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Genetic variability in *Colletotrichum gloeosporioides* (Penz.) isolated from cassava and yam from four agro-ecological zones of Ghana

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ABSTRACT

Anthracnose disease caused by *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. is one of the most important fungal diseases of yam and cassava. To determine the genetic variations in *C. gloeosporioides* from the two crops, twenty-three isolates of the pathogen obtained from anthracnose-infected cassava stem and leaves and twenty-one isolates from yam leaves and vine from four agro-ecological zones of Ghana (Forest, Transition, Guinea and Coastal Savannah) were evaluated. Six species-specific internal transcribed spacer (ITS) primers were used in pairs to identify isolates to species. Amplification of the genomic DNA using specific primers ITS1 paired with ITS4 generated PCR products of band sizes between 500 to 600 bp which compared well with published data. Also eight Random Amplified Polymorphic DNA (RAPD) markers were used to determine genetic relationships among the 44 *C. gloeosporioides* isolates from cassava and yam. A phylogenetic tree constructed based on polymorphic bands generated with eight random primers, using the upgma method grouped forty-three of the isolates into two main clusters. The main clusters separated *C. gloeosporioides* isolates from cassava from those from yam. One isolate from cassava presented profiles completely different from the two main clusters and was grouped separately.

INTRODUCTION

Colletotrichum gloeosporioides (Penz.), the causal organism of anthracnose disease is a facultative parasitic fungus belonging to the Melanconiales (order producing conidia in acervuli). The fungus produces hyaline, one-celled, and ovoid to oblong, slightly curved conidia, 10-15µm in length and 5-7µm in width. The waxy acervuli, which are produced in infected tissue, are sub-epidermal, and with setae (Padman and Janardhana, 2011; Kumar 2014). Identification of isolates in the *C. gloeosporioides* has traditionally been based on morphology, shape and size of conidia, presence or absence of setae and colony growth characteristics (Smith and Black, 1990; Sutton, 1992). However, these characteristics can be highly variable in *C. gloeosporioides* and differentiation based on these characteristics may be uncertain. Kumari *et al.* (2008) stated that pathogen variability is one of the main causes of failure to breed crop varieties or fungicidal control of plant diseases. Genetic variation of a pathogen is therefore a pre-requisite for any resistance breeding programme.

To overcome the limitations in traditional characterization, molecular markers can be used to bring about variations in isolates to the genetic level. Among the different molecular markers, there are some that are relatively economical, reliable and simple to use in assessing variations among plant pathogens. One such reliable molecular marker is the use of species-specific primers for fungus, the Internal Transcribed Spacers (ITS) primer to show the specificity of DNA from *C. gloeosporioides* isolates (White *et al.*, 1990). To show genetic diversity, another economical and simple maker, the Random Amplification of Polymerized DNA (RAPD) can be employed. RAPD uses single primer of arbitrary nucleotide sequence to amplify genomic DNA to detect genetic polymorphism (Weising *et al.*, 1995). The aim of this study was to characterize *C. gloeosporioides* isolates from cassava and yam from four agro-ecological zones of Ghana using molecular tools.

MATERIALS AND METHOD

Single Spore Isolation of Pathogens

Pieces of diseased tissues from leaves, stems and vines from cassava were obtained from four agro-ecological zones of Ghana. The pieces were surface sterilized by immersion in 5% sodium hypochlorite (NaOCl) solution for 5min followed by washing (three times) in sterile distilled water. They were then dried in a sterile Lamina flow hood, placed on PDA in Petri dishes and inoculated at 28 ± 2^{0}C.

Mycelial growths from the plated tissues were transferred to fresh PDA in Petri dishes. They were then sub-cultured until pure cultures of *C. gloeosporioides* were obtained. These were maintained on PDA under Near Ultra Violet (NUV) light for one week.

Spore suspensions were prepared by adding 10ml sterile distilled water to the cultures followed by gentle surface agitation with a sterile inoculation needle. The spore suspensions were serially diluted from 1 x 10^{-1} to 1 x 10^{-5}. From each of the 1 x 10^{-4} and 1 x 10^{-5} spore suspensions, 100µl were pipetted and spread onto the surface of freshly prepared water agar plates in 9-cm diameter Petri dishes and left for 24 hours. Sterile inoculation needles were used to gently pick germinating single conidia of *C. gloeosporioides* onto freshly prepared PDA plates. The plates were incubated under alternating cycles of NUV and darkness at 28^{o}C for seven days. Single spore cultures produced were maintained on PDA.

