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ISOLATION AND MOLECULAR IDENTIFICATION OF DOMINANT FUNGAL ENDOPHYTES FROM GREEN LEAVES OF PHYSIC NUT (*Jatropha curcas*) FROM UNILORIN PLANTATION, ILORIN, NIGERIA

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Abstract

Jatropha curcas is an important crop grown in the plantations of the University of Ilorin for research and commercialization. *J. curcas* has been reported to have high tolerance for stress, pests and diseases. Different parts of *Jatropha* are utilised for various uses both domestically and medicinally, thus, this study was carried out to unravel the dominant endophytic fungi associated with green leaves of *J. curcas* in the said plantation. The dominant fungi were isolated and characterized using the internal transcribed spacer (ITS) region of the ribosomal DNA. Green leaves of *J. curcas* were collected and processed for isolation of endophytic fungi using water agar. The putative endophytic fungi from *J. curcas* were identified using morphological and molecular methods as *Curvularia geniculata* and *Phyllosticta capitalensis*. The results of this study will be an addition to the fungal endophytes identified on *J. curcas* and a potential source of secondary metabolite production. This study constitutes, to the authors' knowledge, the first report of *C. geniculata* and *P. capitalensis* as endophytes in *J. curcas* leaves, in Nigeria.

Keywords: *Curvularia*, Endophytic fungi, ITS, *Jatropha*, *Phyllosticta*, Water agar.

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1. Introduction

The plant *Jatropha curcas* L. is a medium, deciduous multipurpose tree in the family Euphorbiaceae*. J. curcas* is a drought resistant, perennial and highly stress adaptable plant that can grow well in marginal and poor soils (Jongschaap *et al.*, 2007). This plant is propagated for its benefits as a source of biofuel, for soil erosion control, as boundary fences, and for its industrial, medicinal and traditional uses (Kumar & Sharma, 2005).

Previous studies have reported the isolation and identification of endophytic fungi from important plants such as *Pinus* spp. (Huang *et al.*, 2009; Jae-Joon & Ahn-Heum, 2012; Fouda *et al.*, 2015), *Caralluma acutangula*, *Rhazya stricta*, *Moringa peregrine* (Khan *et al.*, 2017)*, Phyllanthus amarus, Centella asiatica, and Zingiber sp*. (Nalini *et al.*, 2014).

Fungal endophytes have been identified from seeds (Ikhatua & Dikoru, 2016) and leaves of *J. curcas* using morphological and molecular techniques (Kumar & Kaushik, 2013). The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA were used by Kumar and Kaushik (2013) to identify endophytes from *J. curcas* in India. This technique provides more accurate identification over the morphological methods of fungal identification. Fungal endophytes previously reported from *J. curcas* includes *Colletotrichum truncatum, Nigrospora oryzae, Fusarium proliferatum, Guignardia cammillae, Alternaria destruens*, *Chaetomium* sp. (Kumar & Kaushik, 2013), *Fusarium* spp., *Phoma* spp., *Colletotrichum* sp. (Ikhatua & Dikoru, 2016), *Curvularia geniculata*, *Lasidiplodia theobromae*, *Trichoderma harzianum*, *Mucor* sp., *Penicillium* sp. (Stephen *et al.*, 2017), *Curvularia*, *Colletotrichum* (Nasiru *et al.*, 2015), and *Fusarium* sp. (Zarafi & AbdulKadir, 2013). However, there has been no report on fungal endophytes from *Jatropha curcas* leaves in Nigeria. This study therefore reports the isolation and molecular characterization, using the ITS1-5.8S-ITS2 region of rDNA, of fungal endophytes from *J. curcas* collected from the plantations of the University of Ilorin, Ilorin, Nigeria. The resulting putative fungal endophyte isolates from this study will be an addition to the list of fungal endophytes identified on *J. curcas* and a potential alternative source of secondary metabolite production.

2. Material and Methods

2.1 Plant sample collection and Isolation of fungi

Healthy green leaves were collected from seven year old plants of *J. curcas* grown within the plantations of University of Ilorin (Unilorin), (80°27'48.0"N 40°38'57.11" **E**), Nigeria. The leaf samples were randomly collected from five different plants. The leaf samples collected were kept in different sterile polythene bags, labelled and transferred to the laboratory for isolation of fungal endophytes.

