Fungal Genomics & Biology
ISSN: 2165-8056
Wamalwa et al., Fungal Genom Biol 2018, 8:2
DOI: 10.4172/2165-8056.1000156
Research Article Open Access

Genetic Diversity of *Fusarium Oxysporum* Races Associated with Cowpea Fields in Kakamega County

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Received date: May 24, 2018; **Accepted date:** September 06, 2018; **Published date:** September 14, 2018

Abstract

Fusarium oxysporum is the most abundant and most damaging species of the genus Fusarium responsible for crop wilt diseases in cultivated fields. It possess risk to production of banana, tomato, onions, beans, peas, palm, wheat, sorghum, maize, potatoes, garlic and cowpea among others. Fusarium involves several species that produce mycotoxins associated with serious animal diseases. Fusarium is a potential threat to global food security. Furthermore, disease incidence of pathogenic Fusarium species could increase due to the effects of the predicted global changes. Limitation of occurrence records and diversity of the races of *F. oxysporum* in Kakamega County necessitated this study. This study aimed to characterize strains of Fusarium pathogens in cowpea fields of Kakamega County. The colonies had sparse to abundant mycelia with colour ranging from white to pale violet. The isolates gave rise to elliptical microchonidia without septa, smooth walled terminal and intercalary chlamydospores at times singly and paired in some cases on microscopy. Further, PCR amplification of ITS gene region in the ten isolates of *F. oxysporum* was performed using universal ITS primers. Fusarium the genus was amplified as a fragment of about 500 bp corresponding to the region between the 18S-28S rRNA intervening sequence for *Fusarium* spp. The selected isolates of *Fusarium* spp. were sequenced and submitted in NCBI database with the accession numbers of KY855504, KY855505, KY855506, KY855507, KY855508, KY855509, KY855510, KY855511, KY855512, KY855513 and KY855514. Eight soil-borne fungal isolates [KY855505, KY855506, KY855507, KY855508, KY855510, KY855511, KY855512 and KY855514] were identified as *F. oxysporum* based on its cultural, morphological and molecular characteristics. KY855504 and KY855509 had molecular identity to Ascotamycota and KY855513 had the molecular identity of *Phoma* sp. This study contributes knowledge on genetic diversity of local pathogenic Fusarium strains useful in crop breeding and disease management of cowpea crop in Kakamega County, Kenya