DNA Extraction

Spore suspensions from forty-four *C. gloeosporioides* isolates from cassava and yam were produced from 10-day-old single spore cultures. Five hundred microlitres (500µl) of each spore suspension was used to inoculate 100ml of V8 Czapek Dox liquid medium. The inoculated medium was kept on an orbital shaker (Stuart Scientific), at 100 rpm for 48 hours after which mycelia produced were harvested. Hundred milligrams (100 mg) of mycelia from each isolate was frozen in liquid nitrogen and ground to powder. DNA was extracted from the powder using a modified protocol for DNA extraction from plant tissue using a commercial QIAGEN extraction kit (Qiagen sciences). The genomic DNA estimated was re-suspended in 50µl Tris-EDTA (10mM Tris-HCl [pH 8.0], 1mM EDTA and stored in a freezer until required (https://www.qiagen.com/resources/molecular-biology-methods/dna/).

DNA Amplification with Specific Primers for Species Identification

Six sets of universal internal transcribed spacer (ITS) primers (obtained from Sigma Aldrich, Germany) were used in pairs for their specificity to amplify the extracted genomic DNA. The ITS specific primers were: ITS1 (5'-CCGTAGGTGAACCTGCGG-3'), ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3'), ITS4 (5'-TCCTCCGCTTATT GATATGC-3'), ITS4-B (5'-CAGGAGACTTGTACACGGTCCAG-3'), 5.8S (5'-TCGATGAAGAACGC AGCG-3'), 5.8SR (5'-TCGATG AAGAA CGCA GCG-3'). Four primer pairing methods were used in the reactions; in the first one, ITS1 was paired with ITS4, in the second, ITS1 was paired with ITS1-F. ITS4 was paired with ITS4-B in the third pairing while 5.8S was paired with 5.8SR for the last.

PCR was performed with a DNA thermocycler (Gen Amp® PCR system 9700 version 3.09). Each PCR reaction mixture contained 2µl of genomic DNA, 25mM $MgCl_2$ 0.9µl, 10 x PCR buffer 1 µl, 10mM each dNTP 0.4µl, 0.05 of Taq DNA polymerase (Biolab) and 0.25µl of each primer. The amplification cycle consisted of initial denaturation at 94^{o}C for 5min followed by 35 cycles consisting of 1min at 94^{o}C, 2-min at 60^{o}C and 2-min at 72^{o}C followed by final extension of 7min at 72^{o}C. PCR products were

separated in a 1.5% agarose gel, stained with ethidium bromide, viewed and photographed on a UV transilluminator (Stanley *et al.,* 1996; Xiao *et al.,* 2004).

DNA Amplification with Random Primers

Eight oligonucleotides: RP2-5'CCTGGGCTTG3'; RP4-5'CCTGGGCTGG3'; RP15- 5'CCTG GGTTTG3'; RP85 -5'GTGCTCGTGC3'; RP95 -5'GGGGGG TTGG3'; RP346 -5'AGGCGA ACG3'; RP362-5'CCGCCTTACA3'; RP400- 5'GCCCTGATAT 3' were used for Randomized Amplification Polymorphic DNA (RAPD) analysis. PCR amplifications were carried out in 25µl mixtures containing 2µl of genomic DNA, 25mM $MgCl_2$ 0.9µl, 10 x PCR buffer 1µl, 10mM each dNTP 0.4µl, 0.05 of Taq DNA polymerase (Biolab) and 0.5µl of primer. The reaction mixtures were incubated on a DNA thermocycler (Gen Amp® PCR system 9700 version 3.09). The PCR profile was of 3 min denaturation at 94°C, followed by 35 cycles of 30sec at 94°C, 30 sec at 45°C and 1min at 72°C, and then a 10min extension at 72°C. PCR products from the RAPD were separated in a 1.5% agarose gel, stained with ethidium bromide, viewed and photographed on a UV transilluminator (Xiao *et al.,* 2004; Vinod and Benagi 2009). The multiple and single RAPD bands produced were scored manually and analyzed as binary data. The presence or absence of monomorphic and polymorphic bands was scored 1 and 0 respectively, and the results obtained for all primers were pooled in a single matrix for analysis.

