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Diversity of Fungi Associated with Mirid (Hemiptera: Miridae) Feeding Lesions and Dieback Disease of Cocoa in Ghana

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ABSTRACT

Dieback disease causes heavy yield losses of cocoa in West Africa and for over a century, control strategies have involved insecticide use against mirids whose feeding punctures provide access for fungal infection. Information on the true identity of the causal pathogens is needed for enhanced understanding of the epidemiology of the disease and for development of integrated control strategies. So, the aim of the research was to address the role and identity of the different fungi associated with dieback disease of cocoa in Ghana. Fungal isolates were extracted from severely infected cocoa tissue from Ghana at Silwood Park Campus, Imperial College London during 2006-2009. Morphological characteristics comprised colour and shape of colonies and sizes of conidia. The genetic diversity was evaluated using the Qiagen DNeasy Plant Mini Kit protocol to extract genomic Deoxyribonucleic Acid (DNA) followed by Polymerase Chain Reaction (PCR) and sequencing of the Internal Transcribed Spacer (ITS) region of ribosomal DNA. The procedure confirmed the presence of 117 fungal isolates. The most frequently isolated genera were *Fusarium* (dominant) and *Lasiodiplodia*. The *Fusarium* isolates were identified as *F. chlamydosporum*, *F. solani*, *F. oxysporum* and *F. proliferatum*. The *Lasiodiplodia* isolates were identified as either *L. pseudotheobromae* or *L. theobromae*. *Fusarium* and two *Lasiodiplodia* species are the causal agents of dieback disease identified so far by the study. Contrary to known facts, *Fusarium decemcellulare* was not isolated from any of the infected wood tissues from Ghana.

Key words: Cocoa, dieback disease, *Fusarium*, *Lasiodiplodia*, mirid, pathogen

INTRODUCTION

Cocoa is an important cash crop in West Africa. The sub-region produces 70% of the world cocoa and nearly one million people, mostly small-holder farmers derive their livelihood from the crop (Baah and Garforth, 2008). Dieback disease constitutes a major constraint to cocoa production in West Africa. Over the last six decades *F. decemcellulare* has been implicated as the major cause of dieback disease in West Africa (Crowdy, 1947). Two ascomycete fungi are now known to cause dieback of cocoa in West Africa (Adu-Acheampong, 2009). Symptoms of dieback disease are typical of fungal infection but are often confused with insect disorders due mainly to the relationship between mirid feeding punctures and disease occurrence. This probably has led to mismanagement of the disease in the past through uncontrolled application of broad-spectrum insecticides. The consequences are the resistance development in mirids (Dunn, 1963; Lowor *et al.*, 2009). As in other disease complexes, variation in pathogenicity among the isolates could have resulted from

genetic differences between strains of a single species. Otherwise, this may have arisen from the presence of multiple species.

Identification of fungi traditionally has relied upon microscopic features and by morphology of macroscopic features, colony characteristics on artificial media and biochemical reactions (Sutton and Cundell, 2004). Such approaches have served well in the past. However, specialist knowledge is needed to differentiate large and complex genera such as *Penicillium*, *Aspergillus* and *Fusarium* (Henry *et al*., 2000; Leslie and Summerell, 2006). The range of isolates described in the present study exhibit considerable morphological diversity. In *Fusarium*, the macroconidial dimensions frequently fell outside the range previously reported for *Fusarium decemcellulare* on cocoa (Leslie and Summerell, 2006). Accurate identification of isolates is a prerequisite in disease resistance studies. The possibility that mixed infections by multiple species might occur makes it more important for reliable identification of all species involved in dieback disease. Molecular methods (Cumagun *et al*., 2007) are currently widely used to characterize organisms as they give quick and more reliable results. In the last decade, numerous DNA-based methods have been developed to improve the diagnosis of fungal infections and the identification of plant pathogenic and other fungi (Gottfredsson *et al*., 1998). These methods are particularly promising because of their simplicity, specificity; sensitivity and potential for scaling up to handle large numbers of isolates. For example, PCR methods targeting different genes have been described elsewhere for identification of *Cryptococcus neoformans* (Tanaka *et al*., 1996), *Aspergillus fumigatus* (Kobayashi *et al*., 1999), *Candida* (Hidalgo *et al*., 2000) and species of smut fungi (Singh *et al*., 2004). A number of studies have also described probes, restriction fragment length polymorphism, or other methods to identify unique ribosomal DNA (rDNA) sequences (Evertsson *et al*., 2000; Izadi and Moosawi-Jorf, 2007). The most common approaches have targeted portions of the fungal rDNA and sequence information on a wide range of fungi has accumulated in the public database (Braun *et al*., 2000). Regardless of the occasional misidentification of deposited sequences, molecular tools represent an invaluable resource with which to either characterize new isolates or to confirm the identity of name isolates. The objectives of this study were to address the role and identity of the different fungi associated with dieback disease of cocoa in Ghana.