Keywords: *Vigna unguiculata*; *Fusarium oxysporum*; Molecular; Diversity; Wilt

Introduction

Fusarium spp. is pathogenic fungi that cause numerous diseases on wide range of host plants [1-3]. This fungus affects a wide variety of hosts of any age by colonizing the vascular tissues and causing wilting of the plant [4]. Some of the pathogenic forms of this fungus include; *F. oxysporum f.sp. Lycopersici* in tomato [5], *Fusarium oxysporum f.sp.Cubence* tropical race 4 in banana [6-8], *Fusarium oxysporum f.sp. Phaseoli* in beans [9,10], *Fusarium oxysporum oxysporum f.sp. Cepae* in onions [11,12], *Fusarium oxysporum f.sp. Batatas* in sweet potato [13], *Fusarium oxysporum f.sp. Cucumerinum* in cucurbits [14], *Fusarium virguliforme* in soy bean [15,16], *Fusarium graminearum* in wheat and other cereals [17,18], *F. oxysporum f.sp. Cumini* in cumin [19], *Fusarium graminearum* (*Gibberella zea)* in corn [20], *F. oxysporum f.sp. Niveum* in water melon [21], *Fusarium oxysporum Schl. f.sp. Tracheiphilum* in cowpea [22-24]. *Fusarium oxysporum* is the most widely distributed species which can be recovered from most soils [2]. Most of the isolates are host specific and hence more than 100 *formae specialis* and races have been described [20]. Diseases caused by *Fusarium* spp. include vascular wilts, dumping off, crown and root rots [2,25]. This fungus was ranked 5th out of the top 10 plant pathogens of scientific and economic importance [26-29]. Worldwide, *Fusarium* spp. is known to cause significant field and vegetable crop losses [20,30,31]. Fusarium involves several species that produce mycotoxins that associate with serious animal diseases like feed refusal syndromes, moldy sweet potato toxicity, and bean hulls poisoning [32]. As a result of this *Fusarium oxysporum* is a potential threat to global food security. Traditionally classification of Fusarium isolates was based on morphological characters like presence or absence of chlamydospores, and size and shape of macro- and micro-conidia [33]. Fusarium isolates were also classified on the basis of vegetative compatibility groups [34] and host specificity, nevertheless all these parameters were not persistent to develop a consensus scheme. With the advancement of molecular biology, fungal classification and phylogenetic studies have shifted to DNA sequence base methods [35]. These methods play an important role in Fusarium identification [36] and in understanding of genetic diversity of members of genus *Fusarium* [37]. Studies on genetic diversity of Fusarium include; Mes et al. [36]; Kim et al. [37]; Bogale et al., [38]; Cha et al. [39]. However, there is a scarce record on occurrence and diversity of this fungus in Kakamega County. In present study, genetic diversity of Fusarium isolates from cowpea fields in four sub-counties of Kakamega County was done by using Internal Transcribed Spacer [ITS] sequences of rRNA gene complex. There is a significant consensus about the use of the ITS sequences in fungus identification as an initial step and as a default region for species identification by international sub-commission on Fungal. This knowledge will be useful for monitoring effects and disease caused by *Fusarium oxysporum* races in cowpea fields in the region.

Materials and Methods

This study involved focused farmer groups to identify farms with cowpea within four sub-counties of Kakamega County (Lurambi, Kakamega East, Kakamega North and Mumias west). Three farmer groups were identified per Sub County, and one farm from each group with successive cowpea crop for at least two consecutive seasons was randomly selected in each sub county. From each selected farm, at least 4 symptomatic cowpea plants were sampled purposively [2]. Soil from the same field was sampled for the purpose of isolating *Fusarium* spp. Recovery of the fungus from the plants was carried out by surface sterilization of different plant parts using 70% alcohol for three minutes followed by 4% sodium hypochlorite for three min. Respective fungal isolates from different parts of the plants were obtained on potato dextrose agar treated with 1% ambicillin to inhibit bacterial growth. The cultures were incubated in an oven at 30°C for 4 days. More cultures of the fungus were generated by culturing soil particles on PDA media treated 1% ambicillin and incubated as that of the plant parts [40]. The cultures with characteristic features of *Fusarium oxysporum* spp. were further purified by making further sub-cultures (3 successive sub cultures) on PDA media treated with 1% ambicillin and incubated for four days at 30˚C to obtain clean single colonies of the fungus.

Sample Name	Colony Characteristic	Source of Culture	Location of Collection and Sub- county	Altitude	Date of Collection	Gene Bank Accession Number
1CLB	Whitish brown dense cottony	Cowpea leaf	Lurhambi sub-county, Shieywe ward, Mr. Manyasi's farm	00.29178˚N 034.73947˚E Elevation 1538 m	3.2.2016	KY855504
1BSW	White cottony	Soil Sample	Lurhambi sub-county, Shieywe ward, Mama Femia's farm	00.28752˚N 034.76547˚E Elevation 1538 m	3.2.2016	-
1ASPP	Very pale violet cottony	Soil sample	Lurhambi sub-county, Shieywe ward, Mama Halima's farm	00.28751˚N 034.76546˚E Elevation 1538 m	3.2.2016	KY855505
1ARPP	Very pale violet cottony velvet	Cowpea root	Lurhambi sub-county, Shieywe ward, Mama Halima's farm	00.28751˚N 034.76546˚E Elevation 1538 m	3.2.2016	KY855506
1ARW	White cottony	Cowpea root	Lurhambi sub-county, Shieywe ward, Mama Halima's farm	00.28751˚N 034.76546˚E Elevation 1538 m	3.2.2016	KY855507
4ARPP	Pale violet cottony velvet	Cowpea root	Kakamega Noth sub county, Ichina village	00.424070N 034.887390E Elevation 1608 m	1.3.2016	KY855508
4BLW1	White feathery	Cowpea leaf	Kakamega Noth sub county, Kimanget village	00.429220N 034.900790E Elevation 1600 m	1.3.2016	KY855509
4BLW2	White feathery	Cowpea leaf	Kakamega Noth sub county, Kimanget village	00.429220N 034.900790E Elevation 1600 m	1.3.2016	KY855510
4BRPP	Pale violet cottony velvet	Cowpea root	Kakamega Noth sub county, Kimanget village	00.429220N 034.900790E	1.3.2016	KY855511

