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MOLECULAR CHARACTERISATION OF FUNGI FROM *ARACHIS HYPOGAEA*

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ABSTRACT

Arachis hypogaea L. (groundnut) is a food and oil crop of global interest. Groundnut seeds are plagued by fungal organisms especially during storage. This reduces the quantity and the commercial value of the products. Molecular techniques for determining the species of a fungus based on the PCR amplification and sequencing of the Internal Transcribed Spacer (ITS) region of the fungus has proven to be a more reliable method of identifying fungal species. This study was carried out to isolate and identify the fungi associated with groundnut seeds using both traditional cultural techniques and molecular method. Fungi were isolated from diseased groundnut seeds obtained from Choba market, Port Harcourt, using both Standard Blotter and Potato Dextrose Agar methods. DNA was extracted using Zymo Fungal/Bacteria DNA Miniprep Kit and was amplified using universal primer pair; 27F and 1525R. PCR products of 236 and 615 base pairs were sequenced and the fungal isolates were identified as *Lasiodiplodia theobromae* and *Aspergillus penicillioides*, respectively. This study has provided information on the fungal species associated with *A. hypogaea* which will help in suggesting ways to reduce the incidence of these organisms. Phylogenetic analysis showed the relationship that exists between the fungal isolates and other closely-related species in Genebank such as *Pediastrum duplex, Penicillium penicillioides, Colletotrichum capsici* and *Lasiodiplodia pseudotheobromae*.

Keywords: *Arachis hypogaea*; fungi; sequencing; phylogeny

INTRODUCTION

Groundnut *(Arachis hypogaea* L.) belongs to the family Leguminosae or Fabaceae (Isleib *et al*., 1994). It is the fifth most produced oil crop worldwide and is also an important food crop (USDA, 2013). It originated from Brazil and was disseminated to Africa, Asia, Europe and the Pacific Islands presumably in the sixteenth and seventeenth centuries with the discovery voyages of the Spanish, Portuguese, British and Dutch (Isleib *et al*., 1994). Groundnut, one of the staple foods in Nigeria, is associated with diseases which can diminish its productivity, economic importance or cause harm to man through consumption as some of these disease-causing organisms produce toxic substances. Plants are generally infected by different diseases. Abbas *et al*. (2004) reported the presence of aflatoxin, carcinogenic, mutagenic secondary metabolites produced due to the invasion of groundnut by *Aspergillus* sp.

There are many molecular methods used in the identification of fungal organisms. Diwakar *et al*. (2015) isolated 187 fungal species from groundnut seeds collected from farmers' field using Amplified Fragment Length Polymorphism (AFLP). Busiswe *et al*. (2015) reported the use of Simple Sequence Repeat (SSR) markers to induce resistance in groundnut against the fungal rust disease caused by *Aspergillus* spp. Identification of fungal organisms by media culturing is not reliable as the problem of misinterpretation may arise. The fact that some species cannot be cultured in the laboratory is also a shortfall of traditional cultural method. Identification of fungal species using modern molecular techniques such as amplification by Polymerase Chain Reaction (PCR) and sequencing has proven to be a more reliable alternative to traditional cultural methods (Horton and Bruns, 2001). This study was carried out to determine the species identity of fungal organisms associated with *Arachis hypogaea* seeds using basic molecular techniques.

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MATERIALS AND METHODS

Study Area and Sample Collection

The study was conducted at the Regional Centre for Biotechnology and Bioresources Research Laboratory, University of Port Harcourt, Choba, Rivers State, Nigeria. The PCR products were sequenced at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. *Arachis hypogaea* seeds with disease symptoms were obtained from Choba market in Port Harcourt, Rivers State in July 2017.

Isolation of Fungi from Groundnut Seeds

Isolation of fungal organisms was carried out using Standard Blotter recommended by International Seed Health Testing Association (ISTA, 2016). Groundnut seeds were surface-sterilized with 70% ethanol for 2-3 minutes and rinsed with sterile distilled water three times. The Petri-dishes were lined with 3 layers of sterilized 9 cm Whatman's filter paper. Five groundnut seeds were plated per Petri-dish of 9 cm in diameter. The plated groundnut seeds were incubated at 25 ± 2^{o}C in the laboratory for 7 days. After incubation, the isolated fungi were sub-cultured on Potato Dextrose Agar to obtain pure cultures of fungi.

