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MOLECULAR CHARACTERISATION OF FUNGI ASSOCIATED WITH SEWAGE-IMPACTED SOIL

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ABSTRACT

The decay of faecal matter from a septic system causes the arousal of fungi in the surrounding soil. These fungi can cause diseases if there is sewage spillage containing untreated or improperly treated wastewaters. Molecular techniques of identification of fungi have shown to be more dependable than traditional methods of identifying fungal species. This study was carried out to identify the fungal species associated with soil obtained from sewage-impacted soil near a septic tank using both traditional cultural techniques and molecular method. Fungi associated with the soil samples were isolated using serial dilution and Potato Dextrose Agar (PDA) method. Deoxyribonucleic Acid (DNA) was extracted from the pure cultures of fungal isolates using Quick DNA Fungal/Bacterial Miniprep kit. Polymerase Chain Reaction (PCR) amplification of internal transcribed spacer (ITS) region of the fungal isolates was carried out using universal primer pair; ITS4 and ITS5. The PCR products were sequenced and the sequences were blasted against National Centre for Biotechnology Information database. The result of the nucleotide sequence analysis revealed the identity of the isolates as *Trichoderma harzanium* with 580 base pairs and *Aspergillus welwitschiae* with 560 base pairs. Sequences of the isolates were aligned and compared with sequences on GenBank and a phylogenetic tree was constructed. The cultural method only aided in suggesting the suspected genera of the isolates while the molecular method was able to identify the isolates to the species level. This study will promote the knowledge of the fungal species associated with sewage-impacted soil and also aid researchers in proffering ways to enhance the prevention/control of diseases associated with sewage spill.

KEYWORDS: Septic tank, fungi, soil, phylogeny, sequencing

INTRODUCTION

Physical, chemical and biological activities take place in the soil. In the same way, the fertility of the soil has three components: physical, chemical and biological fertilities. In biological fertility, the interaction of microorganisms in the soil with other components varies greatly (Johns, 2017). The role of fungi in the soil is an extremely complex one and is fundamental to the soil ecosystem. They play important roles in nutrient cycles which are vital to sustenance of life on earth. They perform ecological services that strongly impact the quality of human life with consequent economic benefits. A septic system is an underground treatment system for wastewater. It is made up of two main parts: a subsurface soil absorption system which is the treatment part and then a septic tank (Adegoke and Stenstrom, 2019). A septic tank is an underground chamber made of concrete fibre glass or plastic through which domestic waste water (sewage) flows for basic treatment (Tiley *et al*., 2014). Septic tanks are constructed in such a way that they hold raw domestic effluent that contain faecal matter and other suspended materials disposed from homes so that the denser solids sediment as a sludge in the septic tank and are partially digested in the absence of oxygen, leaching the effluent into the ground. These septic tanks are not designed to destroy pathogens that may be in the human wastes and thus can allow bacteria, viruses, protozoa, fungi and intestinal parasites to spread and may cause diseases (Pradhan *et al*., 2011) if human come in contact with sewage spill. Any urban development that practises on-site effluent disposal methods of domestic effluent treatment like septic tanks soil absorption systems (ST-SAS) and pit latrines creates a risk to groundwater contamination (Mumma *et al.*, 2011). Most African countries do not

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conscientize their citizens on the need to protect groundwater reservoirs. Public understanding of groundwater, its intricacies and importance is generally poor in Africa. The overreliance on ground water is threatened by overwhelming numbers of septic tanks, improper disposal of household wastes and wastewaters (WHO, 2003). Majority of the populace in most parts of Nigeria depend on groundwater gotten from boreholes for domestic uses including drinking. As of 2018, an estimate of 60% of the total population in Nigeria depends on groundwater as their main source of drinking water (Joint Monitoring Programme for Water Supply, Sanitation and Hygiene, 2019).

