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Using Translation Elongation Factor Gene to Specifically Detect and Diagnose *Fusarium xylaroides*, a Causative Agent of Coffee Wilt Disease in Ethiopia, East and Central Africa

Olal S[1]*, Olango N[1], Kiggundu A[2], Ochwo S[4], Adriko J[2], Nanteza A[4], Matovu E[4], Lubega GW[4], Kagezi G[1], Hakiza GJ[1], Wagoire WW[1], Rutherford MA[3], and Opiyo SO[5,6]

[1]National Agricultural Research Organization, National Coffee Research Institute, Mukono, Uganda
[2]National Agricultural Research Organization, National Agricultural Research Laboratories, Kampala, Uganda
[3]CAB International, UK Centre, Bakeham Lane, Egham, Surrey, United Kingdom
[4]College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, Kampala, Uganda
[5]Molecular and Cellular Imaging Center-Columbus OARDC, The Ohio State University, Columbus, Ohio, USA
[6]Patira Data Science, Westerville, Ohio

Abstract

The present study presents the first report on the application of DNA-based polymerase chain reaction (PCR) for the specific detection and diagnosis of *Fusarium xylarioides* (anamorph: *Gibberrela xylarioides*). *Fusarium xylarioides* is the causative agent of Coffee wilt disease (Tracheomycosis), and the disease is the most important economic constraint in Robusta coffee production in Uganda. The pathogen has two races, one pathogenic to Robusta coffee and the other to Arabica coffee, and not vice versa. Its laboratory diagnosis has been mainly based on microscopy, which is slow, has poor discriminative power, requires high expertise, only applicable on host plants with symptoms, and has since failed to detect the pathogen from the soil. Translation Elongation factor-1α (TEF-1α) gene from a *F. xylarioides* isolated from infected Robusta coffee plant was amplified by Fusarium genus specific primer then the PCR product sequenced. The sequence data was then used to design the specific primer. The primer-BLAST product was found to match only *F. xylarioides* sequences comprising 75% of the race pathogenic to Robusta and 25% to Arabica coffee. *In vitro* test by PCR showed the primer to be specific to only *F. xylarioides* amplifying a 284bp product and was able to differentiate *F. xylarioides* from all closely related species of Fusarium and other plant pathogens tested. More so it was able to amplify DNA from all the *F. xylarioides* isolates from different regions of Uganda, and amplified DNA concentrations as minute as 0.78 ng/μL.

Keywords: *Giberella xylarioides*; Polymerase chain reaction; Specific primer; Coffee wilt disease; Translation elongation factor-1α

Introduction

The genus *Fusarium* comprises a high number of well-known fungal pathogens with significant economic impact on plants causing diseases in several agriculturally important crops, including coffee [1], as well as humans and animals [2], *Fusarium xylarioides* is a typical vascular pathogen known to cause coffee wilt disease (CWD), commonly referred to as tracheomycosis or sometimes carbuncullariosis. The sexual (perfect) form is *Gibberella xylarioides* while *F. xylarioides* is the conidial (asexual) state of this species [3]. In Uganda and the rest of East and Central Africa, the pathogen causes CWD exclusively on Robusta coffee unlike in Ethiopia where the pathogen infects Arabica coffee and no cross-infection occurs in Robusta coffee with pathogen from Arabica coffee and vice–versa. The disease is endemic to Robusta coffee in Uganda yet this provides over 78% of the coffee exported [4]. The disease advent in Uganda was in 1993 and destroyed 44.5% of Robusta coffee (*Coffea canephora*) by 2002 [5], leading to economic losses of over $100M. This had a negative impact on the country's GDP and livelihoods of over 7 million (21%) Ugandans/ 1.7 (42%) million out of the estimated Uganda 42 million agricultural households who depend on coffee growing or employed along the value chain [4]. The disease is still causing severe damage on coffee and of late been reported in Kabale district in Uganda, a situation which could be attributed to effect of climate change.