Occurrence of fungi was determined based on the Score method recommended by Ataga and Akueshi (1986); where numeral '1' represents absence of fungi and '2' represents presence of fungi so that any value above '1' indicates presence of fungi.

Fungal DNA Extraction

Genomic DNA was extracted following the protocol of Quick-DNA^{TM} Fungal/Bacterial MiniPrepKit (Zymo Research Group, California, USA) as described by the manufacturer, with modifications at the Regional Centre for Biotechnology and Bioresources Research Laboratory, University of Port Harcourt, Choba, Rivers State, Nigeria. Half a gram (0.5g) of the groundnut seed was directly used for fungal DNA extraction to know whether a fungal organism can be extracted directly from the seed without going through the process of culturing on media. Fungal DNA was also extracted from the mycelium of each fungal pure culture.

Concentration and Purity of DNA

DNA quantity and purity were measured using NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific Inc. Wilmington, Delaware, USA). DNA purity was determined as the ratio of UV absorbance by DNA at 260 nm and 280 nm. Agarose gel electrophoresis was used to determine the quality of the DNA.

PCR amplification and sequencing

Universal primers 27F, forward (5´-AGAGTTTGATCMTGGCTCAG3´) and 1525R, reverse (5´AAGGAGGTGWTCCARCCGCA-3´) were used to amplify the ITS region of the fungi. PCR was carried out in a final volume of 10 µL containing 2 µL of genomic DNA (10 ng/µL), 0.1µL of Taq polymerase, 1.0µL of 10X PCR buffer, 1.0 µL of DMSO, 0.8 µL of 2.5 mM DNTPs, 1.0µL of 25mM $MgCl_2$ (Promega), 0.5µL of each primer (concentration of 5µM) and 3.1µL of Nuclease-free water. Amplifications were performed in a thermal cycler (Eppendorf) using an initial denaturation step of 94°C for 5 minutes. This was followed by 36 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 52°C and elongation for 45 seconds at 72°C. A final extension for 7 minutes at 72°C was then performed. PCR products were loaded on 1% agarose gel and gel electrophoresis was carried out. The amplicons were sequenced using Sanger sequencing. Single-stranded DNA template, a DNA polymerase, a DNA primer, di-deoxynucleotide triphosphates (ddNTPs) and deoxynucleotide triphosphates (dNTPs) were used for sequencing. Each dideoxynucleotide (ddNTP) incorporated at intervals terminated DNA chain elongation as they lack a 3'-OH group which is responsible for the formation of a phosphodiester bond between two nucleotides. This stopped the extension of DNA.

Phylogenetic Analysis

Sequences obtained were blasted against known sequences in the National Centre for Biotechnology Information (NCBI) database. Best BLAST hits were used for the construction of phylogenetic tree using composite likelihood method (Tamura *et al*., 2004). Evolutionary analysis was conducted on MEGA7 software (Kumar *et al*., 2016).

RESULTS

Fungi associated with *Arachis hypogaea* Seeds.

Two fungal organisms were isolated from groundnut. After one week of incubation, sample 1 had a fluffy dark mouse grey texture with a sky grey colour, and sample 3 had an ash-wooly colour with black spots. The frequency of occurrence of each organism was determined. Sample 1 had a higher frequency of occurrence (1.6) than sample 3 (1.3) as presented in Table.1. Pure cultures of the fungal isolates are presented in Plate 1.

Table 1: Frequency of Occurrence of Fungi Isolated from Groundnut Seeds

Fungal Sample ID	Morphological description	Frequency of Occurrence
1	Grey and wooly	1.6
3	Ash with black spots	1.3

1a 1b

Plate 1a (Sample 1) and b (Sample 3): Pure Cultures of fungal isolates from *Arachis hypogaea* seeds

DNA Extraction, Quantification and Gel Electrophoresis

NanoDrop spectrophotometer revealed the concentration and purity of DNA extracted from fungal species and groundnut seed as presented in Table 2. Gel electrophoresis showed that the extracted DNA of samples 1 and 2 are of good quality. DNA of sample 3 did not show because the quality of the DNA was low (Figure 1).