The assessment of water quality in groundwater reservoirs is essential because it is the main source of water in most developing countries of the world especially in the rural areas. Untreated sewage from pit latrines and septic tanks can seep into hand dug boreholes that are not very deep or that are very close to the septic tanks (Adelekan, 2010). The untreated sewage contains pathogens such as bacteria, viruses, fungi and protozoa that become a potential source of disease and a genuine public health threat. Pathogens including viruses, bacteria and fungi have caused severe health related issues due to septic system failures (Adelekan, 2010).

There is need for proper molecular characterisation of the soil-borne fungi associated with septic tank system. Fast and accurate detection and characterisation of microorganisms is a demanding and important feature in various industries and in medicine. Standard methods such as biochemical tests and culturing of microorganisms on media are known to be time-consuming and laborious. Molecular techniques are quick less laborious and yield broad reports on microorganisms (Franco-Duarte *et al*., 2019). Molecular identification of fungi has been reported to be consistent in the confirmation of the identity of strains that were previously identified using conventional method based on morphological features (Iheanacho *et al*., 2014). The use of PCR amplification and subsequent sequencing of PCR products is a sensitive and specific technique for the identification of microorganisms. Pure and high-quality DNA is extracted using the appropriate kits (Dalla-Costa *et al.*, 2017). Fungal cell walls are strong and are usually difficult to disrupt; so DNA isolation requires steps that will effectively break down the cell wall. Bashing beads which enhances mechanical disruption in order to promote enzymatic digestion during the lysis stage are usually used (Gonzlez-Mendoza *et al.*, 2010; Arvanitis *et al.*, 2014). The result generated from this study will outline some of the fungal species present in sewage-impacted soil and also help sensitize stakeholders on the need to site septic tanks appropriately, away from bore holes or ground water reservoir commonly called “dug well”.

MATERIALS AND METHODS

Study Area and Sample Collection

The study was carried out at the Regional Centre for Biotechnology and Bio-resources Research (RCBBR), University of Port Harcourt Choba, Rivers State, Nigeria. The PCR products were sequenced at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Sewage-impacted soil was obtained from five different points at a sewage spillage near a septic tank within University of Port Harcourt Choba, Rivers State in April 2019 using a hand trowel to a depth of about 2.5cm. Soil samples were transferred into a sterile container and transported to the laboratory for isolation of fungi and subsequent DNA extraction of isolated fungi.

Isolation of Fungi from Sewage-impacted Soil

Fivefold serial dilution of the soil sample was prepared using sterile physiological saline. Sterile pipette was used to collect an aliquot of 0.5ml of the dilute solution from test tube 10^{-3} dilution and this was transferred into sterile Petri dishes containing Potato Dextrose Agar (PDA) medium. The Petri dishes were incubated in a laminar flow cabinet at room temperature for 7days and thereafter observed for fungal growth. Frequency of occurrence for each isolate was determined by counting the number of colonies for each fungal characteristic feature on Petri dishes. The fungal colonies observed on plates were separately sub-cultured to obtain pure cultures. Pure cultures were stored at 4^{o}C prior to Deoxyribonucleic acid (DNA) extraction.

Fungal DNA Extraction and DNA Quality Checks

Extraction of genomic DNA from the fungi was conducted using Zymo Quick DNA Fungal kit. The protocol of the above mentioned kit was used with slight modifications as obtainable at the Regional Centre for Biotechnology and Bio-resources Research Laboratory, University of Port Harcourt, Choba, Rivers State, Nigeria.

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 absorbance at 280 nm. The quality of the DNA was determined through gel electrophoresis using 1% agarose gel.

