *et al*., (2003) also could not identify any correlation between geographical origin and clustering of *Fusarium oxysporum* isolates in Egypt.

Of all taxa examined *F. avenaceum* and *F. nygamai* showed the greatest genetic similarity (GS=58.3). *Fusarium xylarioides* and *F. phyllophilum* displayed very little genetic resemblance with each other although both were recovered from maize LUT. *Fusarium solani* were closest genetically to *F. polyphialidicum* with genetic similarity of 34.13 %. *Fusarium solani* was isolated from all LUTs while *F. polyphialidicum* isolates were recovered from most of LUTs. This study was similar to reports by Szecsi and Dobrovolsky (1985), Walkers *et al*., (2001), Khalil *et al*., (2003), and Beladid *et al*., (2004). However, these results contrasts the findings reported by Fiona *et al*., (2000) that found a strong genetic relationship between *F. torulosum* and *F. avenaceum.*

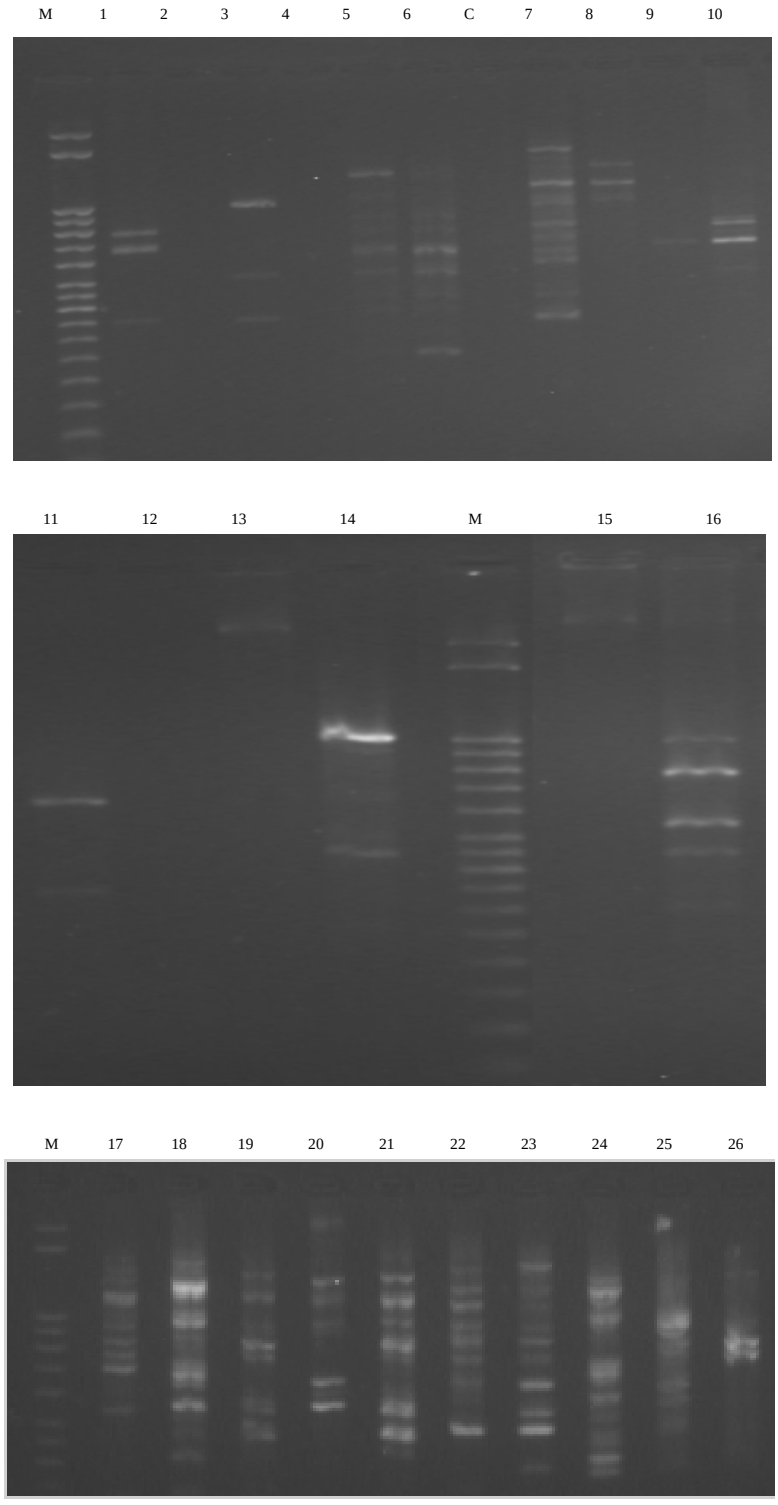

Figure. 1 Patterns of amplification products of *Fusarium* spp.after PCR with primer FS08/06 (5' CAGGCCTTCA 3'): M is the size marker, 1. *F.decemcellulare,* 2. *F.phyllophilum,* 3. *F. polyphialidicum,* 4. *F sp.,* 5. *F.acuminatum,* 6. *F.dlamini,* 7. *F.nelsonii*,8. *F.heterosporum*,9. *F.sporotrichioides,* 10. *F.semitectum,* 11. *F.solani*,12. *F.compactum*,13. *F. lateritium,* 14. *F.xylarioides*,15. *F.scirpi* 16. *F.chlamydosporum,* 17. *F.oxysporum*,18. *F. torulosum,* 19. *F.poae,* 20. *F. beomiforme,* 21. *F.verticillioides,* 22. *F.avenaceum,* 23. *F.nygamai,* 24. *F.redolens,* 25. *F.denticulatum,* and 26. *F. graminearum.*