Table 2: Concentration of DNA extracted from *Arachis hypogaea* seed (sample 2) and fungal isolates from *Arachis hypogaea* seeds.

Sample ID	DNA (ng/µl)	Absorbance at 260/280 (Purity)
1	3.9	1.89
2	70.9	1.85
3	8.3	1.21

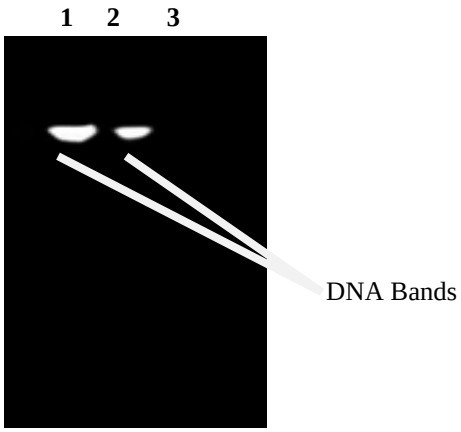

Figure 1: Gel electrophoresis of DNA extracted from groundnut seeds and fungal isolates from groundnut seeds using 1% agarose gel.
The numbers 1 and 2 represent DNA from fungal isolates. The number 3 represents DNA from diseased groundnut seed.

Polymerase Chain Reaction

Only amplified DNA showed on gel as represented by bands in Figure 2. Polymerase Chain Reaction could not amplify sample 3 as the DNA was of low quality. Only amplified samples were sequenced.

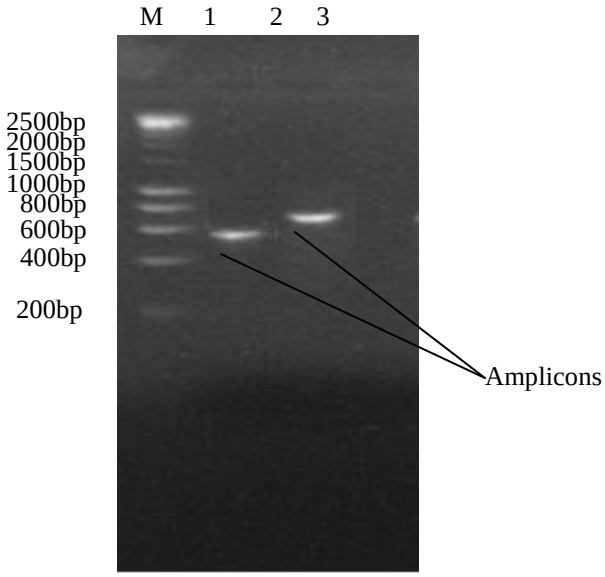

Figure 2: Gel Electrophoresis showing PCR amplification generated from fungal DNA samples using 1% agarose gel.
The numbers 1, 2 and 3 represent PCR products for samples 1, 2 and 3.
M- DNA Marker (1kb Ladder).

DNA Sequencing and Blasting

After sequencing, the blast results revealed the species identity of the fungal organisms associated with groundnut to be: sample 1- *Lasidioplodia theobromae* (Pat.) Griffon & Maubl. and sample 2- *Aspergillus penicillioides* Speg (Table 3). The alignment of the samples are shown in Figures 3 and 4. The alignment scores of both samples as presented with red lines were greater than 200.

Table 3: Putative Taxonomic Affinities of Sequence Types Inferred from Blast Searches of ITS Sequences

Sample ID	Source	Taxonomic affinity (Gene Bank No)	Percentage similarity (%)
1	Fungal isolate	*Lasiodiplodia theobromae* (KX270361.1)	87
2	*A. hypogea* seed	*Aspergillus penicillioides* (GU017496.1)	87