Analysis of Data

The NTSYS PC software (Numerical Taxonomy System Applied Biostatistics, Inc.) (Rholf, 1994) was used for analysis of the genetic similarity of isolates based on Randomized Amplification Polymorphic DNA (RAPD) data. The data was analyzed as a binary matrix, from which a similarity matrix was built using the Darwin v5 – Jaccard coefficient (Jaccard, 1908). Data from the similarity matrix were used to produce dendrograms using the unweighted pair group method with arithmetic mean (UPGMA).

RESULTS AND DISCUSSION

Species Identification with Specific Primers

Of all the primer pairs used, only the ITS1 and ITS4 pair was able to amplify the genomic DNA from the *Colletotrichum gloeosporioides* isolates; no amplification product was observed with the ITS primer pairs ITS1/ITS1-F, ITS4/ITS4-B and 5.8s/5.8SR. Using the two universal primers, ITS1 and ITS4, a unique single band PCR product between 500-600bp was obtained from amplification of the ITS regions of the *C. gloeosporioides* isolates (Fig. 1). Out of the 44 isolates analysed, the specific primer ITS1 and ITS4 pair was able to generate amplification band patterns for 36 (82%) of them. The pairing of primer ITS1 and ITS4 to amplify the genomic DNA samples to generate bands of fragment sizes between 500 and 600 bp is in conformity with literature. For example Adaskaveg and Hartin (1997) paired primers ITS1 and ITS4 to generate 500 pb fragment amplifications specific for *C. gloeosporioides* DNA. Vinnere *et al*. (2002) used ITS1, 5.8s and ITS2 primers to generate a single fragment of 600 bp for *C. gloeosporioides* isolates. The close range of fragment sizes between 500 and 600 bp generated between isolates from yam and those from cassava suggests that the isolates are closely related.

PCR Amplification with Random Primers

RAPD amplifications using the eight oligonuclotide primers yielded scorable bands for all the isolates of the fungal species. Primers RP2, RP4, RP95, RP15, RP85, RP346 and RP368 produced multiple bands (Fig. 2) while primer RP400 produced only a single band (Fig. 3).

Similarity coefficient values between *C. gloeosporioides* isolates from cassava and those from yam ranged from 0 to 39%. There was 39% similarity between isolate C11a (isolate from cassava from forest ecological zone) and Y43b (isolate from yam from the coastal ecological zone) (Table 1). Similarity coefficient values among *C. gloeosporioides* isolates from the same crop ranged from 0 to 92% for *C. gloeosporioides* isolates from cassava (Table 2) and ranged from 0 to 58% for the isolates from yam (Table 3).

The dendrogram obtained from the cluster analysis of RAPD data portrayed two distinct clusters of the 48 *C. gloeosporioides* isolates from cassava and yam (Fig. 4). The first cluster (Cluster A) comprises 22 *C. gloeosporioides* isolates from cassava and three isolates from yam. The second cluster (Cluster B) consists of 18 *C. gloeosporioides* isolates, all from yam. Apart from the two major clusters in the dendogram there is also isolate C43b which is clearly distinguished from all the others. The genetic relatedness and diversity investigated through RAPD analysis separated C. *gloeosporioides* isolates from cassava from those of yam. The two clusters produced: Cluster A, comprising isolates mainly from cassava only and, Cluster B comprising isolates, all from yam is an indication that isolates from the two crops are genetically distinct.

