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Molecular Characterization of Fungi Associated with Cowdung-impacted Soil

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ABSTRACT

Dung is the undigested remains of food taken in by herbivores. It is a combination of faeces and urine at a ratio of 3:1. It mostly consists of cellulose, hemicelluloses and lignin. Cow dung harbours several microorganisms, including various species of fungi. The aim of this study was to isolate and characterize the fungal organisms associated with cow dung-impacted soil using both traditional cultural techniques and molecular method. DNA extraction was carried out using Zymo Quick DNA Fungal/Bacterial Mini prep kit. The Polymerase Chain Reaction (PCR) amplification of the Internal Transcribed Spacer (ITS) genes, using the universal primer pair; ITS4 and ITS5, generated amplicon sizes of 372 and 834 base pairs. The amplicons were sequenced using Sanger sequencing and the isolates were identified as *Lichtheimia ramosa* and *Galactomyces pseudocandidus*. Phylogenetic analysis showed the relationship that exists between the fungal species and other closely-related species in GenBank such as: *Aspergillus amstelodami*, *Absidia corymbifera*, *Mycocladius corymbiferus* and *Geotrichum candidum*. This study has provided information on some of the fungal organisms harboured by cowdung-impacted soil which will help predict the possibility for disease transmission to plants or humans through cow dung.

Keywords: Cowdung, fungi, sequencing, phylogeny

Introduction

Soil is the upper layer of the earth on which plants grow. It is a combination of organic remains, clay and rock particles. Soil provides a medium for plant growth; supplying plants with physical support, nutrients and water. Soils mostly have a dry bulky density between 1.1 and 1.6g/cm³ while the particle density is between 2.6 to 2.7g/cm³ (Yu *et al.*, 2015). The soil is a habitat for several microorganisms. Soil fungi, bacteria and archaea make up the vast majority of the biological diversity on earth (Aislabie and Deslippe, 2013). However, the knowledge of microbial soil diversity is still limited in Nigeria. Soil

microbiology is important because soil microbes are very sensitive and dependable indicators of soil health as they respond quickly to any soil disturbance (Griffiths and Philippot, 2013). Also, soil microbes are considered vital in any ecosystem because they act on the breakdown of soil organic matter, affect the soil physical and chemical properties, and are involved in nutrient cycling. This has a direct influence on soil sustainability and fertility. Soil borne diseases are always difficult to control, even when conventional strategies such as use of chemicals, cultural methods (crop rotation and shifting cultivation) are

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employed (Baysal-Gurel *et al.*, 2012). Most soil borne fungi survive in soil over a long period of time because they produce resistant structures such as sclerotia, melanized hyphae, oospores and chlamydospores (Baysal-Gurel *et al.*, 2012).

Cow dung is a mixture of urine and faeces at a ratio of 1:3; it mainly comprises of cellulose, hemicelluloses and lignin. It contains 24 minerals including potassium and nitrogen with trace amount of iron, sulphur, copper, cobalt, magnesium and manganese (Kartikey *et al.*, 2016). In addition, it improves the mineral status of the soil. Cow dung harbours a great diversity of microorganisms, which includes different species of bacteria (*Lactobacillus* spp., *Bacillus* spp. and *Corynebacterium* spp.), yeast (*Sacchromyces* spp. and *Candida* spp.) and protozoa (Randhawa and Kullar, 2011). Microorganisms present in cow dung when combined with soil could prove useful or harmful. Morphological and biochemical characterization of these microorganisms is not adequate for taxonomy. Therefore, proper characterization of these microorganisms using molecular methods becomes pertinent. There is difficulty in the use of microscopic features in the identification of microorganisms to the species level. Fungal colony morphology and identification of fungi based on microscopic characteristics such as spore producing structures has its setbacks. An incorrect identification can lead to a wrong preventive/control measures which will make the organism persistent in case of a pathogen. Therefore, molecular characterization becomes necessary in the identification of fungal organisms as it is based on the genetic constitution of the isolates. Polymerase Chain Reaction (PCR) has been reported as the most common and most widely used molecular method for the characterization or identification of fungi (Madhavan *et al.*, 2011; Arvanitis *et al.*, 2014) and is regarded as a typical platform in several molecular biology laboratories worldwide because of its reproducibility (Ragheb *et al.*, 2014; Arastehfar *et al.*, 2018). This study was conducted

to determine the species identity of fungal organisms associated with cow dung-impacted soil using molecular techniques.