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				Elevation 1600 m		
4CLPP	Pale violet cottony velvet	Cowpea leaf	Kakamega North sub county, Makhwibuyu village	00.426200N 034.91863E Elevation 1615 m	1.3.2016	KY855512
4CLR	Brown white ringed feathery	Cowpea leaf	Kakamega North sub county, Makhwibuyu village	00.426200N 034.91863E Elevation 1615 m	1.3.2016	KY855513
4CRPP	Pale violet cottony velvet	Cowpea root	Kakamega North sub county, Makhwibuyu village	00.426200N 034.91863E Elevation 1615 m	1.3.2016	KY855514

Table 1: *Fusarium* spp. colonies recovered from Kakamega County.

Morphological characteristics of the fungus recovered was determined by studying cultural characteristics and microscopic features as previous studies had done [2,3,41,42]. This was carried out by making micro cultures using blocks of PDA, slides and slide covers, and Glass Bridge arranged in petridishes. The micro-cultures were incubated at 30°C for 4 days and observation of the colonies stained with bromophenol blue under a microscope at x100. Further observation was carried out after dilution plating where by a small scrape of the fungus colony was grown in 10 ml of sterile water and incubated overnight at room temperature. The fungus culture was then stained with bromophenol blue and observed under a microscope at x100.

Molecular characterization

DNA isolation PCR amplification and sequencing

The fungal DNA was extracted and purified based on the prescribed protocol of the Qiagen mini plant DNA extraction kit. DNA quantification was done by use of a U.S. thermo scientific DNA NanoDrop 2000/2000c Spectrophotometer. PCR was carried out in 0.2 mL tubes with a reaction volume of 25 µL containing: 2.5 µL 10x PCR buffer, 1 µL of both primers, 1 mM of each dNTPs, 0.5 U Taq DNA polymerase, 50 mg DNA. The tubes were placed in an Eppendorf Master Cycler Gradient thermo cycler programmed for initial denaturation at 94°C for 1 min, followed by 35 cycles of 30 sec at 94°C, 30 sec at 55°C, 1 min at 75°C and final extension of 10 min at 72°C. The PCR products were resolved on an agarose gel (1%) using 0.5x TBE containing 1 mg/mL ethidium bromide with a vertical electrophoresis apparatus. The gel was photographed using AlphaImager 2200 under UV trans-illuminator. The resolved products were extracted from the gel and purified using the Qiagen DNA purification kit according to the prescribed protocol. DNA quantification was done by use of a U.S. thermo scientific DNA NanoDrop 2000/2000c Spectrophotometer. Sanger capillary sequencing was performed. This involved Reverse strand synthesis performed on copies of the DNA using a known priming sequence upstream of the sequence to be determined and a mixture of deoxy-nucleotides (dNTPs, the standard building blocks of DNA) and dideoxy-nucleotides (ddNTP, modified nucleotides missing a hydroxyl group at the third carbon atom of the sugar) [43]. The dNTP/ddNTP mixture causes random, non-reversible termination of the extension reaction, creating from the different copies molecules extended to different lengths [44]. Following denaturation and clean-up of free nucleotides, primers, and the enzyme, the resulting molecules are sorted by their molecular weight (corresponding to the point of termination) and the label attached to the terminating ddNTPs is read out sequentially in the order created by the sorting step [45].