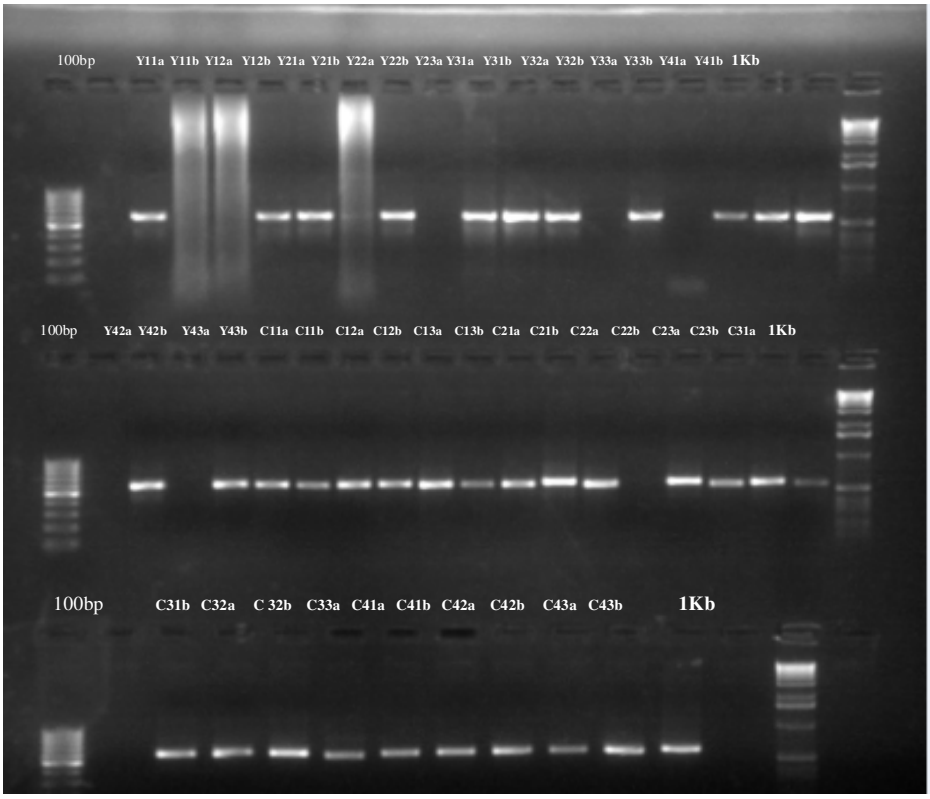

Fig. 1: PCR amplification bands from genomic DNA of *Colletotrichum gloeosporioides* using ITS1 and ITS4

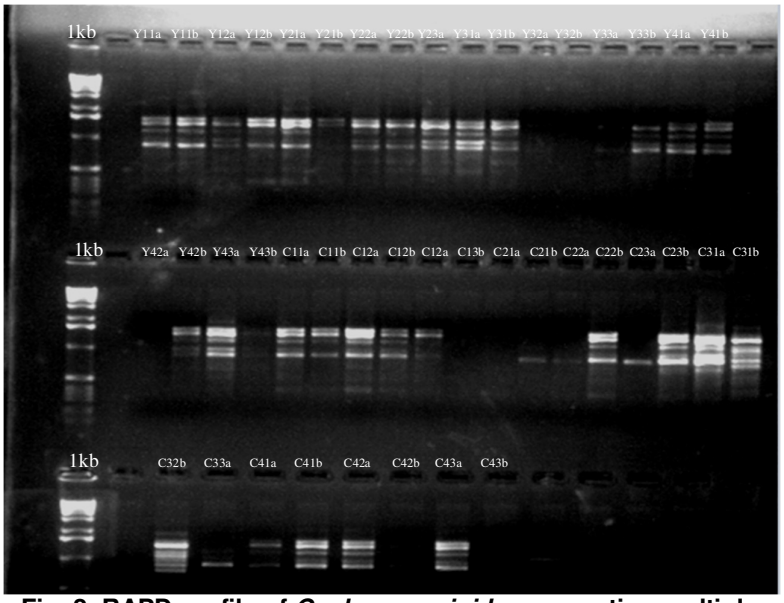

Fig. 2: RAPD profile of *C. gloeosporioides* generating multiple bands with primer RP4

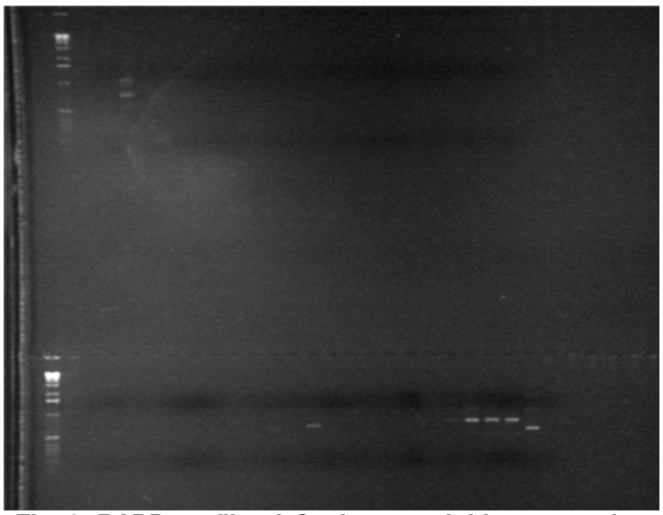

Fig. 3: RAPD profile of *C. gloeosporioides* generating a single band with primer RP400