MATERIALS AND METHODS

Fungal isolates from infected cocoa stems and growth conditions: Experimental samples of *Fusarium* and *Lasiodiplodia* were obtained from severely infected cocoa stems (cut to ~15 cm long) from cocoa farms in the Eastern Region (06°13' N, 00°21' W) of Ghana. Isolations were done at Silwood Park Campus, Imperial College London, UK during 2006-2009. Culture media used were Potato Dextrose Agar (PDA), Potato Carrot Agar (PCA), Carnation Leaf Agar (CLA) and Cocoa Pod Husk Agar (CPHA). Infected stem branches were sterilised with 70% ethanol followed by 0.5% sodium hypochlorite. Sterilised wood pieces were rinsed in sterile de-ionized water, blotted dry and incubated for 14 days at 30.0±2.0°C under 12:12 (light: darkness).

One hundred and seventeen isolates were obtained and they belonged either to *Fusarium* spp. (75 isolates) or *Lasiodiplodia* (36 isolates) (Table 1). Four *Fusarium* isolates received as PDA cultures were collected from diseased cocoa in the Sefwi-Boako area (06°19' N, 02°30' W) of the Western Region of Ghana.

Colony and conidial morphology: Two reference isolates of *F. decemcellulare* (IMI 380504 and IMI 361352) and one *L. theobromae* isolate (IMI 333797) were obtained from the CABI Fungal Genetic Resource Collection. Dr Gary Samuels of the United States Department of Agriculture (USDA) also kindly provided two-reference *F. decemcellulare* isolates (viz. GJS 03-81 and

GJS 01-170). Growth was continuously observed and the macroscopic characters (colour, sector, border and texture) (Chehri *et al*., 2010) were determined. Conidia measurements and spore counts were done using a light microscope (x 400 magnification) and a haemocytometer (Fuchs-Rosenthal), respectively.

Growth rate measurement of colonies: Linear growth rates of the isolates were determined on PDA plates. Plates were incubated in the dark at a temperature range of 10-35 at 5°C intervals. Mycelial growth measurements were recorded at 24 h interval until the colonies reached the edge of the plate.

DNA extraction and amplification: Mycelium was ground into fine powder in sterile mortar with liquid nitrogen. Genomic DNA was extracted and purified using the Qiagen DNeasy Plant Mini Kit (Qiagen Ltd., Qiagen House, Fleming Way, West Sussex, UK) according to the manufacturer's instructions. Presence of DNA was confirmed by running the samples on Tris-acetic acid-EDTA-agarose gel (0.8%, w/v) and their concentrations determined using an automated Eppendorf BioPhotometer.

The Internal Transcribed Spacer (ITS) fragment was amplified by two universal fungal primer, ITSI and ITS4 (White *et al*., 1990; Bandyopadhyay and Raychaudhuri, 2010). Each 25 μL PCR reaction mixture consisted of 12 μL PCR Biomix™ Red (Bioline), 2.5 ng of template genomic DNA, 0.5 μL of each primer (10 pmol), 0.5 μL of $MgCl_2$ (50 mM) and 9 μL of DNase free water. Control amplifications using primers only were made to ensure the reagents used were not contaminated with extraneous DNA. PCR reaction was conducted using a Whatman Biometra T1 Thermocycler according to the following protocol: initial denaturing at 95°C for 5 min, 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 45 sec. These cycles were repeated 35 times with a final step at 72°C for 7 min and then storage at 4°C. The PCR products were detected by agarose gel [0.8% (w/v)] electrophoresis and staining with GelRed™ Nucleic Acid Gel Stain with subsequent visualisation and photography under UV transilluminator.

DNA sequencing: All PCR products were purified from agarose gel before DNA sequence analysis using a Genomics Montage Millipore gel extraction kit (Millipore Corporation, Bedford, MA 01730 USA). The protocol per reaction comprised template DNA 2.0 μL, primer (10 pmol) 1.0 μL, 5×BigDye Buffer 1.75 μL, ABI BigDye (v3.1) Mix 0.5 and ddH_2O 4.75 μL. The samples were run 35 PCR cycles in a Thermal Cycler (Uno II, Biometra). Purified amplicons were then sequenced on both strands using the same primers as described above and BigDye Terminator System, version 3.1 (Applied Biosystems, Foster City, California). Products were analyzed on an automated capillary DNA sequencer (ABI Prism 377) according to the manufacturer's directions.