Endophytic fungi were isolated from the leaves following the method of Rakotoniriana *et al.*, (2008) with modifications. The leaves were washed with tap water, cut into small segments of about 1-2 cm^2, surface sterilised in 70 % ethanol for 3 mins followed by 5 % NaOCl (bleach) for 3 mins and then washed 5 times with sterile distilled water after which the leaves were left to dry under sterile conditions. The leaf segments were then placed on water agar (WA) media and incubated for 2 weeks at room temperature. Imprints of the leaf surfaces were made on PDA (Microxpress, India) to'ascertain the effectiveness of the surface sterilisation procedure used'. Fungal hyphal tips growing from the leaves were transferred to Potato Dextrose Agar (PDA) media to obtain pure cultures.

2.2 Morphological Characters of the Endophyte Isolates

Cultures were grown on PDA for macroscopic and microscopic examination. The isolates were incubated at room temperature (27 ± 2 °C) under alternate 12 hr light and darkness. Macroscopic features such as colony shape and margin, colour (surface and reverse), texture and diameter of the colonies were recorded after 1 - 2 weeks of growth.

Fungal structures were mounted on glass slides in lactophenol cotton blue under a light microscope CH model to observe their microscopic features. The morphological features of the isolates were recorded and used in identifying the isolates to genus level, after which molecular characterization was carried out for species identification of the isolates.

2.3 Fungal DNA extraction, Amplification and Sequencing

Seven day old pure cultures of the morphologically similar isolates were used for the genomic DNA extraction. DNA extraction was carried out with a commercial kit (Zymo research fungal mini prep (USA)) following the manufacturer's instructions. The Polymerase Chain Reaction (PCR) was carried out using the primer pairs ITS 5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.*, 1990) was then carried out to amplify the internal transcribed spacer (ITS) gene regions of the total genomic DNA in a Labnet Multigene thermocycler.

The 25 µl PCR mixture was prepared by adding 12.5 µl of Taq 2X PCR master mix (New England Biolabs), 1 µl of each primer (10 µM) (ITS 5 and ITS 4), 9.5 µl of double-sterilised distilled water (ddH_2O) and 1 µl of the DNA template. For the control reaction, ddH_2O was used as the template instead of the DNA. The PCR programme used was 2 mins at 94 ˚C, followed by 35 cycles at 94 ˚C for 1 min, 55 ˚C for 1 min, 72 ˚C for 1 min and final extension at 72˚C for 10 mins.

Sanger sequencing of the PCR products were carried out in an ABI PRISM sequencer at a private sequencing company (Inqabba Biotec Pvt) in Pretoria, South Africa using the same forward and reverse primers.

2.4 DNA Sequence Assembly and Alignment

After the sequencing results were received, SeqTrace version 0.9.0 (Stucky, 2012) was used to obtain a consensus DNA sequence from both the forward and reverse sequences. Sequence similarity searches were performed using the consensus DNA sequence to compare with similar sequences in the GenBank database maintained by the National Center for Biotechnology Information (NCBI) using the on-line tool BLAST-n on the GenBank website (http://blast.ncbi.nlm.nih.gov).

Similar sequences and outgroup species sequences from reputable sources were downloaded from GenBank (Table 1 and 2). AliView version 1.17-beta1 software (Larsson, 2014) was used to do the alignment and the DNA sequences of our isolates were submitted to GenBank for accession numbers.

2.5 Phylogenetic Analysis

Maximum parsimony and Bayesian analyses were done using PAUP (Phylogenetic Analysis Using Parsimony) software v.4.0b10 (Swofford, 2002) and MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003) respectively.

For PAUP analysis, all characters were equally weighted and gaps were treated as missing data. Parsimonious trees were inferred using the heuristic search option with Tree-Bisection Reconnection (TBR) branch swapping and 1000 random

Table 1. ITS Sequences used in this study for the phylogenetic analysis of *Phyllosticta* species isolated from physic nut (*J. curcas*) with their GenBank accession numbers.