Detection of CWD pathogen from disease plant tissue has been mainly done microscopically after culturing and has since failed to detect the pathogen from the soil and asymptomatic plants [6], it is expensive, slow, and requires high taxonomic expertise [7], On the other hand, field identification basing on symptoms is confusing with other effects like stem borer, drought and other physiological problems, which produce similar symptoms to CWD [6]. A fast and reliable method for detection and identification of the pathogen is therefore needed. Here, we report the first PCR detection method developed specifically for *F. xylarioides* that will offer a fast, sensitive and versatile detection and diagnostic procedure with ability of handling bulk specimens over the conventional method [8,9]. The tool will promote timely and correct mitigation measures; epidemiological, agronomic and breeding for resistance research; quarantine check and support seed inspection and will eventually impact on the socio-economic transformation.

The first stage in development of a molecular diagnostic assay is to select the nucleic acid sequences to be used to identify the organism. This can be done by (i) targeting particular genus specific gene but which have useful sequence variation at species level (ii) *in silico* using sequences in gene banks and (iii) randomly selected DNA fragment amplified with arbitrary primers [10,11]. The principle is that every

***Corresponding author:** Sammy Olal, National Agricultural Research Organization, National Coffee Research Institute, P.O.BOX 185, Mukono, Uganda, Tel: +256 77 2 366 792; E-mail: sammyolal83@gmail.com

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species has a unique DNA sequence that can be used for its specific identification. In this study, we have used the first approach to develop the specific primer to *F. xylarioides*.

The work therefore utilized TEF-1α gene sequence to develop the first specific, fast and robust PCR based detection and identification protocol for coffee wilt disease pathogen and disease respectively for improved Robusta coffee plant health and eventually translating into increased coffee production for socio-economic transformation of the people of Uganda. The motivation for using the TEF-1α gene was that it is considered a housekeeping gene with higher sequence polymorphism and are being more extensively used to design species specific markers and probes for the identification, quantification of pathogenic population of *Fusarium* species [12-17].

Materials and Methods

Fungal species used in the study

Eleven fungal species including *F. xylarioides* isolated from infected Robusta coffee tree and ten other different fungal species namely: *F. solani*, *F. oxysporum*, *F. decemcellulare*, *F. moniliforme*, *F. solani*, *F. lateritium*, *F. pallidoroseum*, *Sclerotia* spp., *Ambrosia* spp. and *Rhizoctobia solani* which were identified culturally and microscopically as described by Booth, et al. [18-20] and available in the culture collection at the National Coffee Research Institute, Kituza, Mukono, Uganda. Apart from *Sclerotia* spp. and *R. solani* which originated from infected bean roots, the rest were isolated from Robusta coffee trees. Positive and negative reference gold standards were acquired from CABI Biosciences England and these were *F. xylarioides* (IMINo: 379925) and *F. oxysporum* (IMI No: 244509) respectively. *Fusarium oxysporum* was chosen as a negative standard because it tested positive with all the primers that were discarded for lack of specificity to *F. xylarioides*.

Reparation of fungal mycelia for DNA extraction

Preparation of cultures for DNA extraction was carried out as described by Olal, et al. 2014 [6]. The stored cultures were sub-cultured on fresh Potato dextrose agar (PDA) medium (OXOID, CMO 139, England) and allowed to grow for 4 days under 12-hour fluorescence light and dark cycles at room temperature. Potato dextrose broth (PDB) comprised 200 g washed unpeeled Irish potato, 20 g dextrose/D-glucose powder (BDH, England) and 1L tap water was autoclaved for 20 min at 121^{O}C at 15 Psi, cooled in the laminar flow hood, suspension allowed to settle and 10 mL suspended in 22 individual 9 cm Petri dishes for mycelia production. Four (4 × 4 mm) plugs taken from the periphery of each of the 11 rejuvenated fungal isolates on PDA were suspended in duplicate in PDB plates. The inoculated PDB plates were incubated in the dark without shaking at room temperature (25 ± 2^{o}C) for 4 days to encourage mycelia mat formation while limiting sporulation. The mycelial mats from each fungal species were put in individual tea strainers, rinsed thoroughly with sterile distilled water and blotted dry with sterile absorbent papers ready for DNA extraction.