The obtained nucleotide sequences were searched for identity with the sequences of identified organisms through BLASTn at GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/).

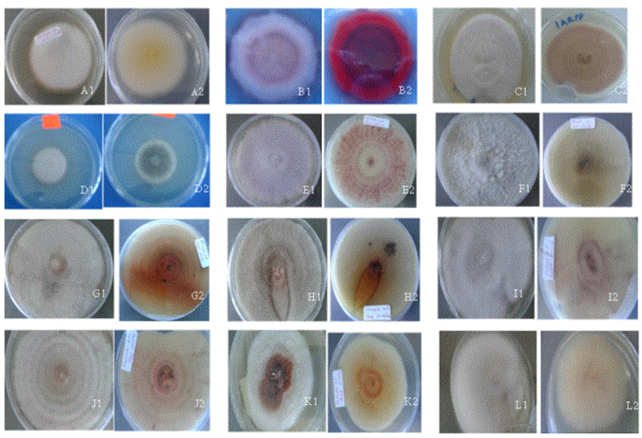

Figure 1: Picture of pure colony cultures of sample 1BSW [A1, A2], 1CLB [B1, B2], 1ARPP [C1, C2], 1ASPP [D1, D2], 4ARPP [E1, E2], 4CRPP [F1, F2], 4BLW1 [G1, G2], 4BLW2 [H1, H2], 4BRPP [I1, I2], 4CLPP [J1, J2], 4CLR [K1, K2] and 1ARW [L1, L2].

Results

Twelve isolates of the fungus were obtained from soil and plant samples collected from Lurambi sub-county and Kakamega North sub-county. The isolates were labelled according to the region of collection and the sample that was cultured as indicated in Table 1. Twelve

Fusarium isolates gave rise to colonies of different colours as shown in Table 1 and Figure 1.

On microscopy, the isolates gave rise to elliptical micro-conidia without septa, smooth walled terminal and intercalary Chlamydophores at times singly and paired in some cases (Figures 2 & 3).

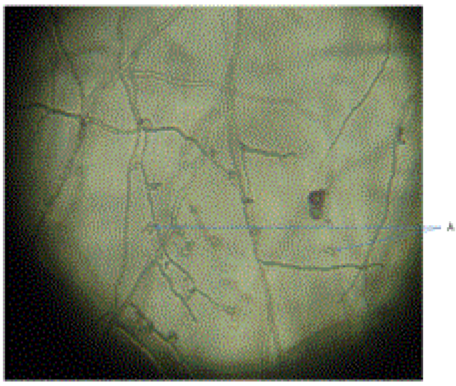

Figure 2: Microconidia *in situ.*

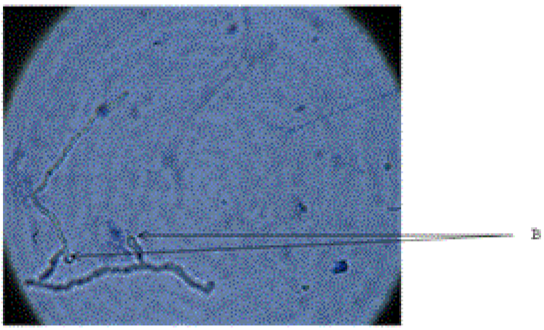

Figure 3: Terminal and intercalary.

Phylogenetic analysis of Fusarium isolates

Fungal ITS sequences generated from the twelve Fusarium isolates were arranged into two clusters (Figure 4). Cluster A comprised of six isolates (1CLB, 4CLR, 4ARPP, 4BRPP, 1ARPP and 4BLW2). Cluster B comprised of five isolates (1ASPP, 1ARW, 4CRPP, 4BLW1 and 4CLPP). Isolate 1BSW was omitted because the DNA did not give a clear resolution on PCR amplification.