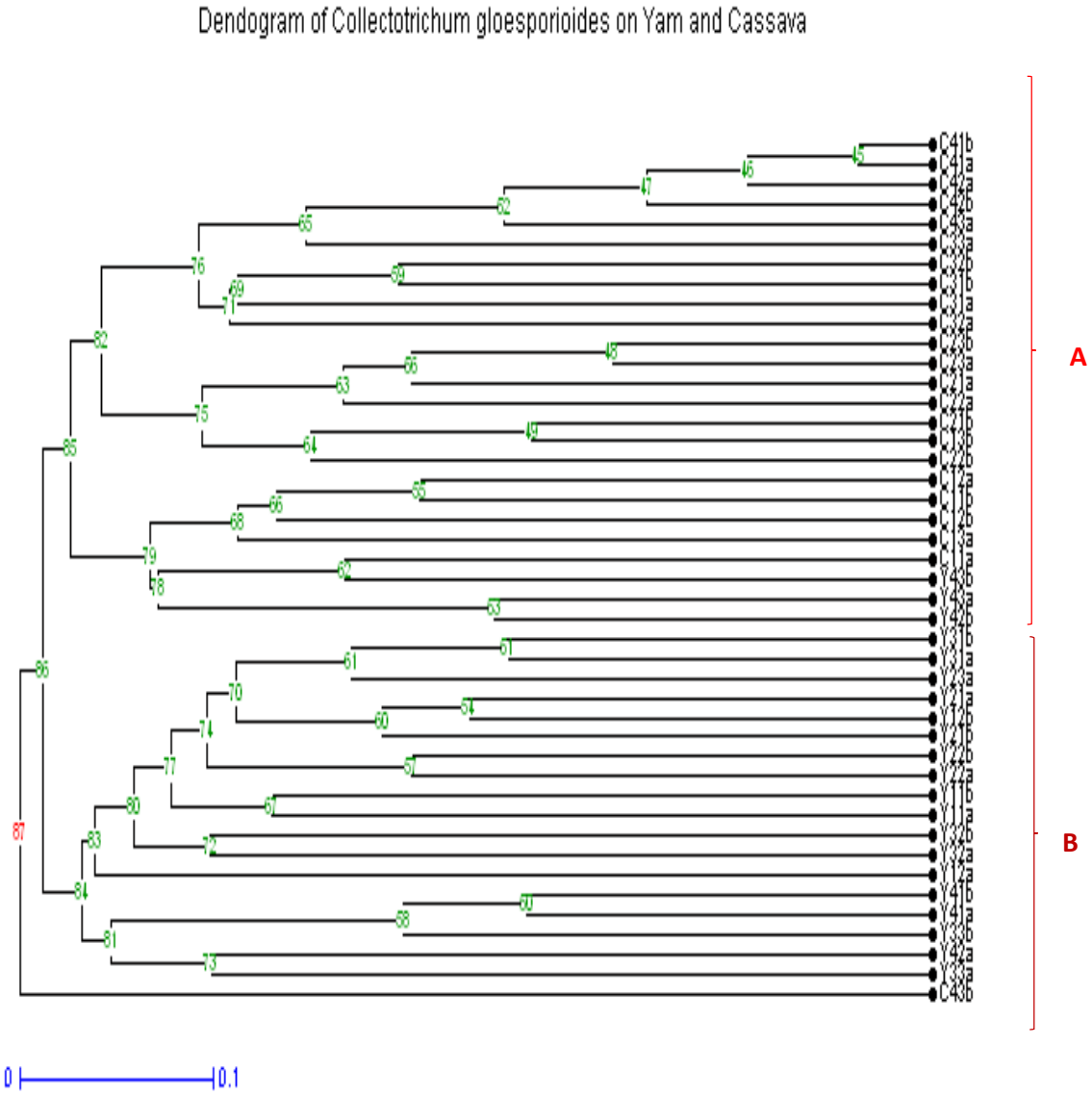

Fig. 4: Dendrogram showing genetic similarity between *C. gloeosporioides* isolates from yam and cassava based on RAPD profile