DNA extraction

To amplify the *F. xylarioides* TEF-1α gene with Fusarium genus specific TEF-1α primers for sequencing and to test the specificity of the designed primers against the 10 test Fungal species, DNA was extracted from each of the 11 isolates using ZR Plant/Seed DNA MiniPrep (ZYMO RESEARCH, USA) according to the manufacturer's instructions. The purity and quantity of the DNA extracted was verified using Nanodrop spectrophotometer (Nanodrop 2000C, Thermo Scientific). The purity of the DNA was found to lie between absorbent readings at wavelength (λ) 260/280 ratio ranging from 1.8 - 2.0 which was quite substantial quality.

Amplification of translation elongation factor (TEF-1α) gene

The TEF-1α gene from *F. xylarioides* isolates from Robusta coffee were amplified with TEF-1αgene specific primer pair, TEF-Fu3f: 5'GGTATCGACAAGCGAACCAT3', TEF-Fu3r: 5'TAGTAGCGGGGAGTCTCGAA3' [13]. The PCR was performed in a 40 µl reaction mixture containing 4 µl of 50 ng/µl of genomic DNA, 20 µl of 2x Green GoTag PCR master mix (Promega), 1.2 µl of 10 mM of each primer (forward and reverse), and 1.6 µl ddi sterile water. The amplification conditions were as follows; initial denaturation at 95°C for 8 min, followed by thirty five (35) cycles of 95°C for 15 sec, 53°C for 20 sec, 72°C for 1 min and final extension at 72°C for 5 min (Eppendorf AG 22331 Hamburg, England). Amplification product was loaded in four (4) wells of 10 µl each and separated by electrophoresis (Sub-cell model 96. BIO-RAD) on a 1.4% agarose gel in TAE buffer. The gel was then stained for 15 min in 0.5 µg/ml solution of ethidium bromide (BIO-RAD, USA) and gel photograph was taken using gel documentation system (SyngeneG:Box). The characteristic bands from all the four wells were then excised from the gel, purified with GeneJET PCR Purification Kit. Thermo SCIENTIFIC, Lithuania according to the manufacturer's instruction and sequenced directly from Inqaba Lab. LTD, Republic of South Africa.

Primer design and specificity test

Primer-BLAST was used to design and test the specificity of the primers *in silico* [21]. The primers were selected for syntheses based on similarity in melting temperatures (Tm). The designed primers were synthesized from Ingaba Lab, Republic of South Africa. The specificity of the primers was predetermined according to the Primer-BLAST output and confirmed *in-vitro* by polymerase chain reaction (PCR). One critical property observed when selecting the primer was that it should only amplify the intended target.

Primer specificity testing *in-vitro* by PCR

Specificity of the synthesized primers was tested by PCR of the genomic DNA from all the fungal isolates used in the study namely: *F. xylarioides, F. solani*, *F. oxysporum*, *F. decemcellulare*, *F. moniliforme*, *F. lateritium*, *F. pallidoroseum*, *Sclerotia* spp., *Ambrosia* spp. and *R. solani*. The PCR was performed in a 20 µl reaction mixture containing 1 µl of 50 ng/µl of genomic DNA, 10 µl of 2x One tag Hot Start PCR master mix (New England), 0.3 µl of 10 mMof each primer (forward and reverse), and 8.4 µl ddi sterile water. The amplification conditions were as follows: Initial denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 58°C 1 min, and 68°C for 1 min and final extension at 68°C for 5 min (Eppendorf AG 22331 Hamburg, England). Amplification products were separated by electrophoresis (Sub-cell model 96, BIO-RAD) on a 1.4% agarose gel in TAE (Buffer containing Tris base, acetic acid and EDTA) buffer. The gel was then stained for 15 min in 0.5 µg/ml solution of ethidium bromide (BIO-RAD, USA) and gel photograph was taken using gel documentation system (Syngene G: Box).

Sensitivity of the primers

This was done to determine the minimum amount of DNA which can be amplified by the primer. 1 µL containing 50 ng/µL of DNA was serially diluted in a series of seven concentrations ranging from 0.001 ng/mL to 50 ng/µL and a negative control. The same reaction mixture and cycling processes described above for DNA amplification were used for the sensitivity tests.

The diagnostic potential of the primers

This was done to find the robustness of the primers to be used in any pathogen strain across the country. The primers were tested on DNA isolated from the pathogen originating from different regions in the country.