Sequence alignment and phylogenetic analysis: The sequence chromatograms were edited using Sequencher 4.8 software (Genecodes Corp., Ann Arbor, Michigan) and searches were carried out in BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to determine the closest matches in the public database. The ITS sequences of *Fusarium* and *Lasiodiplodia* were manually aligned with MacClade 4.06 OSX. A likelihood bootstrap analysis was performed with 100 replications using RAxML 7.0.4 (Stamatakis *et al*., 2008).

RESULTS

Pathogen isolation and description: One hundred and seventeen isolates were studied. Microscopic examination showed that 81 isolates were *Fusarium* spp. while 36 were *L. theobromae*

Table 1: Characteristics of *Fusarium decemcellulare, Fusarium* spp. and *Lasiodiplodia* theobromae isolates on PDA

			Morphological characteristics		
Fungus	Isolate	Geographic origin	Colony colour	*Conidium length[a]±SE	*Conidium width[a]±SE
F. decemcellulare	IMI 380504	Ghana	Pinkish	66.00±0.086	9.10±0.044
	IMI 361352	Malaysia	Pinkish	66.00±0.106	9.10±0.057
	GJS 03-81	Brazil	Pinkish	66.22±0.065	9.25±0.055
	GJS 01-170	Cameroon	Pinkish	65.85±0.117	9.12±0.048
Fusarium species	AC 037	Eastern, Ghana	Creamy-white	39.77±0.074	3.17±0.055
	AC 110	Eastern, Ghana	Creamy-white	37.90±0.044	3.42±0.053
	AC 120	Eastern, Ghana	Creamy-white	37.97±0.065	3.30±0.067
	AC 174	Eastern, Ghana	Creamy-white	37.85±0.062	3.36±0.062
	AC 197	Eastern, Ghana	Creamy-white	38.92±0.039	3.20±0.057
	AC 206	Eastern, Ghana	Creamy-white	36.57±0.101	3.47±0.048
	AC 229	Eastern, Ghana	Creamy-white	38.45±0.092	3.22±0.055
	AC 255	Eastern, Ghana	Creamy-white	37.80±0.074	3.50±0.044
	AC 276	Eastern, Ghana	Creamy-white	35.87±0.048	3.35±0.051
	AC 284	Eastern, Ghana	Creamy-white	36.77±0.074	3.43±0.053
	AC 328	Eastern, Ghana	Creamy-white	38.90±0.044	3.27±0.048
	AC 330	Eastern, Ghana	Creamy-white	34.80±0.074	3.50±0.044
	AC 402	Eastern, Ghana	Creamy-white	39.90±0.044	3.02±0.039
	AC 451	Eastern, Ghana	Creamy-white	38.80±0.074	3.41±0.044
	AC 593	Eastern, Ghana	Creamy-white	38.85±0.071	3.32±0.039
	AC 638	Eastern, Ghana	Creamy-white	37.97±0.042	3.35±0.033
	AC 656	Eastern, Ghana	Creamy-white	35.92±0.039	3.22±0.039
	AC 705	Eastern, Ghana	Creamy-white	38.85±0.062	3.40±0.044
	AC 773	Eastern, Ghana	Creamy-white	38.87±0.059	3.42±0.039
	AC 914	Eastern, Ghana	Creamy-white	37.82±0.073	3.31±0.044
	AC 317	Eastern, Ghana	Creamy-white	41.92±0.063	3.65±0.033
	AC 511	Eastern, Ghana	Creamy-white	41.97±0.042	3.47±0.042
	AC 787	Eastern, Ghana	Creamy-white	41.90±0.057	3.42±0.039
	AC 806	Eastern, Ghana	Creamy-white	41.87±0.059	3.50±0.044
	AC 031	Western, Ghana	Creamy-white	42.92±0.063	3.15±0.048
	AC 392	Western, Ghana	Creamy-white	41.85±0.071	3.32±0.039
	AC 748	Western, Ghana	Creamy-white	42.02±0.065	3.30±0.060
	AC 767	Western, Ghana	Creamy-white	43.97±0.065	3.02±0.065
	AC 076	Eastern, Ghana	Pinkish-white	61.80±0.074	4.67±0.048
	AC 211	Eastern, Ghana	Pinkish-white	59.95±0.078	4.02±0.065
	AC 219	Eastern, Ghana	Pinkish-white	58.05±0.069	3.97±0.042
	AC 361	Eastern, Ghana	Pinkish-white	62.02±0.065	4.25±0.033
	AC 551	Eastern, Ghana	Pinkish-white	60.95±0.060	4.95±0.033
	AC 580	Eastern, Ghana	Pinkish-white	60.87±0.069	5.03±0.033
	AC 739	Eastern, Ghana	Pinkish-white	58.85±0.062	4.90±0.044
	AC 768	Eastern, Ghana	Pinkish-white	61.84±0.078	4.55±0.048
	AC 995	Eastern, Ghana	Pinkish-white	60.87±0.059	4.50±0.044
Lasiodiplodia species	IMI 333797	Nigeria	Grey to black	22.44±0.133	12.25±0.11
	AC 008	Eastern, Ghana	Grey to black	22.02±0.065	12.10±0.156
	AC 036	Eastern, Ghana	Grey to black	21.90±0.057	11.90±0.057
	AC 064	Eastern, Ghana	Grey to black	21.97±0.082	12.02±0.042
	AC 068	Eastern, Ghana	Grey to black	21.92±0.095	11.97±0.065