S/N	Isolate name	GenBank Accession Number	Source of Isolation
1.	***Phyllosticta capitalensis***	**MF595520**	***Jatropha curcas* (this study)**
2.	*P. elongata*	KC172070	*Atractylodes lartoea*
3.	*P. elongata*	KX424992	Unidentified
4.	*P. capitalensis*	KP998485	*Diospyros kaki*
5.	*P. mangiferae*	AY816311	*Citrus* sp.
6.	*P. camelliae*	GU066668	*Citrus latifolia*
7.	*P. mangiferae*	JN791605	*Citrus maxima*
8.	*P. mangiferae*	HM807531	*Citrus maxima*
9.	*P. cammeliae*	FJ462743	Unidentified
10.	*P. capitalensis*	KU671305	*Flacourtia inermis*
11.	*P. capitalensis*	KJ883595	*Coffea Arabica*
12.	*P. capitalensis*	KR056285	*Sonneratia alba*
13.	*P. musicola*	FJ538334	*Citrus maxima*
14.	*P. musicola*	NR137716	*Citrus* sp.
15.	*P. mangiferae*	EU273524	Unidentified
16.	*P. mangiferae*	AM403717	*Centella asiatica*
17.	*P. vaccinii*	GU066692	Fruit
18.	*P. mangiferae*	AB731125	*Quercus myrsinaefolia*
19.	*P. citricarpa*	GQ906476	*Citrus maxima*
20.	*P. citricarpa*	KC556921	*Citrus sinensis*
21.	*P. citricarpa*	HQ008221	*Citrus sinensis*
22.	*P. citricarpa*	GU060465	*Catharanthus roseus*
23.	*P. gaultheriae* (Outgroup)	NR144916	*Gaultheria humifusa*
24.	*P. bidwellii* (Outgroup)	KF851312	*Citrus* sp.
25.	*P. camelliae*	GU066719	Fruit

sequence additions. Maximum trees were set up to maximum 10000, branches of zero length were collapsed and all multiple parsimonious trees were saved. The robustness of the most parsimonious tree was evaluated by 1,000 bootstrap replications resulting from maximum parsimony analysis, each with 10 replicates of random stepwise addition of taxa (Felsenstein, 1985).

Tree descriptive statistics such as tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated. The Kishino-Hasegawa tests (Kishino & Hasegawa, 1989) were performed to determine whether the trees inferred under different optimality criteria were significantly different.

For Bayesian analysis using MrBayes, the GTR model (no rate variation) was selected and used for the Bayesian inference analysis of four simultaneous Markov Chains Monte Carlo (MCMC) chains for 2 000 000 generations and trees were sampled every 1000th generation. The trees generated were viewed and annotated in TreeGraph 2 (Stöver & Müller, 2010).

The morphological characters of the pure cultures of *Phyllosticta* grown at room temperature (27 ± 2 °C) for 2 weeks on PDA (Plate 1) showed that they were slow growing , had a diameter of 3-4 cm, was grey to dark grey colour in the centre with overlapping ridges and grey to whitish towards the margin, was irregular in shape; the mycelium was moderately aerial, smooth and woolly; the reverse side of the colony was black at the centre ,grey towards the margin and whitish at the margin.

Table 2. ITS Sequences used in this study for the phylogenetic analysis of *Curvularia* species isolated from physic nut (*J. curcas*) with their GenBank accession numbers.

S/N	Isolate name	GenBank Accession Number	Source of Isolation
1.	***Curvularia geniculata***	**MF595521**	***Jatropha curcas* (this study)**
2.	*C. lunata**	NR 138223	Culture from neotype
3.	*C. inaequalis*	AY941256	Human
4.	*C. lunata*	DQ836800	Grain
5.	*C. lunata*	GU966505	Environmental sample
6.	*C. senegalensis*	HG779001	Clinical specimen
7.	*C. geniculata*	KP131873	Toe nail
8.	*C. geniculata*	KX022493	*Dracaena surculosa*
9.	*C. geniculata*	KY310634	Clinical sample
10.	*C. geniculata*	KT150263	*Calamus thwaitesii*
11.	*C. geniculata*	KX022497	*Dracaena fragrans*
12.	*C. geniculata*	KJ909781	*Sorghum bicolor* Seeds
13.	*Cochliobolus geniculatus*	HE861840	Clinical sample
14.	*C. fallax*	JQ360963	*Oryza sativa* (Rice)
15.	*Alternaria alternata* (Outgroup)	FJ196306	*Triticum aestivum* (Wheat)

3. Results

3.1 Isolation and Morphology of the Endophytes

Endophytes in the genus *Phyllosticta* and *Curvularia* were isolated and identified. These isolates were dominantly observed growing out of all the leaves inoculated on agar media.