Phylogenetic analysis

BLAST [22] was used to search for similar sequences comparing Fusarium xylarioides sequence used in primer design with those in the Gen Bank database and local sequence databases, the cut off for score were 952 and E-value 0.0). Multiple sequence alignment of the identified sequences from Blast analysis was performed using Clastal X/W version 2.0 [23]. Phylogenetic analysis of the aligned sequences was performed by Maximum likelihood (ML) using the MEGA 6 phylogenetic package [24].

Result

In silico primer specificity test

In silico test was a pre-specificity test and showed that the primer matched only *F. xylarioides* TEF-1α gene sequences available in the gene banks. This is a positive indication that the primer is not likely to amplify TEF-1α gene of any organism whose TEF-1α gene sequence is currently available in the gene banks, yet *F. xylarioides* is one of the least organism that has been studied at molecular level [25]. It is also possible that the primers can detect *F. xylarioides* race pathogenic to Arabica since 25% of the sequences matched belong to the race pathogenic to Arabica coffee. The result also indicates possibility of using the primer regionally since the pathogen sequences from all the country where currently CWD exist namely Tanzania, Uganda, the DRC and Ethiopia were matched (Table 1).

In vitro specificity test using PCR

The PCR reaction was a confirmatory test for the specificity of the primer. The primer was able to amplify only DNA from *F. xylarioides* and not from any test species The specific primer to *F. xylarioides* is therefore OSTF15 F (5' GACCTGGCGGGGTATTTCTC) and OSTF15R (5'AATGGGAGAGGGCAGAAACG) (Figure 1).

Diagnostic potential of primer OSTF15

This was done to test the robustness of the primer to detect isolates from different parts of the country. The result showed that the primer was able to amplify DNA from all the *F. xylarioides* isolates from different part of the country tested. This means there is no variation in the pathogen across the country at TEF-1αgene level (Figure 2).

In vitro sensitivity test

This was done to find the minutest amount of DNA which can be amplified by the primer. The result showed that the minimum amount of DNA amplifiable by the primer was as minute as 0.78 ng/µL (Figure 3) and this is vital for the detection of the minute amount of the pathogen DNA available in infected asymptomatic plants and soil (Figure 3).

Accession number	Strain detected	Target gene	Strain source	Host species	Country of origin
KJ173606.1	*G. xylarioides*	Partial TEF-1α	IMI392680	Coffee spp	Ethiopia
KJ173605.1	*G. xylarioides*	Partial TEF-1α	IMI392273	Robusta	DRC
KJ173604.1	*G. xylarioides*	Partial TEF-1α	IMI392279	Robusta	TZ
KJ173603.1	*G.xylarioides*	Partial TEF-1α	IMI392277	Robusta	TZ
KJ173602.1	*G. xylarioides*	Partial TEF-1α	IMI 392274	Robusta	TZ
KJ173601.1	*G. xylarioides*	Partial TEF-1α	IMI392266	Robusta	Uganda
KJ173600.1	*G. xylarioides*	Partial TEF-1α	IMI392261	Robusta	Uganda
KJ173599.1	*G. xylarioides*	Partial TEF-1α	IMI392259	Robusta	Uganda
KJ173598.1	*G. xylarioides*	Partial TEF-1α	IMI392255	Robusta	Uganda
KJ173597.1	*G. xylarioides*	Partial TEF-1α	IMI392254	Robusta	Uganda
KJ173596.1	*G. xylarioides*	Partial TEF-1α	IMI392249	Robusta	DRC
KJ173595.1	*G. xylarioides*	Partial TEF-1α	IMI392248	Robusta	DRC
KJ173594.1	*G. xylarioides*	Partial TEF-1α	IMI389567	Arabica	Ethiopia
KJ173593.1	*G. xylarioides*	Partial TEF-1α	IMI375909	Arabica	Ethiopia
KJ173592.1	*G. xylarioides*	Partial TEF-1α	IMI375908	Arabica	Ethiopia
KJ173591.1	*G. xylarioides*	Partial TEF - 1α pseudogene	IMI375907	Arabica	Ethiopia
AM295281.1	*G. xylarioides*	Partial TEF - 1α pseudogene	BBA 62458	Arabica	Ethiopia
AY707130.1	*G. xylarioides*	Partial TEF - 1α pseudogene	FRC L-394	Robusta	Uganda
AY707122.1	*G. xylarioides*	Partial TEF-1α	FRC L-126	Robusta	Uganda
AY707119.1	*G. xylarioides*	Partial TEF-1α	FRC L-96	Robusta	Uganda
All the primers matched exclusively *G. xylarioides* (Anomoph. *F. xylarioides)* strains with 75% originating from Robusta coffee. The table was a specificity Primer-BLAST product for primer OSTF15.					