Table 1: Continued

Fungus	Isolate	Geographic origin	Morphological characteristics		
			Colony colour	*Conidium length[a]±SE	*Conidium width [a]±SE
	AC 280	Eastern, Ghana	Grey to black	22.05±0.048	12.00±0.061
	AC 318	Eastern, Ghana	Grey to black	22.10±0.067	12.07±0.063
	AC 322	Eastern, Ghana	Grey to black	22.07±0.053	12.05±0.048
	AC 329	Eastern, Ghana	Grey to black	22.05±0.060	12.02±0.055
	AC 360	Eastern, Ghana	Grey to black	22.00±0.100	12.02±0.065
	AC 371	Eastern, Ghana	Grey to black	22.10±0.067	12.10±0.067
	AC 375	Eastern, Ghana	Grey to black	22.05±0.085	12.10±0.057
	AC 383	Eastern, Ghana	Grey to black	21.95±0.069	11.97±0.065
	AC 407	Eastern, Ghana	Grey to black	22.12±0.085	12.07±0.053
	AC 450	Eastern, Ghana	Grey to black	22.05±0.048	12.05±0.048
	AC 492	Eastern, Ghana	Grey to black	22.12±0.059	12.10±0.057
	AC 536	Eastern, Ghana	Grey to black	22.07±0.053	12.05±0.048
	AC 564	Eastern, Ghana	Grey to black	22.17±0.063	12.10±0.044
	AC 568	Eastern, Ghana	Grey to black	22.12±0.063	12.15±0.062
	AC 581	Eastern, Ghana	Grey to black	22.05±0.048	12.02±0.042
	AC 640	Eastern, Ghana	Grey to black	22.17±0.063	12.17±0.063
	AC 644	Eastern, Ghana	Grey to black	22.05±0.048	12.00±0.061
	AC 680	Eastern, Ghana	Grey to black	22.02±0.065	12.00±0.070
	AC 742	Eastern, Ghana	Grey to black	22.20±0.054	12.20±0.054
	AC 810	Eastern, Ghana	Grey to black	22.27±0.074	12.20±0.074
	AC 845	Eastern, Ghana	Grey to black	22.07±0.053	12.07±0.053
	AC 857	Eastern, Ghana	Grey to black	22.07±0.039	12.05±0.048
	AC 972	Eastern, Ghana	Grey to black	22.15±0.062	12.15±0.062
	AC 220	Eastern, Ghana	Grey to black	22.17±0.053	12.15±0.062
	AC 420	Eastern, Ghana	Grey to black	22.27±0.074	12.22±0.074
	AC 456	Eastern, Ghana	Grey to black	22.17±0.063	12.15±0.071
	AC 487	Eastern, Ghana	Grey to black	22.10±0.057	12.02±0.055
	AC 496	Eastern, Ghana	Grey to black	22.12±0.048	12.07±0.063
	AC523	Eastern, Ghana	Grey to black	22.05±0.048	12.02±0.055
	AC 639	Eastern, Ghana	Grey to black	22.15±0.062	12.12±0.069
	AC 673	Eastern, Ghana	Grey to black	22.20±0.065	12.00±0.061
	AC 718	Eastern, Ghana	Grey to black	22.20±0.054	12.05±0.069

[a]All measurements are in µm; values are Means of 20 macro-conidial* measurements

(Table 1). *Fusarium decemcellulare* was not isolated. All isolates grew on all the four test media but they grew best on PDA followed by PCA medium. Cocoa-pod-husk agar and CLA generated lower numbers of fungal colonies and growth was also slow.

Growth of *Fusarium* isolates was slow. *Fusarium decemcellulare* colony was initially white and later creamy to pinkish-red due to production of pinkish or reddish pigmentation. The species also produced moist sporodochia and copious oval to cylindrical, hyaline and 0-1 septate micro-conidia. Macro-conidia were, sickle shaped, 5 to 9 celled and 65.8 to 66.0×9.1 to 9.2 µm. Colonies of the other *Fusarium* spp. were creamy white to pinkish; macro-conidia (3 to 6 septate) measured 34.8 to 62.0×3.0 to 5.0 µm.