The study of the genetic distances revealed some close relationships in the Fusarium isolates. Isolate 1ARW was more closely related to isolate 1ASPP, 4CLPP and 4CRPP with genetic distances of 0.013, 0.009 and 0.004 respectively (Table 2). Isolate 1ARPP indicated closer relationships with isolates 4ARPP, 4BLW2 and 4BRPP with genetic distances of 0.006, 0.013 and 0.009 respectively. Closer relationships were also realized between isolate 1ASPP and isolates 1ARW, 4CLPP and 4CRPP with genetic distances of 0.013, 0.011 and 0.013 respectively. Isolate 4ARPP indicated close relationships to isolates 4BLW2 AND 4BRPP with genetic distances of 0.020 and 0.002 respectively. Fusarium isolate 4BLW2 was closely related to 4BRPP with genetic distances of 0.022. Isolate 1CLB indicated closer relationship with 4CLR with genetic distance of 0.013 while isolate 4CLPP showed closer relationship to isolate 4CRPP with genetic distance of 0.009 (Figure 5).

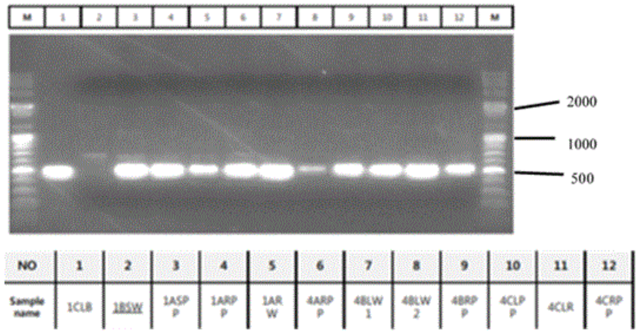

Figure 4: PCR products of Fusarium isolates' DNA using ITS1 and ITS4 primers, M indicates the 1Kb ladder from Bioneer Laboratories.

Species 1	Species 2	Genetic distances	Std. Err
1CLB	1ASPP	1.496	2.814
1CLB	1ARPP	0.415	0.559
1ASPP	1ARPP	1.254	2.505
1CLB	1ARW	1.513	2.826
1ASPP	1ARW	0.013	0.006
1ARPP	1ARW	1.265	2.512
1CLB	4ARPP	0.412	0.556
1ASPP	4ARPP	1.272	2.51
1ARPP	4ARPP	0.006	0.004
1ARW	4ARPP	1.274	2.515
1CLB	4BLW1	1.577	3.219
1ASPP	4BLW1	0.425	0.543
1ARPP	4BLW1	1.556	2.853
1ARW	4BLW1	0.428	0.626
4ARPP	4BLW1	1.556	2.855
1CLB	4BLW2	0.427	0.581
1ASPP	4BLW2	1.289	2.593
1ARPP	4BLW2	0.013	0.006
1ARW	4BLW2	1.301	2.599
4ARPP	4BLW2	0.02	0.007
4BLW1	4BLW2	1.569	2.863
1CLB	4BRPP	0.416	0.558
1ASPP	4BRPP	1.268	2.501

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1ARPP	4BRPP	0.009	0.004
1ARW	4BRPP	1.27	2.506
4ARPP	4BRPP	0.002	0.002
4BLW1	4BRPP	1.55	2.846
4BLW2	4BRPP	0.022	0.007
1CLB	4CLPP	1.5	2.834
1ASPP	4CLPP	0.011	0.005
1ARPP	4CLPP	1.274	2.518
1ARW	4CLPP	0.009	0.004
4ARPP	4CLPP	1.274	2.52
4BLW1	4CLPP	0.416	0.527
4BLW2	4CLPP	1.311	2.604
4BRPP	4CLPP	1.27	2.511
1CLB	4CLR	0.013	0.005
1ASPP	4CLR	1.487	2.801
1ARPP	4CLR	0.423	0.565
1ARW	4CLR	1.515	2.814
4ARPP	4CLR	0.427	0.564
4BLW1	4CLR	1.592	3.195
4BLW2	4CLR	0.42	0.574
4BRPP	4CLR	0.431	0.566
4CLPP	4CLR	1.513	2.822
1CLB	4CRPP	1.496	2.82
1ASPP	4CRPP	0.013	0.006
1ARPP	4CRPP	1.263	2.51
1ARW	4CRPP	0.004	0.003
4ARPP	4CRPP	1.272	2.513
4BLW1	4CRPP	0.428	0.543
4BLW2	4CRPP	1.299	2.598
4BRPP	4CRPP	1.268	2.504
4CLPP	4CRPP	0.009	0.004
4CLR	4CRPP	1.498	2.808