Materials and Methods

Study Area and Sampling

The study was carried out at the Regional Centre for Biotechnology and Bio-resources Research, University of Port Harcourt, Choba, Rivers State, Nigeria. The PCR products were sequenced at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Cowdung-impacted soil was obtained from Choba abattoir in Port Harcourt, in April 2019. Soil mixed with cowdung was collected using a trowel at different points in the abattoir at a depth of 0 to 5cm. The soil was pulled together in a ziploc bag and transported to the lab for DNA isolation of fungi.

Isolation of Fungi from Cowdung-impacted Soil

Fungal organisms were isolated using serial dilution method. Five-fold serial dilution of the cowdung-impacted soil was prepared. 0.1ml aliquot of the 10^{-3} dilution was transferred aseptically into sterilized Petri-dishes containing Potato Dextrose Agar (PDA). The plated soil containing cowdung was incubated at room temperature ($27\pm 2^{\circ}\text{C}$) in the Microbial culture laboratory in Regional Centre for Biotechnology and Bio-resources Research for 7 days. After incubation, the isolated fungi were sub-cultured on PDA to obtain pure culture. The morphological characteristics of the isolates were observed on PDA plates and pure cultures were stored in PDA agar slant until when needed.

Fungal DNA Extraction, DNA Quantity and Quality Check

Genomic DNA was extracted following the protocol of Quick-DNATM Fungal/Bacterial MiniPrepKit (Zymo Research Group, USA), with slight modifications. A sterilized surgical blade was used to scrap off the fungal mycelium. This was homogenized in a sterilized mortar using 750 μl of

Bashing Bead Buffer and liquid nitrogen. The protocol of the above-mentioned kit was then followed from this point.

DNA quantity and purity were measured using NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific Inc., USA). DNA purity was determined as the ratio of Ultraviolet light (UV) absorbance by DNA at 260 nm to that at 280 nm. 1% agarose gel was used to ascertain the quality of the DNA through gel electrophoresis. 0.5g of agarose powder mixed with 50ml of TrisBoris EDTA (TBE) 1X in a measuring flask was microwaved for 2 minutes. 5µL of EZ viewing dye (Blue Light) was added to the content in the conical flask and then poured into the casting tray with a comb in place. The gel electrophoresis unit was set up and molecular weight marker (1Kb DNA Ladder) was loaded into the first lane of the gel. The DNA samples were separately loaded into the other wells. Each DNA sample (3µL) was mixed with 3µL of loading dye (2X) and then loaded in one lane on the gel. The set up was allowed to run for 40 minutes at 100volts. At the end of the running time, the DNA fragments were visualized with UV transilluminator (Gel Documentation microDOC TM, Cleaver Scientific Ltd, UK).

PCR Amplification and Sequencing

The primer pair: ITS4: forward (5'- TCCTCCGC TTATTGATATGS-3') and ITS5: reverse (5' GGAAGTAAAAGTCGTAACAAGG-3) was used to amplify the ITS1-2 gene sequence of the fungi. The PCR cocktail consisted of 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 MgCl₂ (Promega), 0.5µL of each primer (concentration of 5µM) and 3.1µL of Nuclease-free water, making a final volume of 10 µL. A thermal cycler (Eppendorf, Germany) was used to perform amplifications and the PCR conditions consisted of an initial denaturation step of 94°C for 5 minutes, 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. 1% agarose gel was used to ascertain whether amplifications occurred through gel electrophoresis. 5µL of each PCR product and 1kb DNA ladder were separately loaded on gel. The amplicons were sequenced using Sanger sequencing. Single-stranded DNA template, a DNA polymerase, a DNA primer, 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 required for the formation of a phosphodiester bond between two nucleotides. This addition of ddNTP resulted in the termination of DNA.