PCR Amplification and Sequencing

Primers ITS4, forward (5-TCCTCCGCTTATTGATATGS-3) and ITS5, reverse (5-GGAAGTAAAAGTCGTAACAAGG-3) were used to amplify the ITS1-2 region of the fungi. PCR was carried out in a final volume of 25µl containing 2.5µl of 10x PCR buffer, 1.0µl of 25Mm $MgCl_2$,1.0µl each of each primer, 1.0µl of DMSO, 2.0µl of 2.5M DNTPs, 0.1µl of 5µ/µlTaq DNA polymerase and 3.0µl of genomic DNA (10ng/µl) and 13.4µl nuclease free water. Amplifications were performed in a thermal cycler (Eppendorf, Germany) 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 54°C and elongation for 45 seconds at 72°C. A final extension for 7 minutes at 72°C was then performed. Electrophoresis of PCR-amplified products was performed in 1.5% agarose gels. Amplified products were sequenced on ABI 3500 Genetic Analyzer (Thermo Fisher Scientific, Massachusetts, United States).

Phylogeny

Sequences were trimmed on MEGA X and blasted on NCBI database for identification of species. The sequences of the ITS1-2 gene of the isolates were compared with sequences in GenBank. Best BLAST hits were aligned and used for the construction of neighbour-joining phylogenetic tree using the maximum composite likelihood method. Evolutionary analysis was conducted on MEGA X software (Kumar *et al*., 2018).

RESULTS

Fungi associated with Sewage-impacted Soil

Two fungal organisms were isolated and found to be associated with the sewage-impacted soil (Figure 1). The frequency of occurrence of each organism was determined. Sample 2 had a higher frequency of occurrence (2.25) than sample 1 (2.00) as presented in Table 1.

Table 1: Frequency of occurrence of fungi isolated from sewage-impacted soil.

Sample ID	Description	Frequency of occurrence
1	Yellow to brown dark spores	2.00±0.71
2	Green spores	2.25±0.25

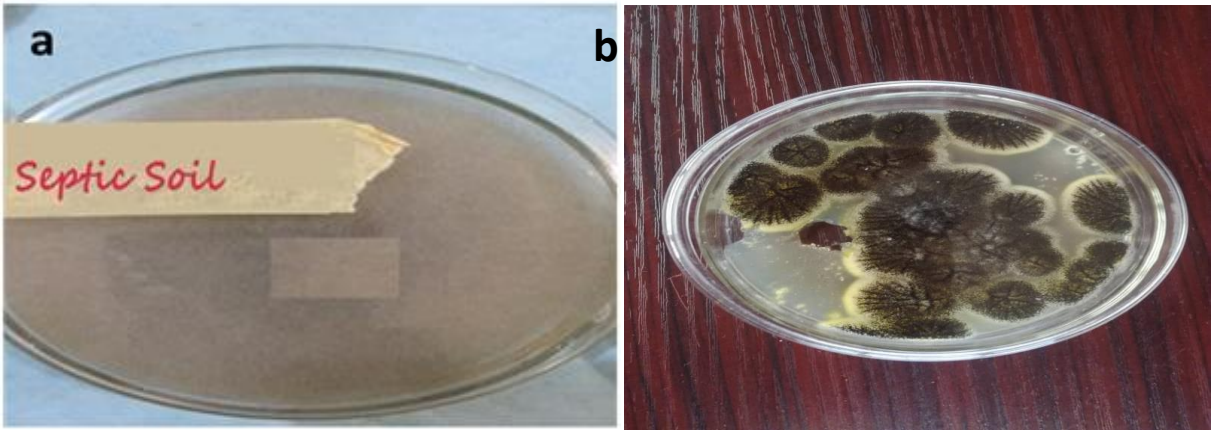


Figure 1: Pure cultures of Sample 1(a) and 2(b) isolated from sewage-impacted soil.

DNA Extraction, Quantification and Gel Electrophoresis

The concentration and purity of genomic DNA from the fungal species is presented in Table 2. Gel electrophoresis showed that the extracted DNA of the isolates were of good quality (Figure 2).