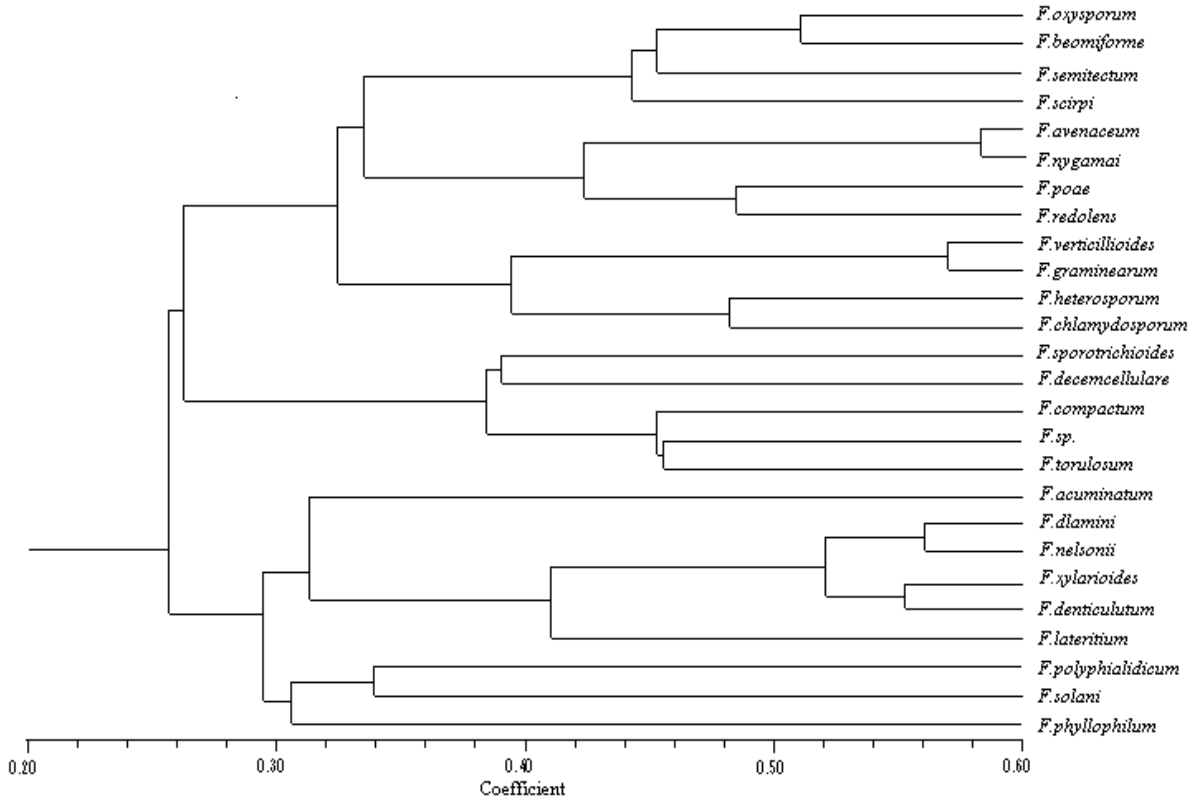

Figure 2. UPGMA dendrogram showing genetic relationships among *Fusarium* species based on Jaccard's similarity coefficients from RAPD data.

High genetic diversity may indicate sexual reproduction within species that have sexual state, for example, *F. graminearum*. Some *Fusarium* species are known to have multiple mating populations, for example, *F. lateritium* (Leslie and Summerell, 2006). Therefore, new genotypes are constantly being produced and diversity should remain high even in a limited spatial area. Bowden and Leslie (1996) hypothesized that high levels of genotypic diversity occurring in a small spatial area indicate high levels of sexual recombination. While limited difference may be explained by the occurrence of mutations, more research is needed to determine the origin and nature of the genetic variation. Nevertheless, migration and sexual recombination are likely to be contributing to *Fusarium* diversity.

CONCLUSION

There is a high genetic variability among *Fusarium* spp. isolated from Taita Taveta. RAPD-PCR analysis gives comprehensive information regarding the genetic variability among the *Fusarium* community and can be used effectively as a DNA fingerprinting technique for *Fusarium* spp.

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