Phylogenetic Analysis

Sequence were edited and trimmed on MEGA X. Sequences were blasted for identification of species on National Centre for Biotechnology Information (NCBI) database. The sequences were compared with other ITS1-2 gene sequences in GenBank. Sequences were aligned using Clustal W. Using the Maximum composite likelihood method, best BLAST hits were used for the construction of neighbor-joining phylogenetic tree. Evolutionary analysis was performed on MEGA X software version 10.0.1 (Kumar *et al.*, 2018).

Results

Fungi Associated with Cowdung-impacted Soil

Two fungi were isolated from the cowdung-impacted soil. The morphological features of the isolates are shown in Table 1. The frequency of occurrence of each organism was determined. Sample 1 occurred more (2.75±0.25) than sample 2 (2.25±0.48) as presented in Table 1. Pure cultures of the isolates are presented in Plate 1.

DNA Quantification and Gel Electrophoresis

NanoDrop Spectrophotometer revealed the purity and concentration of DNA extracted from the fungal species associated with cowdung-impacted

soil (Table 2). Gel electrophoresis showed that the extracted DNA of both samples was of good quality as shown in Plate 2.

Table 1: Frequency of occurrence of fungal isolates from cow dung-impacted soil

Fungal sample ID	Description	Frequency of occurrence
1	Pale grey spores	2.75±0.25
2	Dark brown spores	2.25±0.48

Table 2: DNA Concentration of fungal isolates

Fungal Sample ID	DNA concentration (ng/µl)	Absorbance at 260nm/280nm (purity)
1	13.7	2.5
2	41.3	1.92

Polymerase Chain Reaction

PCR products of the amplified ITS 1-2 genes viewed under UV light showed clear amplicons. The size of each amplicon obtained from the PCR amplification of the ITS gene are presented in Plate 3.

DNA Sequences and Blasting

The nucleotide lengths of the DNA sequences were determined to be: 834bp and 372bp for samples 1

and 2 respectively. The alignments of the sequences are shown in Figures 4a and 4b. The ITS 1-2 gene sequences obtained for each fungal isolate was more than 200 base pairs.

The sequences were blasted on NCBI database and the isolates were identified as *Lichtheimia ramosa* for sample 1 and *Galactomyces pseudocandidus* for sample 2. The taxonomic affinity of the isolates on GenBank is shown in Table 3.

The sequences were submitted on GenBank and accession numbers were assigned to the isolates as follows:

Sample 1: *Lichtheimia ramosa* (KP132382.1) strain RCBBR_AEAPR7

Sample 2: *Galactomyces pseudocandidus* (MK883033) strain RCBBR_AEAPR8

Phylogenetic Analysis

The phylogenetic tree constructed showed that *Aspergillus amstelodami*, *Absidia corymbifera*, *Mycocladius corymbiferus*, *Galactomyces geotrichum* and *Galactomyces candidus* were most closely related to the fungi isolated from cowdung-impacted soil as shown in Figure 5. The vertical lines on the trees signify the difference between the branches. The longer the vertical line, the more the difference between the branches.

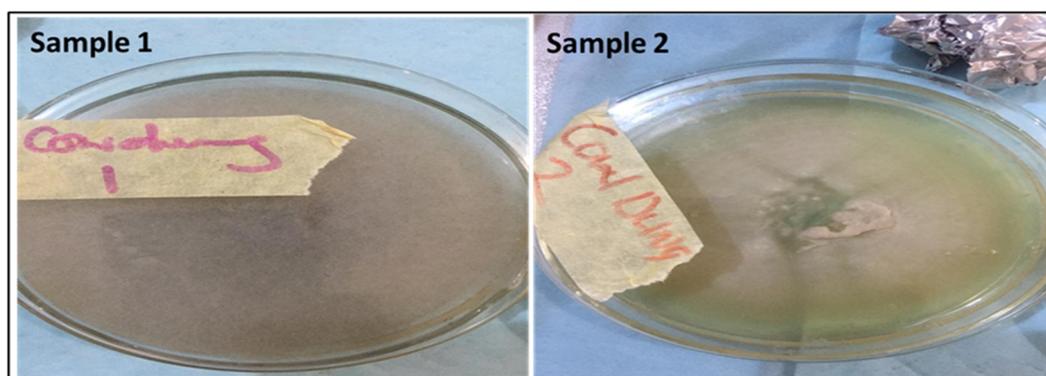


Plate 1: Pure cultures of fungal sample 1 (a) and sample 2 (b) isolated from cowdung-impacted soil