Table 1: *Insilico* specificity test of the primer OSTF15

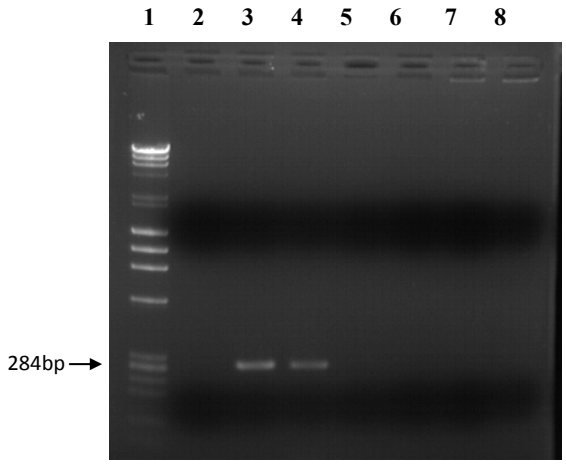

Figure 1: *In vitro* specificity test of primer SOTF15 (284bp) by PCR. Lane 1,100bp DNA Ladder; Lane 2, Negative control (H_20); Lane 3, *F. xylarioides* (Used in primer development;Lane 4, *F. xylarioides* (Gold standard);Lane 5, *F.oxysporum (*Gold standard negative control*);* Lane6, *F. solani*; Lane7, *F. decemcellulare* and; Lane 8, *F.lateritium.*

Phylogenetic analysis

The sequences use to design the specific primer was blasted and the selected species from the blast product was used to generate the tree. It can be clearly seen that *F. xylarioides* was grouped separately from other species and hence the specific primers may not amplify DNA from any of those related species used in the analysis (Figure 4).

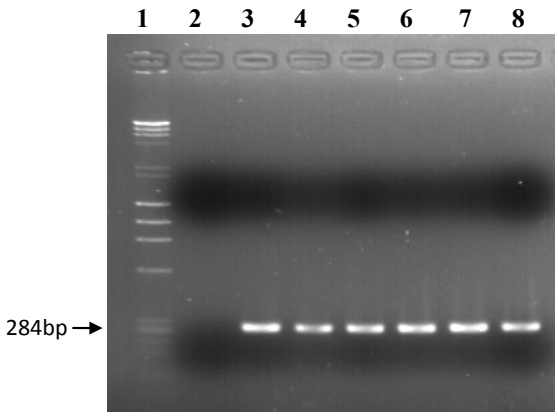

Figure 2: Diagnostic potential of primer OSTF15**.** Lane 1, 100bp DNA ladder; Lane 2,Central (Kituza-Mukono; Lane 3,Western (Bundibidgyo); Lane 4, South western (Rukungiri); Lane 5, West Nile (Arua); Lane 6,Kalangala; Lane 7,Eastern (Iganga) and; Lane 8,Kabale. The primers were able to amplify the DNA from all isolates obtained from different part of the country (Uganda).