Table 2: Concentration of DNA extracted from fungal isolates

Sample ID	Nucleic Acid Conc.(ng/µl)	Absorbance at 260/280 (Purity)
1	105.0	1.82
2	100.5	1.73

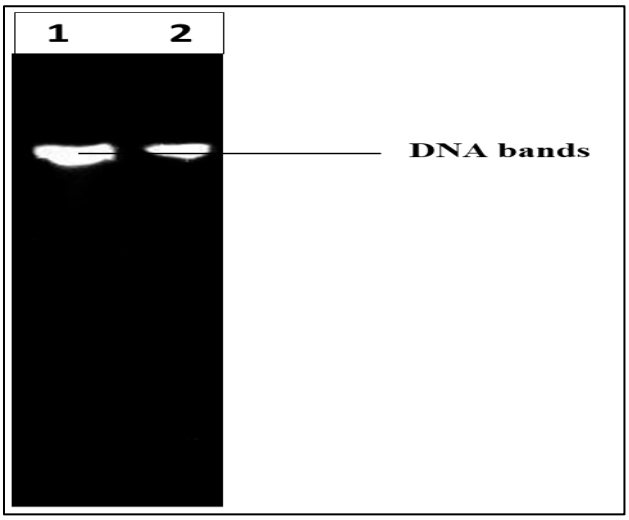


Figure 2: Gel Electrophoresis of genomic DNA of fungal isolates extracted from sewage-impacted soil

Amplified Products Obtained from ITS1-2 Region

The PCR products of the two fungal isolates are presented in Figure 3. The fragment sizes of the PCR products were between 600 to 650 base pairs

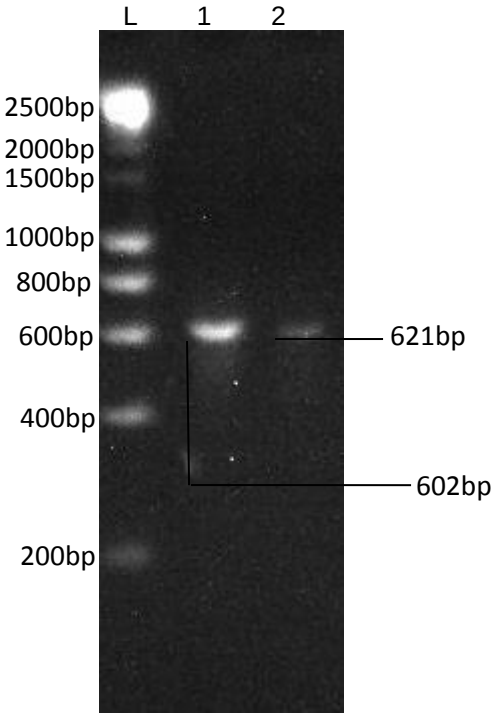
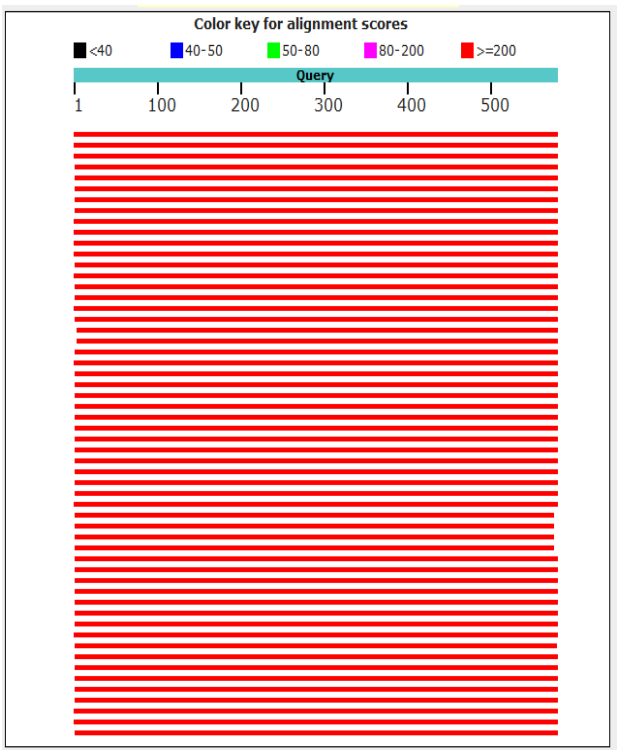


Figure 3: Gel Electrophoresis of PCR amplification of the ITS1-2 gene sequences of the fungal isolates

DNA Sequences and Identification of Fungal Isolates

The number of base pairs of the isolates after sequencing was determined to be 621bp and 602bp for Sample 1 and 2 respectively. The sequence alignments of the samples are shown in Figures 4 and 5.