Table 1: Similarity coefficient values of 44 *C. gloeosporioides* isolates from cassava and yam

Unit	Y11 a	Y11 b	Y12 a	Y12 b	Y21 a	Y21 b	Y22 a	Y22 b	Y23 a	Y31 a	Y31 b	Y32 a	Y32 b	Y33 a	Y33 b	Y41 a	Y41 b	Y42 a	Y42 b	Y43 a	Y43 b
C11a	0.15	0.13	0.11	0.19	0.21	0.10	0.19	0.15	0.08	0.06	0.10	0.00	0.06	0.07	0.12	0.08	0.11	0.14	0.25	0.25	0.39
C11b	0.14	0.08	0.08	0.14	0.16	0.23	0.29	0.24	0.13	0.07	0.05	0.00	0.04	0.09	0.10	0.10	0.09	0.06	0.15	0.18	0.25
C12a	0.07	0.10	0.11	0.12	0.14	0.15	0.19	0.15	0.11	0.03	0.05	0.00	0.03	0.04	0.04	0.04	0.07	0.05	0.19	0.26	0.14
C12b	0.29	0.19	0.09	0.19	0.21	0.15	0.23	0.16	0.15	0.11	0.11	0.00	0.05	0.17	0.06	0.10	0.13	0.03	0.13	0.15	0.11
C13a	0.19	0.11	0.08	0.14	0.21	0.08	0.17	0.08	0.09	0.07	0.11	0.00	0.00	0.09	0.05	0.05	0.09	0.06	0.25	0.25	0.11
C13b	0.17	0.14	0.00	0.06	0.10	0.03	0.07	0.04	0.09	0.06	0.13	0.00	0.03	0.23	0.04	0.04	0.08	0.05	0.00	0.00	0.05
C21a	0.05	0.03	0.04	0.11	0.08	0.09	0.09	0.04	0.03	0.08	0.08	0.00	0.04	0.10	0.05	0.05	0.05	0.00	0.18	0.13	0.00
C21b	0.10	0.06	0.00	0.03	0.04	0.04	0.04	0.04	0.06	0.04	0.08	0.00	0.04	0.16	0.05	0.00	0.05	0.06	0.00	0.00	0.00
C22a	0.10	0.06	0.05	0.07	0.08	0.09	0.14	0.05	0.07	0.04	0.06	0.00	0.00	0.05	0.11	0.05	0.10	0.07	0.20	0.13	0.00
C22b	0.05	0.03	0.00	0.04	0.04	0.05	0.00	0.05	0.03	0.04	0.06	0.00	0.05	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C23a	0.05	0.03	0.04	0.07	0.04	0.04	0.04	0.00	0.00	0.04	0.03	0.00	0.04	0.05	0.00	0.00	0.00	0.00	0.08	0.06	0.00
C23b	0.09	0.03	0.00	0.07	0.04	0.00	0.00	0.00	0.00	0.04	0.03	0.00	0.04	0.10	0.00	0.05	0.00	0.00	0.00	0.00	0.12
C31a	0.15	0.12	0.00	0.03	0.08	0.09	0.09	0.09	0.06	0.04	0.05	0.00	0.04	0.10	0.11	0.00	0.05	0.06	0.00	0.00	0.06
C31b	0.14	0.15	0.08	0.06	0.12	0.08	0.08	0.04	0.06	0.00	0.05	0.00	0.00	0.09	0.05	0.00	0.04	0.06	0.07	0.11	0.05
C32a	0.17	0.20	0.04	0.13	0.10	0.11	0.20	0.12	0.12	0.06	0.10	0.00	0.03	0.13	0.08	0.08	0.12	0.00	0.06	0.10	0.10
C32b	0.11	0.13	0.05	0.04	0.04	0.05	0.10	0.00	0.03	0.00	0.03	0.00	0.00	0.06	0.06	0.00	0.05	0.08	0.11	0.15	0.07
C33a	0.09	0.09	0.09	0.10	0.12	0.13	0.13	0.09	0.03	0.04	0.00	0.00	0.13	0.05	0.05	0.05	0.04	0.06	0.08	0.06	0.19
C41a	0.17	0.14	0.04	0.13	0.10	0.15	0.20	0.12	0.12	0.06	0.10	0.00	0.11	0.23	0.04	0.04	0.12	0.10	0.06	0.05	0.15
C41b	0.16	0.13	0.03	0.12	0.10	0.15	0.19	0.11	0.11	0.06	0.10	0.00	0.10	0.22	0.04	0.04	0.12	0.10	0.06	0.04	0.14
C42a	0.13	0.14	0.04	0.09	0.11	0.16	0.16	0.12	0.09	0.07	0.10	0.00	0.07	0.18	0.04	0.00	0.04	0.05	0.06	0.05	0.10
C42b	0.14	0.11	0.04	0.10	0.07	0.13	0.17	0.08	0.13	0.00	0.08	0.00	0.00	0.14	0.00	0.00	0.04	0.00	0.07	0.05	0.05
C43a	0.10	0.06	0.04	0.07	0.04	0.04	0.09	0.00	0.03	0.04	0.05	0.00	0.04	0.10	0.00	0.00	0.10	0.06	0.08	0.06	0.00
C43b	0.11	0.06	0.00	0.04	0.09	0.05	0.10	0.05	0.03	0.09	0.03	0.00	0.05	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.07