Figure 3: Alignment scores of all aligned sequences of sample 1

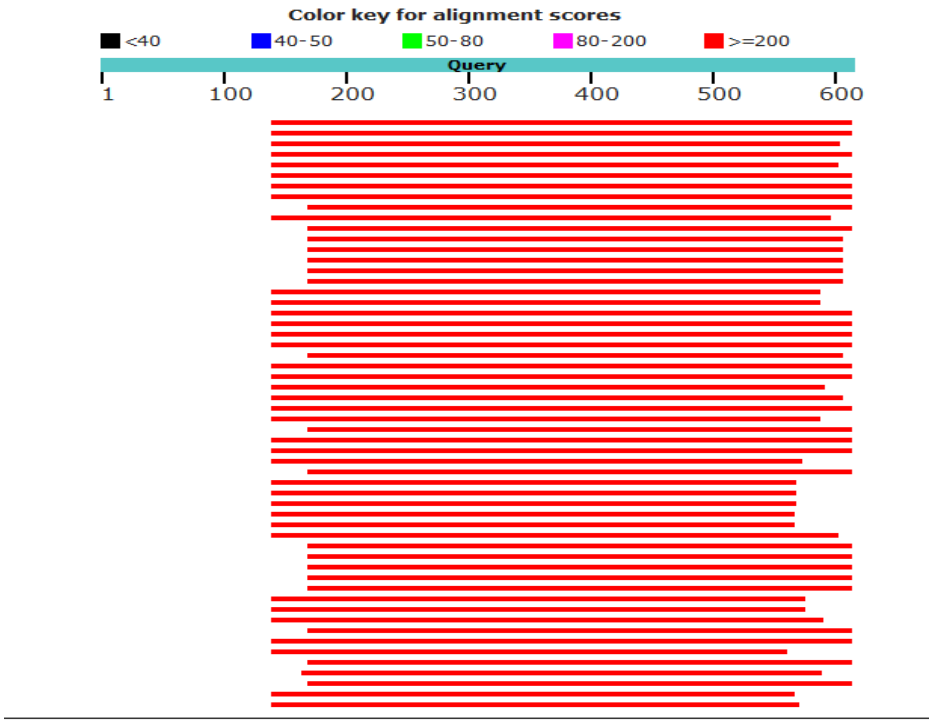

Figure 4: Alignment scores of all aligned sequences of sample 2

The nucleotide lengths of the DNA samples were determined to be: 236 base pairs for *Lasidioplodia theobromae* (sample 1) and 615 base pairs for *Aspergillus pennicillioides* (sample 2). The sequences of the DNA obtained were submitted to GeneBank and accession numbers were assigned to them as follows:

1. Sample 1- *Lasiodiplodia theobromae* (MH793523) strain RE1
2. Sample 2 - *Aspergillus penicillioides* (MH793524) strain RE2

Phylogenetic Analysis

The phylogenetic analysis showed that *Pediastrum duplex*, *Penicillium penicilliodes*, *Colletotrichum capsici* and *Lasiodiplodia pseudotheobromae* were most closely related to the fungal isolates obtained from *Arachis hypogaea* as presented in Figure 3. The vertical lines on the trees indicate the difference between the branches. The greater the length of the vertical line, the more the difference between the branches.