Table 2: Genetic distances.

The results of the polymorphic data revealed that the nucleotide diversity was relatively low (0.40662) while heterozygosity and gene diversity was high with a value of 1. The results on evolutionary rates of all the isolates showed that all had different evolutionary rate at P=0 (Table 3).

	InL	Parameters	+G	+I
With Clock	-116317.8	15	n/a	n/a
Without Clock	-2333.723	24	n/a	n/a

Table 3: Results from a test of molecular clocks using the Maximum Likelihood method.

Sample Sequence ID	Closely Related organism	Identity	NCBI ID
1CLB-ITS1	Uncultured Ascomycota clone 4M1 CO7	99	EU489900.1
1ASPP-ITS1	*Fusarium oxysporum* strain GIFO charna	100	KJ938022.1
1ARPP-ITS1	*Fusarium oxysporum* isolate FJAT-31101	100	KU931552.1
1ARW- ITS1	*Fusarium oxysporum* strain J7	100	KU321556.1
4ARPP-ITS1	*Fusarium oxysporum* isolate MC-17-F	99	KU527801.1
4BLW1-ITS1	Ascomycota spp. QRF361	99	KP278172.1
4BLW2-ITS1	*Fusarium verticillioides* isolate ASU1	100	KT587649.1
4BRPP-ITS1	*Fusarium oxysporum* isolate FU05	99	HM152535.1
4CLPP-ITS1	*Fusarium oxysporum* isolate 59	100	KT719193.1
4CLR-ITS1	*Phoma* spp. F226	100	KM979787.1
4CRPP-ITS1	*Fusarium oxysporum* isolate GIFUUHFA4	99	GQ121287.1
1CLB-ITS4	Ascomycota spp. QRF361	99	KP278172.1
1ASPP-ITS4	*Fusarium oxysporum* isolate F84-Kr1t9	99	KC304806.1
1ARPP-ITS4	*Fusarium oxysporum* strain A3	99	KR708632.1
1ARW-ITS4	*Fusarium oxysporum* isolate F87-Kr1t9	99	KC304806.1
4ARPP-ITS4	*Fusarium oxysporum* strain G01	99	KT884661.1
4BLW1-ITS4	Ascomycota spp. shz-102	99	EU682958.1
4BLW2-ITS4	*Fusarium pseudonygamai* isolate wxm62	99	HM051063.1
4BRPP-ITS4	*Fusarium oxysporum* strain IHB F 2901	99	KM817207.1
4CLPP-ITS4	*Fusarium oxysporum* isolate F50-MB2P1a	99	KC304808.1
4CLR-ITS4	*Phoma* spp. F130	99	KM979923.1

4CRPP-ITS4	*Fusarium oxysporum* strain YQ1	99	KU746659.1
Source: Data obtained from NCBI website.			

Table 4: Closely related organisms to the Fusarium isolates.

Sequence comparison in the GenBank DNA database showed that some of the determined sequence share 99%-100% sequence identity with that of *F. oxysporum* (Table 4).