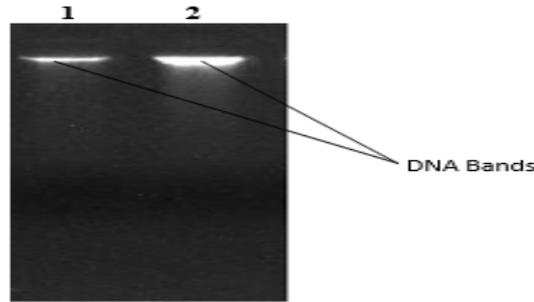


Plate 2: Genomic DNA of fungal isolates as viewed under UV light. (The numbers, 1 and 2 represent the fungal samples)

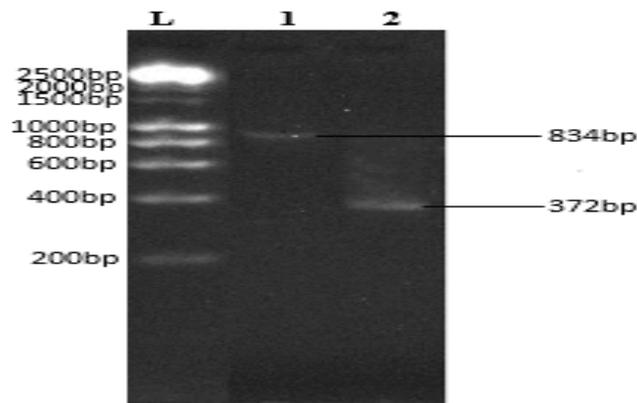


Plate 3: PCR amplification of ITS 1-2 gene sequences of the fungal isolates. L: 1kb DNA Ladder; numbers 1 and 2 represent PCR products for samples 1 and 2.



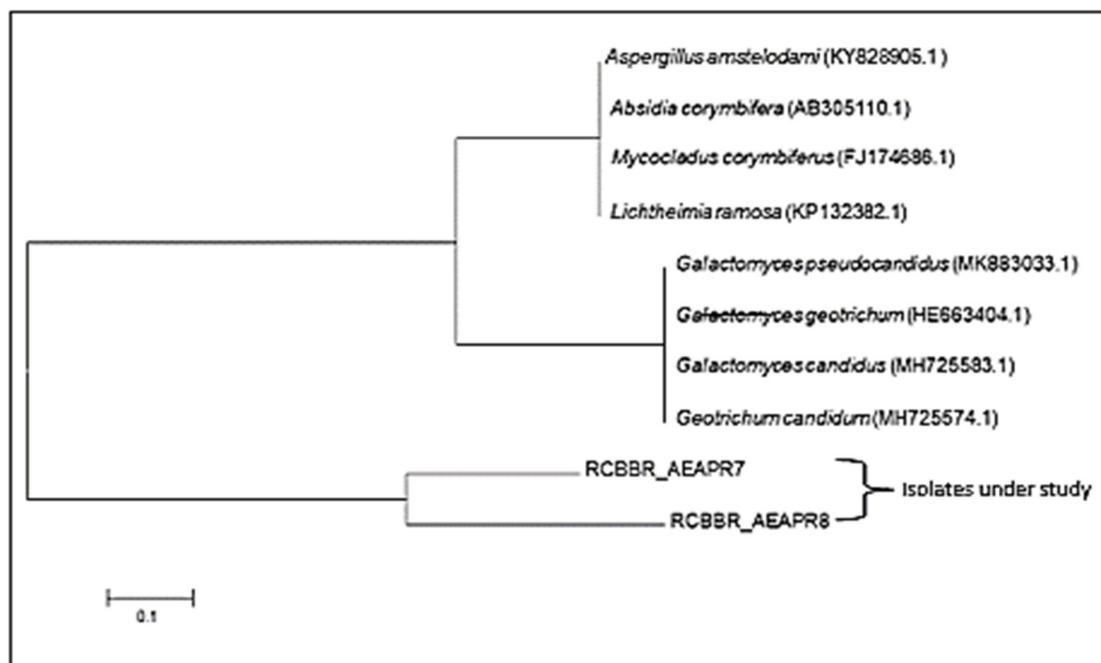
Figure 4a: Sequence alignment of sample 1