Colonies of *Lasiodiplodia* grew faster and mycelia were dense. Mycelia were initially white becoming either grey when under light conditions or sooty black under continuous darkness. Fruiting bodies (pycnidia) were produced as superficial outgrowths only under light. Micro-conidia

were hyaline and unicellular and rounded to ellipsoidal. Macro-conidia were two-celled, dark brown and measured 21.9 to 22.4×11.9 to 12.2 μm. The cultural characteristics of all 36 *Lasiodiplodia* obtained from infected cocoa tissue from Ghana were similar on PDA and identical to reference isolate IMI333797 identified by CABI. The observed characteristics conform to published descriptions of the species (Pavlic *et al.*, 2004).

Growth rate studies: All isolates grew within the temperature range of 15-35°C (Fig. 1). Radial growth rates in *Lasiodiplodia* isolates were higher (mean 14.75 mm per day) than in *F. decemcellulare* (1.50 mm per day) or the other *Fusarium* spp. (2.00 mm per day). All isolates had growth optima at 30°C.

DNA amplification, sequencing and comparative GenBank data analysis: Two phylogenetic trees were constructed using RAxML for *Fusarium* and *Lasiodiplodia*. An assessment of the action of the primers on the extracted genomic DNA indicated that the primers amplified products of the test DNA samples and resolved prominent bands of around 550 bp at the selected annealing temperature of 50°C. Figure 2 shows PCR products (amplicons) of some of the fungal isolates on agarose gels. The 18S ribosomal RNA gene, partial sequence; internal transcribed spacer

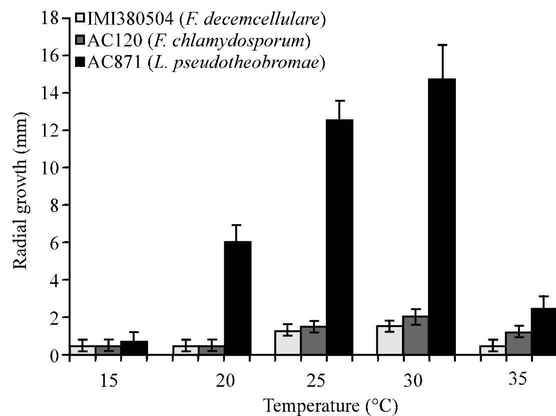

Fig. 1: Radial growth rates (mm per 24 h) of fungi from cocoa stems. Mean of 5 replicates. Bar indicates standard error

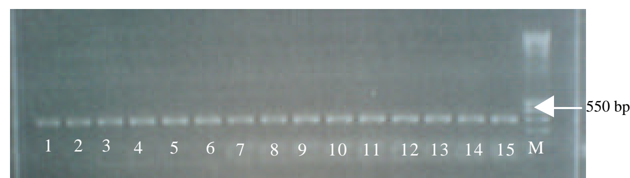

Fig. 2: Amplification products of some *Fusarium* and *Lasiodiplodia* isolates by primers ITS 1 and ITS 4; lane M, 100-bp DNA length ladder; (hyper ladder iv); lane 1, isolate IMI 380504; lane 2, isolate IMI 361352; lane 3, isolate AC 995; lane 4, isolate AC806; lane 5, isolate AC 511; lane 6, isolate AC 551; lane 7, isolate AC 638; lane 8, isolate, 773; lane 9, isolate IMI 333797, lane 10, AC 492; lane 11, AC 845; lane 12, AC 972; lane 13, AC 857; lane 14, AC 680; lane 15, AC 810