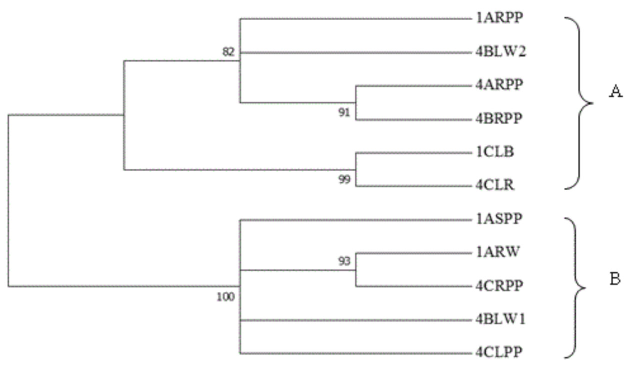

Figure 5: Phylogenetic relationships of Fusarium isolates.

Discussion

The morphological features of the isolated fungus in Kakamega County were consistent to those identified by other researchers. The mycelia in this study varied in morphology with color ranging from white to pale violet a result that is consistent with the findings of Leslie and Summerell [2]. However, they also reported that *Fusarium oxysporum* readily mutate forming a flat wet mycelia colony with a yellow to orange appearance on PDA. Leslie and Summerell [2] reported that presence of elliptical and not septate microconidia as characteristic of *Fusarium oxysporum*, consistent with the findings of this study. This study also found that some chlamydophores were formed singly consistent with other findings. Some were paired and at times clustered consistent with Hussain et al. [33] Although the morphological characteristics of the Fusarium isolates in this study were consistent with other studies on *Fusarium oxysporum*, we could not identify the different strains of *F. oxysporum* from other species of Fusarium based on morphological features. It is almost impossible to identify pathogenic races or *formae speciales* of *Fusarium oxysporum* using morphological features. The ITS regions were used as targets for phylogenetic analysis because they generally display sequence variation between species, but only minor variation within strains of the same species [46]. The results of BlASTn program [47] was used to find homology of consensus sequences obtained from multiple sequence runs, with already reported sequences present in nucleotide database; gave a confirmation of isolates as *Fusarium oxysporum*. The low nucleotide diversity observed in this study is consistent with that observed among Fusarium strains as reported by Naqvi et al. [48]. This study however reports a higher gene diversity/heterozygosity could be attributed to an isolate-breaking effect [48]. This finding is in agreement with the findings of Leslie and Summerell [2] who reported that *Fusarium oxysporum* readily mutate especially on PDA. Although literature on Fusarium wilt of cowpea in Kenya is scarce; this study reports that cowpea fields in Kakamega County have a diversity of the races of this fungus. This could be hypothesized to the effects of climate change, that climate change may lead to changes in the quality, quantity and diversity of plant and soil microbial communities and therefore plant pathogen development. Similarly, Chitarra et al. [49] reports that the disease incidence of pathogenic *Fusarium* species could increase due to the effects of the global changes that have been predicted for the future. This therefore could mean that the pathogenic races of *Fusarium oxysporum* may be reported in new regions where they have never been a problem. To support this further, new disease reports on Fusarium have been submitted in the Agricultural research literature. These include occurrence of Fusarium wilt of *Bougainvillea glaba* in Italy; Fusarium wilt of *Ocimum* minimum in Portugal and first report of *Fusarium oxysporum f.sp. Radilis-cucumerinum* on cucumber in Turkey. Although there is scarcity of information on molecular characterization of *Fusarium oxysporum*, the advancement of molecular biology has enabled a shift of fungal classification and phylogenetic studies to DNA sequence base methods [35]. These methods play an important role in Fusarium identification [36] and in understanding of genetic diversity of members of genus *Fusarium* [37]. This study has established nucleotide sequences of eleven isolates from Kakamega County that will contribute towards understanding of genetic make-up of local pathogenic Fusarium strains and may contribute significantly in crop breeding and disease management of cowpea crop in Kakamega County, Kenya.

Acknowledgement

We acknowledge the technical support in the laboratory offered by Peter Nyongesa and the donation of DNA extraction kits by Dr. Jo Messing the Director of Waksman Institute

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