Table 2: Similarity coefficient values of 23 *C. gloeosporioides* isolates from cassava

Unit	C11a	C11b	C12a	C12b	C13a	C13b	C21a	C21b	C22a	C22b	C23a	C23b	C31a	C31b	C32a	C32b	C33a	C41a	C41b	C42a	C42b	C43a	C43b
C11a	1.00																						
C11b	0.21	1.00																					
C12a	0.17	0.47	1.00																				
C12b	0.17	0.32	0.32	1.00																			
C13a	0.28	0.20	0.38	0.26	1.00																		
C13b	0.04	0.00	0.04	0.28	0.24	1.00																	
C21a	0.11	0.14	0.11	0.17	0.23	0.27	1.00																
C21b	0.00	0.00	0.00	0.23	0.00	0.58	0.27	1.00															
C22a	0.18	0.15	0.12	0.08	0.15	0.13	0.44	0.18	1.00														
C22b	0.00	0.00	0.06	0.14	0.08	0.33	0.22	0.38	0.00	1.00													
C23a	0.11	0.07	0.11	0.13	0.14	0.27	0.56	0.27	0.44	0.38	1.00												
C23b	0.10	0.06	0.05	0.12	0.13	0.33	0.36	0.25	0.27	0.33	0.67	1.00											
C31a	0.05	0.14	0.11	0.13	0.07	0.27	0.08	0.27	0.18	0.22	0.17	0.15	1.00										
C31b	0.10	0.13	0.16	0.12	0.06	0.11	0.14	0.14	0.36	0.08	0.23	0.13	0.33	1.00									
C32a	0.13	0.17	0.14	0.28	0.05	0.14	0.06	0.12	0.06	0.07	0.06	0.00	0.27	0.31	1.00								
C32b	0.06	0.18	0.21	0.09	0.08	0.07	0.10	0.10	0.25	0.00	0.10	0.00	0.22	0.44	0.23	1.00							
C33a	0.22	0.31	0.24	0.12	0.06	0.00	0.07	0.00	0.17	0.00	0.15	0.07	0.15	0.31	0.18	0.20	1.00						
C41a	0.18	0.24	0.14	0.19	0.11	0.09	0.19	0.06	0.20	0.07	0.19	0.11	0.19	0.31	0.33	0.14	0.43	1.00					
C41b	0.17	0.22	0.18	0.18	0.16	0.14	0.18	0.05	0.19	0.13	0.25	0.17	0.25	0.29	0.32	0.13	0.40	0.92	1.00				
C42a	0.14	0.25	0.20	0.15	0.18	0.15	0.20	0.06	0.21	0.15	0.29	0.19	0.29	0.33	0.28	0.15	0.36	0.77	0.85	1.00			
C42b	0.10	0.20	0.16	0.16	0.13	0.11	0.23	0.07	0.25	0.08	0.23	0.13	0.14	0.38	0.31	0.18	0.31	0.75	0.69	0.67	1.00		
C43a	0.11	0.14	0.11	0.13	0.14	0.06	0.27	0.00	0.30	0.00	0.27	0.15	0.08	0.33	0.19	0.22	0.25	0.58	0.54	0.50	0.60	1.00	
C43b	0.06	0.08	0.00	0.14	0.08	0.14	0.00	0.10	0.00	0.00	0.00	0.00	0.22	0.08	0.23	0.00	0.09	0.07	0.06	0.07	0.00	0.00	1.00