The morphological characters of pure cultures of *Curvularia* at room temperature (27 ± 2 °C) after 7 days (Plate 2) showed fast growth, had a diameter of 8 cm, comprised white aerial mycelia with the reverse side of the colony deep brown at the centre and light brown towards the margin.

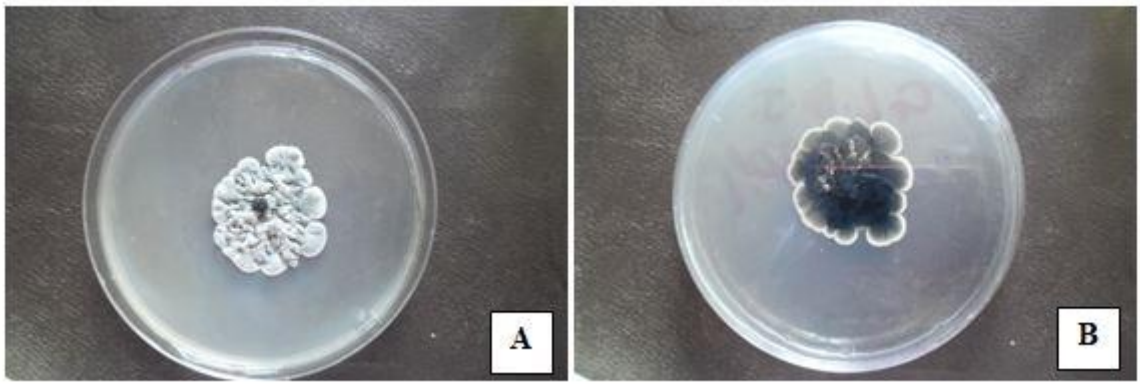

Plate 1. Pure cultures of *Phyllosticta capitalensis* after 2 weeks of growth. A. Front view, B. Reverse view.

3.2 Molecular Identification and Phylogenetic Analysis of the Isolates

Molecular characterization was done to confirm the identity of the isolates to the species level. The DNA sequence of the ITS regions of *Phyllosticta* and *Curvularia* isolates were 623 bp and 469 bp respectively. A BLAST-n search in the GenBank using these ITS sequences showed over 99–100 % identity with several sequences of *P. capitalensis*, *Guignardia capitalensis*, *P. mangiferae*, *P. musicola* and *P. citricarpa* for the *Phyllosticta* isolate while *Cochliobolus geniculata*, *Curvularia geniculata*, *C. affinis*, and *C. clavata* all showed 99 % similarity score with the *Curvularia* isolate.

The molecular phylogenetic relationships of the isolates were further inferred with phylogenetic analysis software (PAUP and MrBayes) using the ITS sequence regions (ITS1-5.8S-ITS2). For *Phyllosticta*, 25 sequence dataset were analysed (Table 1) (including two outgroup sequences) which comprised 697 characters after alignment, 83 characters were parsimony informative, 72 were variable and parsimony uninformative, and 542 were constant/conserved. Parsimony analysis was set to maximum trees 10000 (Tree length (TL) = 195, Consistency index (CI) = 0.9179, Retention index (RI) = 0.9295,