Figure 4b: Sequence alignment of sample 2

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

Fungal Sample ID	Taxonomic affinity (GenBank No)	Similarity (%)
1	<i>Lichtheimia ramosa</i> (KP132382.1)	97
2	<i>Galactomyces pseudocandidus</i> (MK883033.1)	92

**Figure 5:** Phylogenetic tree showing the fungal isolates obtained from cowdung-impacted soil using Composite likelihood method.

Discussion

This present study characterized fungal organisms from cowdung-impacted soil using molecular techniques. Molecular techniques have proven to be more reliable than traditional methods as it allows for the comparison of DNA sequence information between known and unknown fungal species. Traditional methods commonly used for identification of fungi have led to the misrepresentation of many isolates. The molecular techniques employed for identification of fungi in this study led to the successful characterization of two (2) fungi isolated from cowdung-impacted soil. This study revealed the

identity of the isolated fungal organisms to be *Lichtheimia ramosa* and *Galactomyces pseudocandidus*. In line with previous studies on isolation of *Lichtheimia ramosa* and Ascomycetous Yeasts Strains (Abu-Mejdad *et al.*, 2019; Nsa *et al.*, 2020), the result has shown that *Lichtheimia ramosa* and *Galactomyces pseudocandidus* occur in the soil.

Lichtheimia ramosa is a mucoralean fungus and has adverse effect on human health and also on animals as it has been identified to cause mucormycosis (Patrick *et al.*, 2012). *L. ramosa* was discovered in a patient with burn wounds to cause primary cutaneous mucormycosis (Kaur *et*

al., 2014). *Lichtheimia ramosa* is said to be one of the most common cause of mucormycosis in Europe (Volker, 2014). Mucormycosis is an infrequent fungal infection with a very high fatality potential. It occurs in immunocompromised patients, diabetic patients with ketoacidosis and immunocompetent patients after trauma and exposure to contaminated soil (Gracia-Hermoso *et al.*, 2009).

Galactomyces candidum is a yeast-like fungus usually isolated from water, soil, air, milk, plant tissues, cheese, silage, digestive tract in humans and other mammals. *G. candidum* rarely infects plants and animals. However, *Galactomyces pseudocandidus* is an ingredient in the cosmetics industry where it is used as a moisturizing agent. It helps to improve the skin's moisture barrier, protecting the skin from environmental stress. *Galactomyces* fermented filtrate increases the skin's response to stimulus by improving the skin's barrier proteins (Takei *et al.*, 2015). The isolation of other fungal species from other genera in cow dung has been recorded by some scholars. Mohammed *et al.* (2017) reported the presence of fungi including *Agaricus campestris*, *Panaeolus papilionaceus*, *Agaricus bisporus*, *Protothopharia semiglobata*, *Deconica coprophila*, *Copelandia cyanescens* and coprophilous mycoflora in cowdung. Ninety-one (91) *Penicillium* species and nine (9) *Talaromyces* species were isolated from herbivore dung in Spain using molecular method (Guevara-Suarez *et al.*, 2020). These fungi are saprophytic on herbivores and are referred to as coprophilus fungi.

Conclusion

Fungi belonging to different genera have been isolated from cowdung-impacted soils. This study revealed two fungi associated with cowdung-impacted soil. Molecular characterization techniques employed in this study were effective in identifying the two fungal isolates (*Lichtheimia ramosa* and *Galactomyces pseudocandidus*) to the

species level. *Lichtheimia ramosa* as a pathogen responsible for mucormycosis poses a threat to herbivores since most times, herbivores feed directly on grasses where cows might have given out their dung. *Galactomyces pseudo-candidus* is a beneficial organism that can be isolated from cow-dung impacted soil and produced on a large scale for the cosmetics industry. The organisms obtained from this study have thrown more light on some of the fungal organisms present in cow-dung impacted soil making this substrate an area of great concern for the cosmetics and drug industries.

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Conflict of Interest

No conflict of interest exists.

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