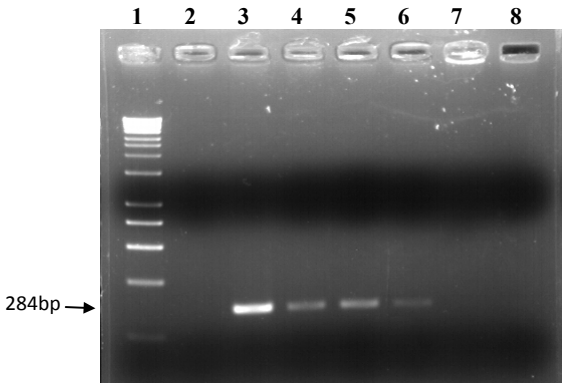

Figure 3: Sensitivity of the OSTF15.Lane 1, 100 bp DNA Ladder; Lane 2, Negative control (H_20); Lane 3, 50 ng/µL; Lane 4, 12.5 ng/µL, Lane 5; 3.124 ng/µL; Lane 6, 0.78 ng/µL, Lane7, 0.2 ng/µL; Lane8, 0.05 ng/µL. The sensitivity stopped at DNA concentration of 0.78 ng/µL. Serial dilution of 50 ng/µL by 4 folds was used progressively in ascending well numbers. Bands were separated on 1.4% agarose gel.

Discussion

This study represents the first application of PCR based detection and diagnostic test for *F. xylarioides* using primers amplifying 284bp region of the translation elongation factor gene (TEF-1α). This *F. xylarioides*-specific amplification product differentiated *F. xylarioides* from all other fungal species used in the study as shown above. The primer was also able to amplify DNA from *F. xylarioides* isolates from different parts of the country meaning it can be used robustly on all the strains within the country. The *F. xylarioides*-specific primers will thus allow rapid and simple diagnoses of pathogen in diverse habitats. The output of the primer-BLAST showed that the primer sequence matched only *F. xylarioides* (100%), 75% from the race pathogenic to Robusta coffee and 25% to Arabica coffee, a clear evidence of the race differences as had been reported by other workers that there exist two host-race specificity in *F. xylarioides* one for *Coffea canephora* and one for *Coffea arabica* [26,27]. The primers were able to amplify DNA from all isolates tested from different region (Figure 2) meaning the primers can be used with high reliability to detect the pathogen from any part of the country; this further emphasized uniformity in the CWD pathogen as suggested by Lewis, et al. 2003 [28]. The primers were also found to be quite sensitive amplifying DNA concentrations as low as 0.78 ng/µL of DNA. This fast, specific and sensitive contemporary method for detection and identification of *F. xylarioides* will reinforce the conventional method that has been in use before. Since the primer sequence matched also the race pathogenic to Arabica coffee, it is possible that the primers can be used to detect the pathogen from Arabica coffee as well.

Translation elongation factor 1α (TEF-1α) gene was used to design the primers because it is considered a housekeeping gene with higher sequence polymorphism and are being more extensively used to design species specific markers and probes for the identification, quantification of pathogenic population of Fusarium species [12-16].

Evolutionary history of an organism is recorded in its genes and the development of sequence analysis techniques has allowed reconstruct biological evolution by comparing the structure of genes or gene-products in different species [29]. In this study, phylogenetic analyses were performed using BLAST results of the nuclear (translation elongation factor 1-alpha, *EF-1α*) sequence used to design the primers (Figure 4). The analysis categorized the organisms into three clusters. The upper cluster consisted of mainly fungus that infects leaves, the middle cluster comprised mainly *F. oxysporum* and the lower cluster comprised *F. xylarioides* strains in the lower sub-cluster and those of *F. odum, F. indica* and *F. phyllophilum* in the upper sub-cluster. The two sub-clusters in the lower cluster are very close relatives. All the species in the upper sub-cluster belong to the *G. fujikuroi* species complex (GFC) [30]. The GFC is a monophyletic and diverse group of several phylogenetic species divided into three sub-clades, often referred to as African, American and Asian, based on the putative geographic origin of most of the species within them [31]. Some authors had grouped *F. xylarioides* as belonging to the African clades of the *G. fujikuroi* species complex (Rutheford, 2006) while others have refuted it suggesting that recent outbreaks of vascular wilt in Uganda may have been caused by a new pathogen [28]. Clades are characterized by shared possession of uniquely-derived evolutionary novelties or "synapomorphies" (literally "together, derived shape"). The so-called "African Clade" is the largest of the three clades with 23 phylogenetic lineages, of which four represent biological species (Capable of undergoing sexual cycle) namely: *F. verticillioides*, *F. thapsinum*, *F. nygamai* and *F. xylarioides* [32]. The agriculturally important pathogenic ones include *F. verticillioides*, *F. denticulatum*, *F. thapsinum, F. nygamai*, *F. lactis*, *F. phyllophilum*, *F. udum*and, and *F. xylarioides* [30]. The close relationship between *F. phyllophilum, F. udum and F. xylarioides* was demonstrated by Lima,et al. [33]. One of the attribute of GCF membership is the presence of chlamydospore [29] of which *F. xylarioides* does not have and this could be one of the reason why it is put in a different lower sub-cluster. The analysis shows that the members in the two sub-clusters are very close relatives supported at a high bootstrap value of 846 / 84.6% (Figure 4), but since they have not been clustered together, there may be no false positive reaction.