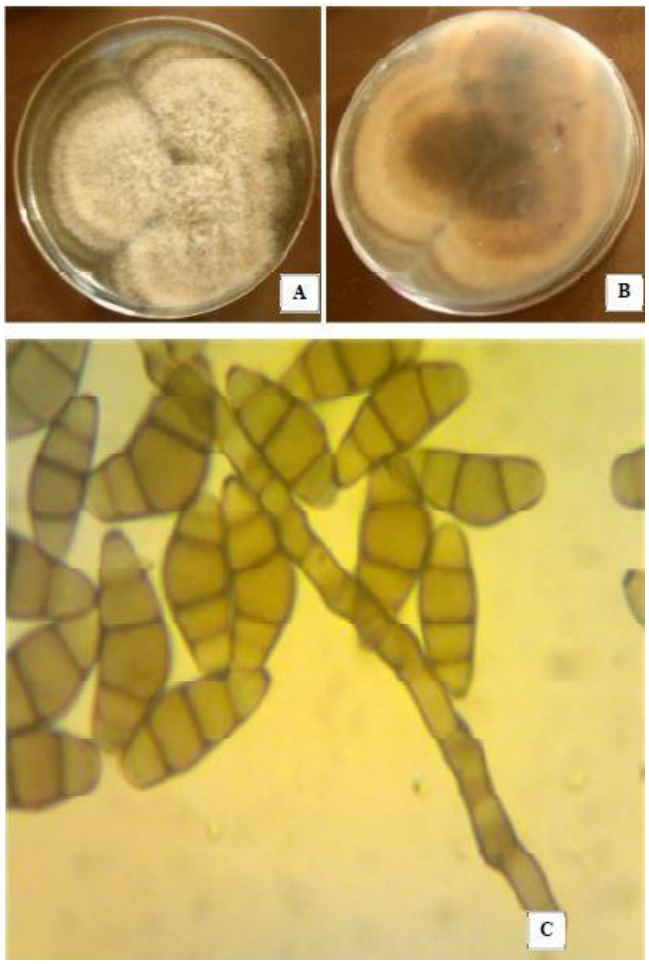

Plate 2. Pure cultures of *Curularia geniculata* after 2 weeks of growth. A. Front view, B. Reverse view.C. Conidia with conidiophores, X1000

Rescaled consistency index (RC) = 0.8532, Homoplasy index (HI) = 0.0821) and the bootstrap majority-rule consensus tree is shown in Fig. 1.

GTR+I+G model was selected in Mr-Modeltest 2.3. (Nylander, 2004). MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003) was used to generate phylogenetic trees under optimal criteria. The Markov Chain Monte Carlo (MCMC) analysis used four chains which were sampled every 1000th generation and the analysis was stopped at average standard deviation of split frequencies below 0.01.

For *Curvularia*, 15 sequences (Table 2.) were analysed in MrBayes including the outgroup *Alternaria alternata*. GTR+I+G model was selected in Mr-Modeltest 2.3. (Nylander, 2004), MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003) was used to generate phylogenetic trees under optimal criteria.

The Markov Chain Monte Carlo (MCMC) analysis used four chains and was sampled every 1000th generation and the analysis was stopped at average standard deviation of split frequencies below 0.01. The consensus tree is shown in Fig. 2.

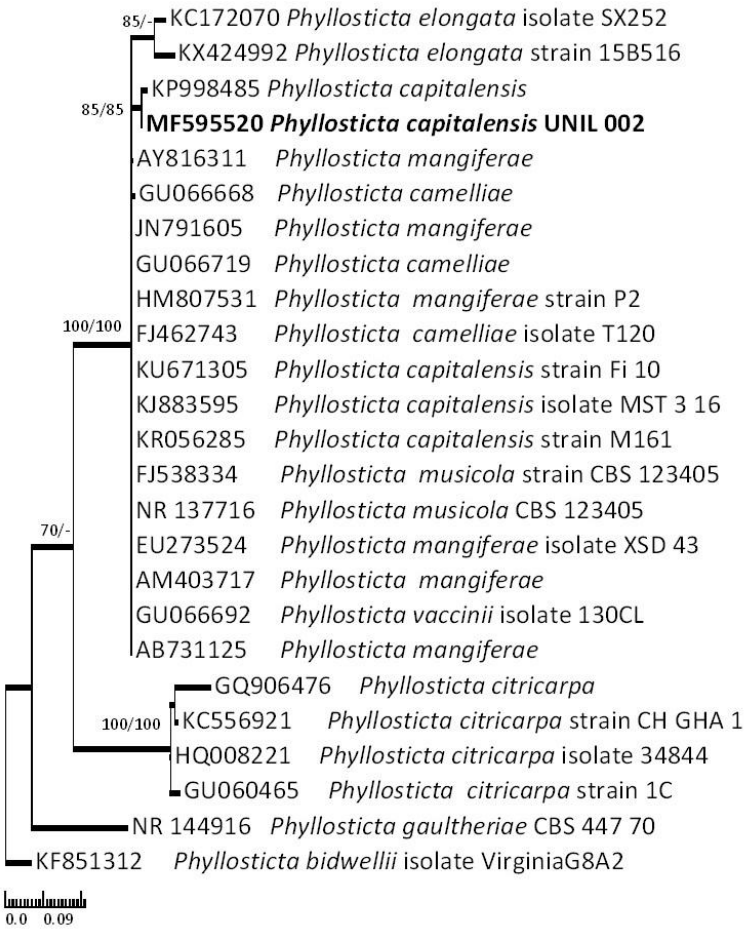

Figure 1: Bootstrap majority-rule consensus tree generated from the ITS (ITS1, 5.8S and ITS2) gene sequences of 25 strains showing the relationship of the isolate from this study (in bold) with other *Phyllosticta* species. Given at the nodes are the Maximum Parsimony Bootstrap support values (MPB) greater than 50% (top) and Bayesian Posterior Probabilities (BPP) (bottom).