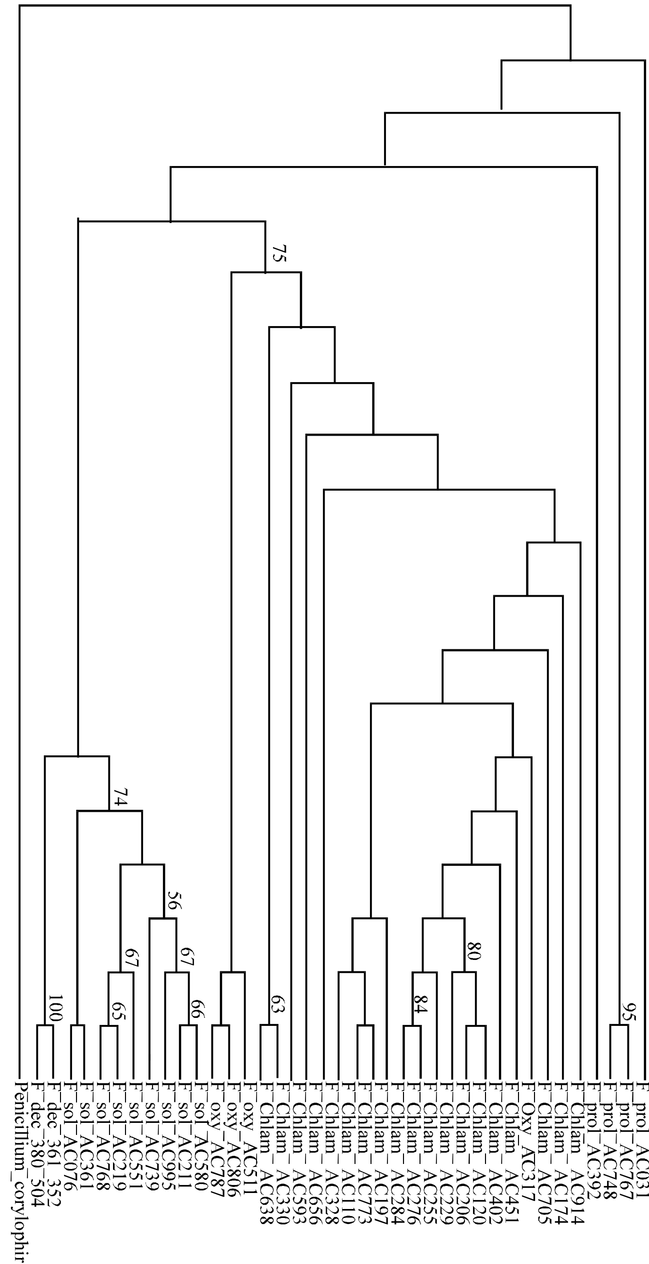

Fig. 3: Cladogram revealing the relatedness of isolates of *Fusarium* based on 400-500 nucleotides from ITS1 and ITS2 rDNA. The DNA sequences were aligned with MacClade and trees were constructed with RAxML. The tree was rooted with *Penicillium corylophilum*. Numbers given on branches are bootstrap values indicating the confidence level from a 100-replicate bootstrap sampling. (Frequencies below 50% are not included)

1, 5.8S ribosomal RNA and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene (Abdel-Motaal *et al.*, 2009), partial sequence of all isolates from Ghana and the CABI reference isolates were successfully amplified from DNA by the fungus-specific universal primer pair ITS1-ITS4.

Identification of *Fusarium* isolates by ITS sequence analysis: The two *F. decemcellulare* isolates were confirmed as *Nectria rigidiuscula* (anamorph *F. decemcellulare*) by likelihood cladogram analysis in the current study. The ITS sequence of the remaining 37 isolates showed close matches to the ITS sequence of *F. chlamydosporum*, *F. oxysporum*, *F. proliferatum* and *F. solani*). Together with *F. decemcellulare*, the phylogenetic tree divided them into four clades (Fig. 3).