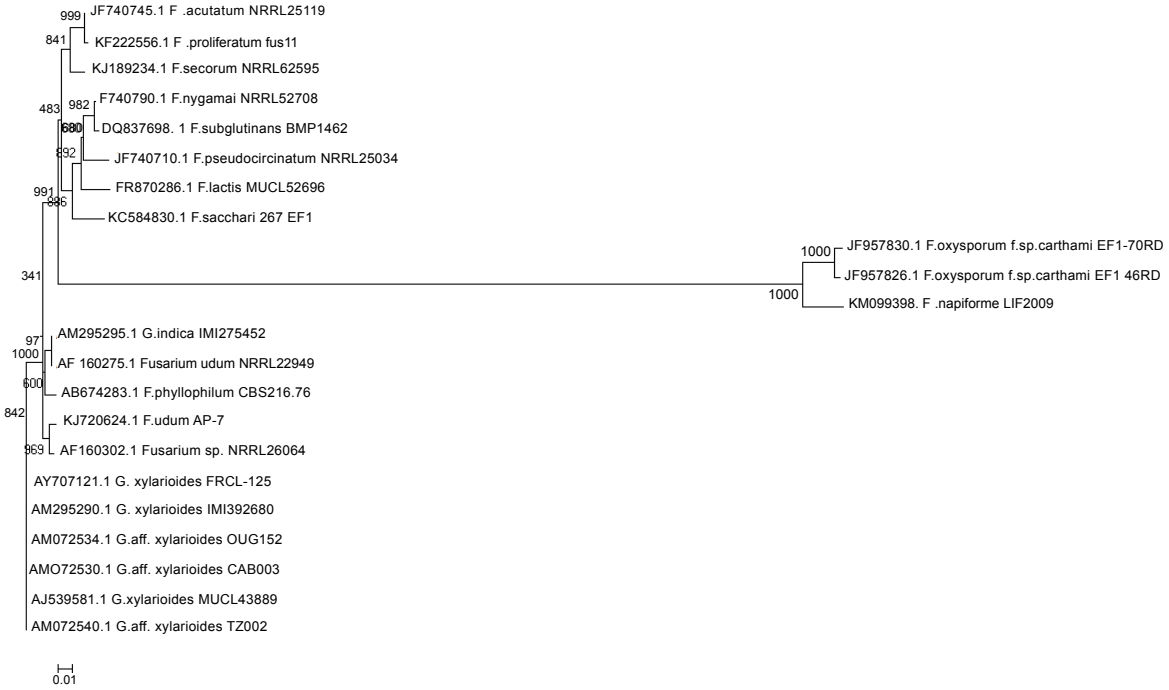

Figure 4: Phylogenetic relationship of ***F. xylarioided*** and other related specie generated by the blast output. The sequences were aligned using Clastal X and tree created using Mega 6 software package. The Numbers below branches represent bootstrap values based on 1000 replicates.

Conclusion

The salient outcome of this study is the presentation of the first report on the application of PCR for sensitive and specific detection of *F. xylarioides,* the cause of CWD. This will be a great opening for research areas that were not possible before, including alternative host, effect of agronomic studies on the pathogen load, seed system certification and quarantine check on materials. This study contributes to impact on production and improvement in livelihoods and socioeconomic development in people involved in coffee production value chain. The primers can be robustly used since *in silico* test indicated the ability to detect the Arabica coffee pathogen strain as well. These primers can only detect the presence of the pathogen so there is need to develop quantitative PCR (qPCR) as well to quantify the pathogen load in soil and plant tissues.

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