Based on the molecular phylogenetic analysis of the ITS regions of the isolated fungi in this study, they were identified as *P. capitalensis* (Figure 1.) and *C. geniculata* (Figure 2.).

4. Discussion

Phyllosticta species have not earlier been reported on *Jatropha*, but have been isolated from several plants such as *Guazuma tomentosa* (Srinivasan *et al.*, 2010), *Hugonia mystax* (Abirami & Boominathan, 2016),

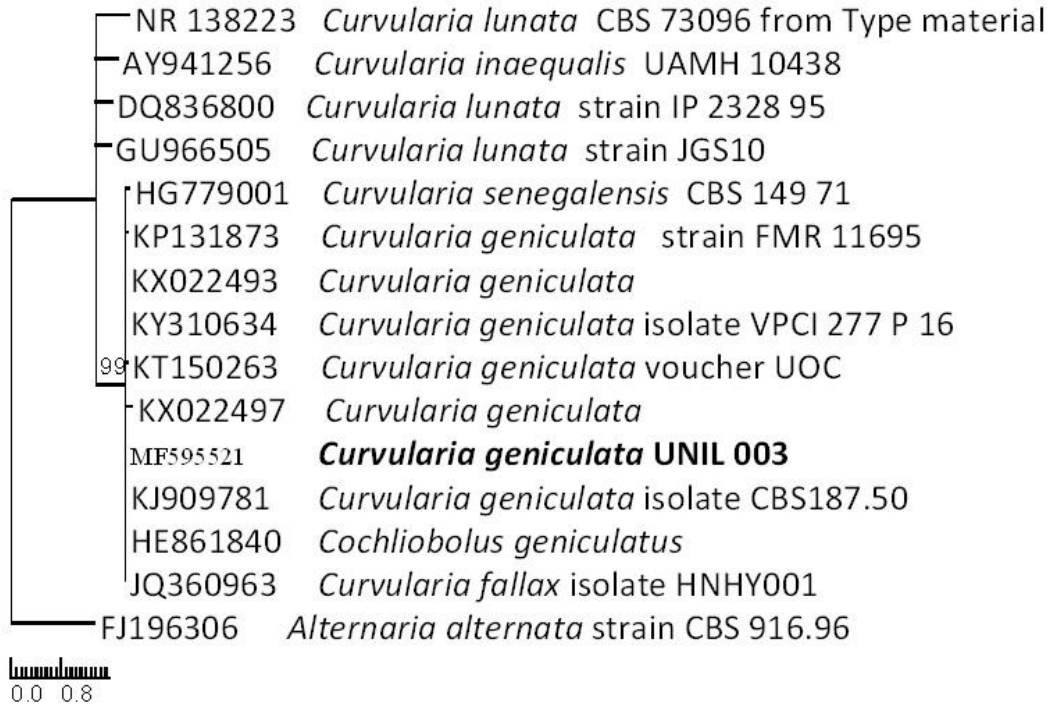

Figure 2: Consensus tree generated from the ITS (ITS1, 5.8S and ITS2) gene sequences of 15 strains showing the relationship of the isolate from this study (in bold) with other *Curvularia* species with *Alternaria alternata* as the outgroup. Given at the nodes are the Bayesian Posterior Probabilities (BPP).

Pure cultures of the isolates were deposited in the Department of Plant Biology, University of Ilorin culture collection. The DNA sequence of *Phyllosticta capitalensis* and *Curvularia geniculata* were deposited in the GenBank with accession numbers MF595520 and MF595521 respectively.

Abies concolor, *Aloe ferox*, *Citrus maxima*, *Taxus baccata*, *Mangifera indica* and *Leucothoe caterbaei* (Wikee *et al.*, 2013) as either endophytes plant pathogens or saprobes (Baayen *et al.*, 2002; Glienke-Blanco *et al.*, 2002; Okane *et al.*, 2003; Srinivasan *et al.*, 2010; Wikee *et al.*, 2013; Abirami & Boominathan, 2016).