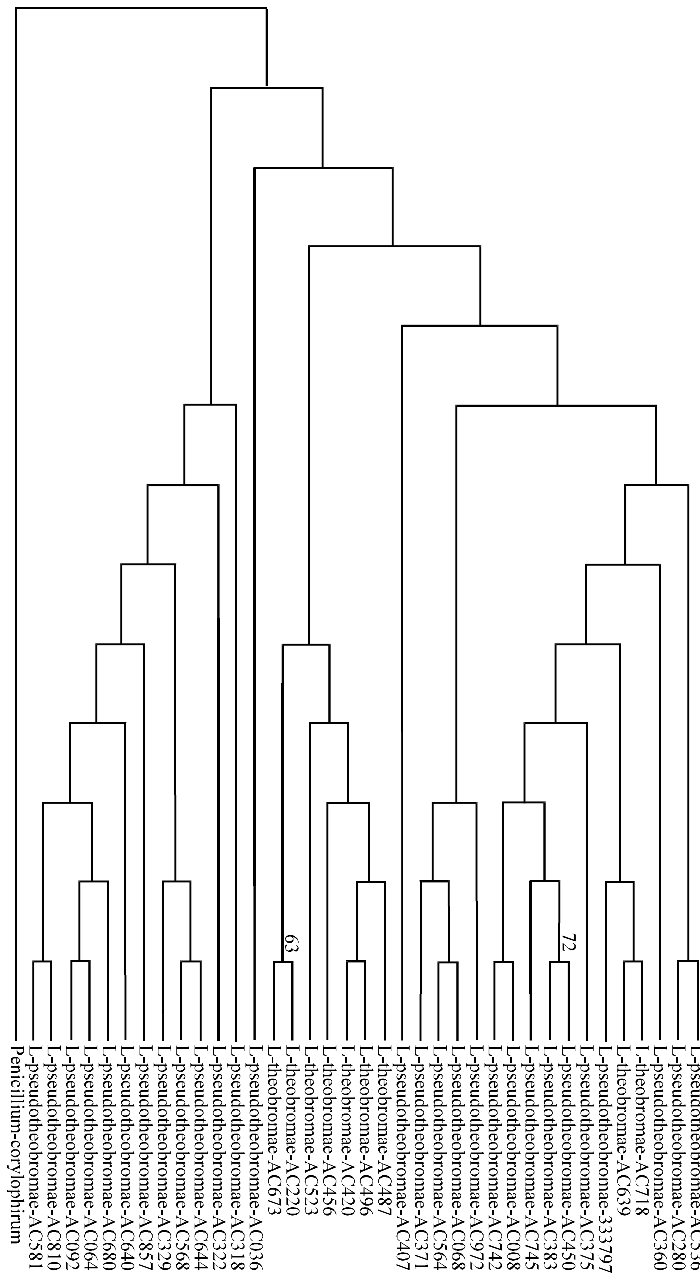

Fig. 4: Cladogram revealing the relatedness of isolates of *Lasiodiplodia* based on 400-500 nucleotides from ITS1 and ITS2 rDNA. The tree was rooted with *Penicillium corylophilum*. Numbers given on branches indicate the confidence level from 100-replicate bootstrap sampling. (Frequencies below 50% are not included)

Identification of *Lasiodiplodia* isolates by ITS sequence analysis: Phylogenetic analysis using MacClade alignment and RAxML tree construction supported the results of BLAST analyses of the ITS sequences. All the *Lasiodiplodia* sequences were closely related to each other and belonged to either *L. theobromae* or *L. pseudotheobromae* (Fig. 4). Twenty-seven of these isolates most consistently aligned with *L. pseudotheobromae* with up to 99% sequence identity whilst the rest were classified as *L. theobromae*.

DISCUSSION

Various selective media are used for fungal isolation, in particular especially so for large groups such as *Fusarium* (Thrane, 1996). The most widely used medium employs the soil fungicide PCNB which partially inhibits many fungal contaminants but allows the normal development of *Fusarium* spp. Other media for *Fusarium* isolation incorporate dichloran a mixture of dichloran and iprodione (Abildgren *et al*., 1987) and Rose Bengal, benomyl and captan.

In the current study, the fungi grew best on PDA medium followed by PCA. Carnation leaf agar and cocoa pod husk agar were less suitable. The reason for this observation is unknown but the energy from carbohydrate in PDA may have contributed to its suitability. The use of complex selective media (other than the addition of streptomycin) was not found necessary in the present study as compared with soil, cocoa is already a selective substrate allowing the growth of only a few species.

None of the fungi was identical to *F. decemcellulare*. This was in contrast to observations published previously from Ghana by Crowdy (1947) citing *F. decemcellulare* as the most frequently isolated fungal pathogen from dieback lesions. There is no doubt however, that both *Fusarium* and *L. theobromae* are present at all times during the development of dieback in cocoa. The present role of *F. decemcellulare* in dieback disease in Ghana is not certain, as there was no obvious reason for its absence from the infected tissues collected from the field. Possibly, changes in fungal succession over the years might have affected its occurrence but this must await a better understanding of how succession in fungi occurs in the cocoa ecosystem in relation to dieback disease. Only isolates from the Eastern and Western Regions of Ghana were available in the present study. Future studies need to consider collection of isolates in other regions and should strive to sample all accessible cocoa growing areas. Such tests will increase our understanding of the present role that *F. decemcellulare* has in cocoa dieback disease in Ghana and other West African cocoa-growing regions. A study of competitiveness between fungi in cocoa stem tissue might be a fertile area in the future for possible discovery of antagonistic species for biological control.

Previous studies by Owen (1956), did not state the importance of other *Fusarium* species as being responsible for dieback disease. It is likely that other *Fusarium* species were present at the time but remained unnoticed due to over action of *F. decemcellulare*.

The morphology of *F. decemcellulare* isolates from Brazil and Cameroon (Table 1) was the same as the Ghanaian isolate procured from CABI. Their micro-conidia were oval and non-septate. Chlamydospores were absent and they grew more slowly on artificial media. *Fusarium decemcellulare* produced a mixture of pink and red pigments in the agar and most characteristically, they produced yellow sporodochia from which droplets of exudate formed. This observation conforms to the characteristics published by Leslie and Summerell (2006). Moreover, conidial measurements were within the size ranges published by Crowdy, 1947. The other *Fusarium* isolates produced white to creamy-pink with considerable differences in spore morphology. Their macro-conidia were smaller than those of *F. decemcellulare* but with a continuum of dimensions that gave no clues to identity or whether a single or multiple species were represented.