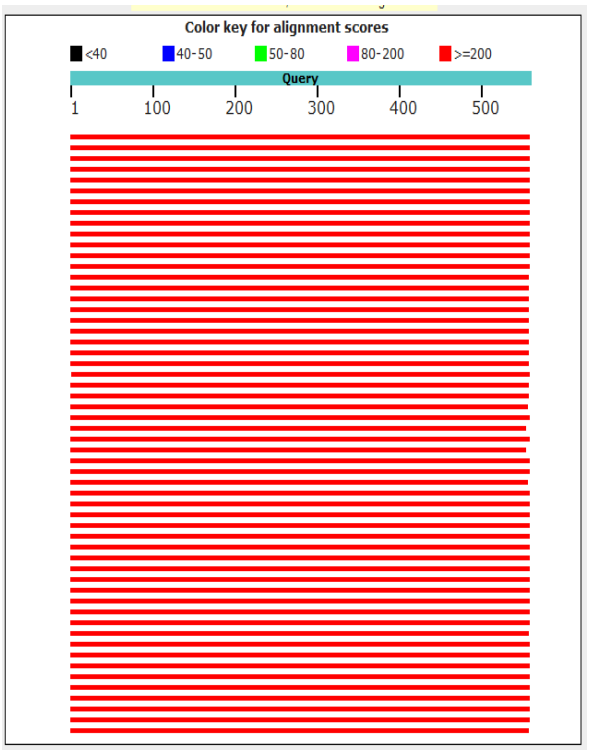


Figure 4: Sequence alignment of isolate 1 **Figure 5: Sequence alignment of isolate 2**

The BLAST results revealed the identity of the fungal organisms as *Trichoderma harzanium* and *Aspergillus welwitschiae* for samples 1 and 2 respectively. Table 3 shows the taxonomic affinities of the isolates from Basic Local Alignment Search Tool (BLAST) searches.

Table 3: Putative taxonomic affinities of sequence type inferred from BLAST search

Sample ID	Taxonomic affinity (GenBank no.)	Similarity (%)
Trichoderma harzanium	KY807124.1	91%
Aspergillus welwitschiae	MG669190.1	95%

Sequences of the DNA obtained were submitted to GenBank and they were assigned accession numbers (in parenthesis) as follows:
Sample 1- *Trichoderma warzanium* (MN443786) strain RCBBR_AEASP4
Sample 2- A*spergillus welwitschiae* (MN443787) strain RCBBR_AEASP5.

PHYLOGENETIC ANALYSIS

The phylogenetic tree constructed showed the relationship between the isolates obtained from the sewage-impacted soil and other fungal isolates on GenBank. The vertical lines on the tree indicate the difference between the branches. The greater the length of the vertical lines, the more the difference between the branches. The isolates from the sewage-impacted soil were shown to be most closely-related to *Trichoderma warzanium* and A*spergillus welwitschiae*; and these two organisms are in turn related to *Trichoderma lixii* and *Aspergillus niger* (Figure 4).