They have been specifically reported as only endophytes from Citrus (Glienke-Blanco *et al.*, 2002), Mango (Glienke-Blanco *et al.*, 2002; Wikee *et al.*, 2013), and Guava (Glienke-Blanco *et al.*, 2002). In this study, *Phyllosticta capitalensis* was isolated as a putative endophyte from *Jatropha curcas* leaves. The isolation of *P. capitalensis* from *Jatropha curcas* leaves represents the first report on their occurrence as an endophyte on this plant in Nigeria. Some species of *Phyllosticta* have also been reported to produce useful secondary metabolites with antimicrobial and antioxidant properties (Srinivasan *et al.*, 2010; Abirami & Boominathan, 2016).

Curvularia species have been reported as a pathogen on *Jatropha curcas* leaves (Nasiru *et al.*, 2015; Stephen *et al.*, 2017), and *Sorghum bicolor* (Akram *et al.*, 2014), as endophytes in *Aquilaria malaccensis* (Premalatha & Kalra, 2013), *Tabernaemontana heyneana* (Manasa & Nalini, 2014), *Musa acuminata* (Zakaria *et al.*, 2016), *Artemisia lavandulifolia, A. tangutica, A. brachyloba, A. subulata, A. argy, A. scoparia* (Cosoveanu *et al.*, 2016), *Hugonia mystax* (Abirami & Boominathan, 2016), *Macaranga* sp. (Lateef *et al.*, 2016) and *Ricinus communis* (Sandhu *et al.*, 2014). In this study, *C. geniculata* was isolated as an endophyte from the leaves of *J. curcas*. The isolation of *C. geniculata* as an endophyte from healthy *J. curcas* suggests that this fungal species although present as endophytes in the leaves, could cause a diseased condition when plants are subject to stress.

Fungi in the genera *Phyllosticta* and *Guignardia* were formerly named separately, but with molecular method of identification (van der Aa & Vanez, 2002; Wulandari *et al.*, 2010), it was discovered that *Phyllosticta* and *Guignardia* were the asexual and sexual states of the same fungi, and following the rules of the International Code of Botanical Nomenclature (ICBN), *Phyllosticta* was preferred (Glienke *et al.*, 2011) being the oldest name (Seifert & Rossman, 2010). Similarly, as seen in the phylogenetic tree above, *Cochliobolus* and *Curvularia* both refers to the sexual and asexual states of the same fungus in which *Cochliobolus* is the old name being used for *Curvularia* species.

Phyllosticta capitalensis (Evidente *et al.*, 2008a) and *Curvularia geniculata* (Gunatilaka, 2006) have been reported to be useful as a source of secondary metabolites such as phyllostin, phyllostoxin, phyllostictines A–D (Evidente *et al.*, 2008a), and phyllosinol (Wijeratne *et al.*, 2008) and Taxol (Kumaran *et al.*, 2010) which are all of pharmaceutical, medical and agricultural application. *Phyllosticta* species have also been used as biocontrol agent in nematode control (Yan *et al.*, 2011) and also as a herbicide (Evidente *et al.*, 2008a, 2008b).

Isolation of *C. geniculata* and *P. capitalensis* from *J. curcas* and other numerous medicinal plants is an indication of their common occurrence in plants with therapeutic abilities. However, these fungi can assume a pathogenic condition when the host plant is under stress.

Molecular identification of these two fungi (*P. capitalensis* and *C. geniculata*) aided their identification accurately to the species level which is an advancement over the morphological method of identification.

Following this study, the biotechnological applications, such as metabolites production, plant growth stimulation and biocontrol potentials, of the two isolated fungal species would be carried out. This findings of this study would eventually lead to the discovery and use of indigenous isolates for biotechnological applications.

5. Conclusion

This study revealed the occurrence and molecular identification of *P. capitalensis* and *C. geniculata* on leaves of *Jatropha curcas* collected from a plantation in the University of Ilorin, Nigeria The molecular identification of the fungi was based on the nucleotide sequences of their ITS regions. This study also represents the first report of *P. capitalensis* from a new host plant, *Jatropha curcas* and also of *P. capitalensis* and *C. geniculata* as putative endophytes from *J. curcas* in Nigeria. Further investigations are required to confirm that they have an endophytic life style in *Jatropha curcas.* The ecological and biotechnological applications of these fungi will be of great importance in further studies.

Acknowledgment

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