Table 3: Similarity coefficient values of 21 *C. gloeosporioides* isolates from yam

Unit	Y11a	Y11b	Y12a	Y12b	Y21a	Y21b	Y22a	Y22b	Y23a	Y31a	Y31b	Y32a	Y32b	Y33a	Y33b	Y41a	Y41b	Y42a	Y42b	Y43a	Y43b
Y11a	1.00																				
Y11b	0.31	1.00																			
Y12a	0.14	0.27	1.00																		
Y12b	0.21	0.29	0.17	1.00																	
Y21a	0.29	0.25	0.23	0.52	1.00																
Y21b	0.17	0.23	0.13	0.45	0.41	1.00															
Y22a	0.21	0.17	0.13	0.17	0.23	0.29	1.00														
Y22b	0.18	0.12	0.13	0.11	0.23	0.35	0.46	1.00													
Y23a	0.20	0.27	0.10	0.25	0.31	0.38	0.42	0.34	1.00												
Y31a	0.12	0.21	0.06	0.25	0.28	0.22	0.11	0.23	0.31	1.00											
Y31b	0.14	0.27	0.04	0.27	0.24	0.28	0.22	0.26	0.49	0.56	1.00										
Y32a	0.00	0.18	0.06	0.18	0.13	0.21	0.03	0.10	0.20	0.28	0.30	1.00									
Y32b	0.06	0.26	0.09	0.23	0.18	0.23	0.06	0.03	0.15	0.29	0.31	0.25	1.00								
Y33a	0.15	0.15	0.03	0.08	0.03	0.10	0.10	0.10	0.14	0.06	0.24	0.11	0.17	1.00							
Y33b	0.15	0.13	0.00	0.12	0.13	0.10	0.19	0.15	0.14	0.21	0.21	0.07	0.14	0.12	1.00						
Y41a	0.11	0.13	0.07	0.09	0.10	0.07	0.10	0.11	0.14	0.17	0.18	0.07	0.22	0.16	0.40	1.00					
Y41b	0.19	0.12	0.03	0.11	0.09	0.06	0.21	0.18	0.24	0.19	0.26	0.03	0.17	0.24	0.50	0.58	1.00				
Y42a	0.04	0.05	0.04	0.00	0.00	0.00	0.08	0.08	0.06	0.11	0.14	0.08	0.21	0.25	0.04	0.14	0.18	1.00			
Y42b	0.10	0.06	0.15	0.11	0.18	0.09	0.14	0.10	0.07	0.08	0.06	0.00	0.04	0.00	0.05	0.11	0.05	0.14	1.00		
Y43a	0.08	0.08	0.12	0.09	0.15	0.07	0.21	0.17	0.09	0.07	0.08	0.00	0.03	0.04	0.14	0.19	0.17	0.17	0.55	1.00	
Y43b	0.13	0.08	0.00	0.06	0.07	0.07	0.12	0.12	0.09	0.03	0.05	0.00	0.07	0.13	0.04	0.09	0.08	0.17	0.06	0.22	1.00

CONCLUSION

The use of ITS primers and Random Amplified Polymorphic DNA (RAPD) molecular markers were able to detect the variations among *C. gloeosporioides* isolates from cassava and those from yam. The pairing of ITS primers (ITS1 and ITS4) to generate PCR products of band sizes between 500 to 600 bp made the pair species-specific primers for the amplification of *C. gloeosporioides* isolates. The use of RAPD analysis with simple primers had generated results that are reliable. The two major groups produced by the dendrogram obtained from the cluster analysis of RAPD data is an indication that *C. gloeosporioides* isolates from cassava is genetically not the same as the *C. gloeosporioides* isolates from yam.

Isolate C43b stood out as a genetically distinct lineage from all the others. Further molecular analyses using other genes and more sensitive approaches are however required for sequence analyses of the pathogen.

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