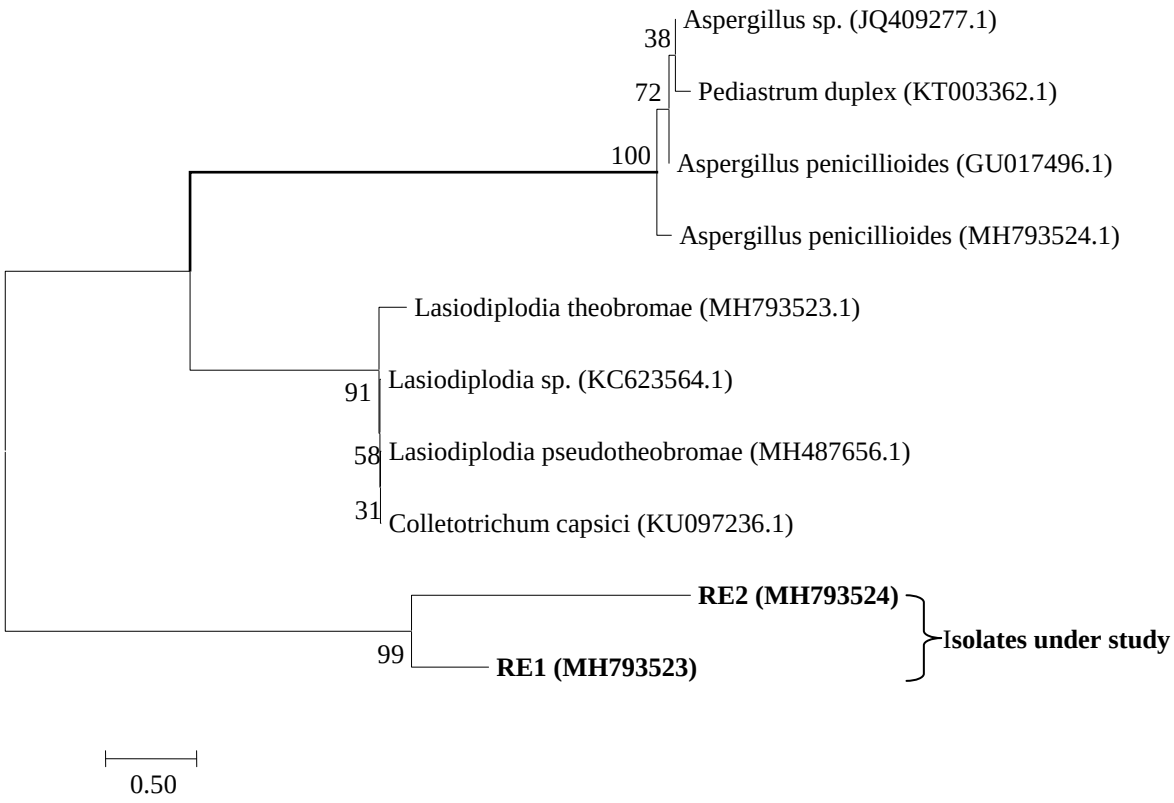

Figure 3: Phylogenetic tree showing the fungi associated with *Arachis hypogaea* using Composite likelihood method

DISCUSSION

Two fungal organisms, *Lasiodiplodia theobromae* and *Aspergillus penicillioides*, were isolated from *Arachis hypogaea* seeds. The DNAs of samples 1 and 2 were successfully amplified using universal primer pair 27F and 525. The DNA of sample 3 was of low quality on agarose gel and so did not amplify. Agarose gel showed the different fragment sizes of the PCR products.

Fungi have been known to infect groundnut either in the field or in storage causing different diseases. Ihejirika *et al*. (2005), Aliyu and Kutama (2007), Hedayati *et al*. (2010) and Akinnibosun and Osawaru (2015) reported that *Fusarium* spp., *Rhizopus* spp., *Mucor* spp. and *Penicillum* spp. were the most abundant fungi associated with groundnut seeds. Rasheed *et al*. (2004) isolated *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum*, *Macrophomina phaseolina* and *Rhizoctonia solani* from seven (7) samples of groundnut collected from different localities in Pakistan. *Lasiodiplodia theobromae* was responsible for dieback disease of *Mangifera indica* in Egypt (Ismail *et al*., 2012) and stem canker disease of *Hevea brasiliensis* in Indonesia (Febbiyanti *et al*., 2019).

Fungi were isolated using traditional cultural techniques and identified using molecular method. Sequences were aligned and a phylogenetic tree was constructed to show the fungal organisms that are most closely related to the fungi isolated from groundnut. The fungal isolates, *Lasiodiplodia theobromae* and *Aspergillus penicillioides* are most closely related to *Colletotrichum capsici* and *Pediastrum duplex*, respectively.

CONCLUSION

Two fungal species were found to be associated with diseased seeds of *Arachis hypogaea*. PCR amplification of the ITS 1-2 regions of the fungal genome led to the successful identification of *Lasiodiplodia theobromae* and *Aspergillus penicillioides* associated with *A. hypogaea.* Phylogenetic analysis showed that *Pediastrum duplex*, *Penicillium penicillioides*, *Colletotrichum capsici* and *Lasiodiplodia pseudotheobromae* were most closely related to the fungal isolates. This study will help add to the existing information on the fungi associated with groundnut seeds which will give researchers an insight into proffering ways of preventing and controlling these organisms on groundnut.

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