In many studies, the Polymerase Chain Reaction (PCR) has proved powerful in detecting obscure genetic variation (El-Siddig *et al*., 2011); for instance, in the DNA of symbionts that cannot be cultured and separated from their co-symbionts and in the microbial floristic composition (Hanson and Hanson, 1996). For systematic and ecological studies, sets of universal primers have been designed which may be used across a range of taxa (Chow and Hazama, 1998). The primer sequences of the Internal Transcribed Spacer (ITS) regions of the nuclear ribosomal DNA have been widely used for resolving phylogenetic relationships at the species or generic levels (White *et al*., 1990). Previous reports have documented the amplification of fungal DNA sequences from plant foliage (Klein and Smith, 1996; Prathepha, 2008) and employing phylogenetic analysis, Camacho *et al*. (1997) was able to identify the endophytic fungi in *Picea* based on the ITS nucleotide sequences. Blast analysis of the rDNA genome sequence defined by the universal fungal primers ITS1 and ITS4 suggested that some of the pathogenic isolates are *F. solani* while others belong to the species *F. chlamydosporum*, *F. oxysporum* and *F. proliferatum* (sexual stage *Gibberella intermedia* (O'Donnell *et al*., 1998)). The sequence data provided evidence that *Fusarium* isolates from cocoa and the two reference isolates from CABI (*F. decemcellulare* IMI380504 and IMI362352) were separate species. What was remarkable in the current study was that, contrary to prior expectation, none of the *Fusarium* isolates from cocoa was *F. decemcellulare*.

The dominance of *L. theobromae* over *F. decemcellulare* may also be partly explained by the practice in Ghana where farmers now use cassava (*Manihot esculenta* Crantz) as a temporary shade food crop during the establishment of new cocoa farms (Osei-Bonsu *et al*., 1998). Cassava may also have complementary effects on dieback disease of cocoa as the harvested residue supports the development of *L. theobromae* (Onyeka *et al*., 2005). If planted into a farm with a history of cassava, or if cassava is used for temporary shading, young cocoa will be exposed to an elevated number of infective macro-conidia of *L. theobromae,* as cassava is known to be a host of this pathogen.

All *Lasiodiplodia* species' sequences were identified as either *L. theobromae* or *L. pseudotheobromae* by BLAST analysis. Based on the ITS phylogeny obtained in the present study, five clades could be recognised for these species that have anamorphs related to the Botryosphaeriaceae. The CABI reference *Lasiodiplodia* isolate (IMI333797) that originally was identified as *L. theobromae* isolated from cassava in Nigeria, phylogenetically was linked to the *L. pseudotheobromae* group in this study.

Conidia of these two species are similar and the CABI isolate was identified as *L. theobromae* because *L. pseudotheobromae* was only differentiated in 2008 by Alves *et al*. (2008). Very recently it has been shown that *Botryosphaeria* comprises several different phylogenetic lineages (Alves *et al*., 2008) that correlate well with morphological features of the anamorphs but observable morphological differences were lacking in the isolates studied. The studies by Phillips *et al*. (2006) suggested *Botryosphaeria* constitutes a relatively small genus consisting of only *B. dothidea* (Moug. Fr.) Ces. and De Not. (the type species of the genus) and *B. corticis* (a species restricted to *Vaccinium* spp.). The remaining lineages within what was known as *Botryosphaeria* now consist of the anamorph genera *Diplodia* (including *Sphaeropsis*), *Lasiodiplodia*, *Neofusicoccum*, *Pseudofusicoccum*, *Macrophomina*, *Neoscytalidium* and *Dothiorella* (Crous *et al*., 2006). It was recently determined that *Diplodia seriata* De Not. was the correct name for the anamorph of *Botryosphaeria obtusa* (Phillips *et al*., 2007).

The type species *L. theobromae*, geographically widespread in the tropics and subtropics, has been associated with approximately 500 hosts (Punithalingam, 1980). This apparently

unspecialized plant pathogen has been reported to cause numerous diseases, including dieback, root rot, fruit rots, leaf spot and witches' broom amongst many others. It is also said to occur as an endophyte (Rubini *et al*., 2005). Pavlic *et al*. (2004) have described a new species, *L. gonubiensis* Pavlic, Slippers and M. J. Wingf. while Burgess *et al*. (2006) described three new *Lasiodiplodia* species (*L. crassispora, L. venezuelensis* and *L. rubropurpurea*).

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