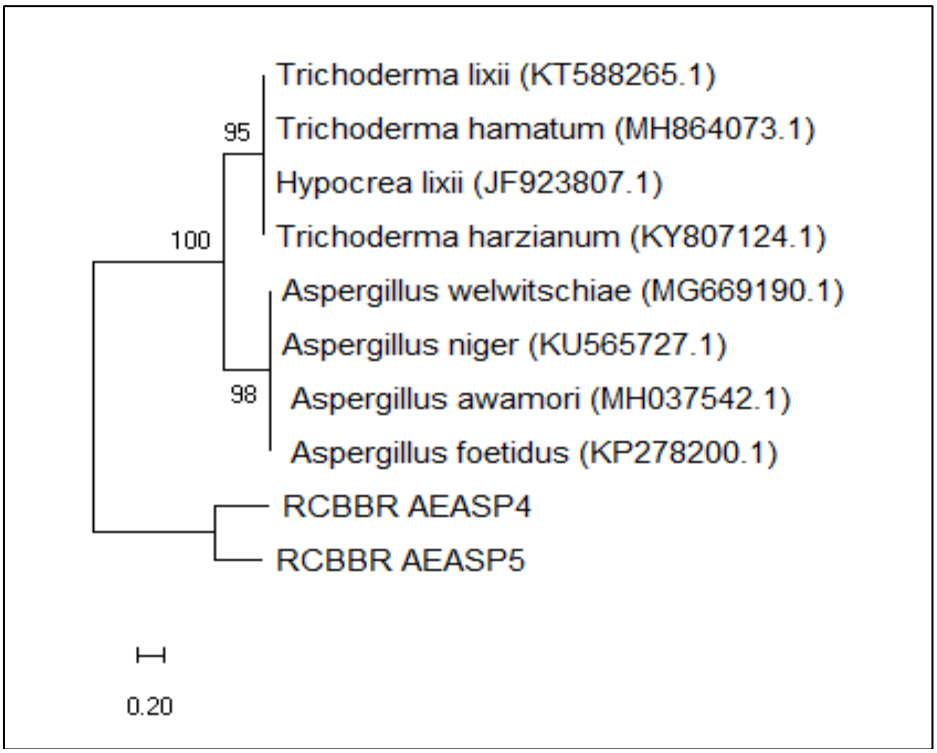


Figure 4: Neighbor joining phylogenetic tree of the fungal isolates and other fungal species

DISCUSSION

Traditional methods of identifying fungi result in species list that misinterprets and misrepresents the fungal community. Molecular techniques used in the identification of microorganisms have shown to be more dependable than traditional methods and many researchers in recent years have resorted to these techniques. The molecular techniques employed in this study led to the identification of the two isolated fungi as: *Trichoderma harzanium* and *Aspergillus welwitschiae*. Sequences were aligned and phylogenetic tree was constructed to show the fungal species that are most closely related to the fungi isolated from the study.

Trichoderma species have long been identified and characterized as potential opportunistic, avirulent plant symbionts and biological agent against different soil-borne pathogens (Ponnusamykonar *et al*., 2011; Barari and Foroutan 2013; Asad *et al*., 2014). The genus *Trichoderma*, commonly called weed mould in mushroom industry are responsible for causing green mould disease of oyster mushrooms (*Pleurotus ostreatus*). This pathogenic fungus completely colonizes the substrates and in certain cases grows on the surface of the emerging mushrooms. *Trichoderma* species produce various mycotoxins which retard the growth of mushroom mycelium and fruit bodies (Jayalal and Adikaram, 2007; Heydari and Pessaraki, 2010). Some mushrooms show symptoms of wateriness, shrinking and drying of fruiting bodies as well.

Aspergillus species have been detected in indoor dust (Visagie *et al*., 2014) and even in clinical specimens from the ear canal in humans, causing otomycoses (Szigeti *et al*., 2012a; Szigeti *et al*., 2012b). *Aspergillus welwitschiae* was reported as the causal agent of the sisal bole rot disease (Duarte *et al*., 2018). *A. welwitschiae* has been detected in different environmental substrates such as; outdoor air (Lee *et*

al., 2016), soils (Palumbo *et al*., 2016), caves (Nováková *et al*., 2018), sea salts (Biango-Daniels and Hodge, 2018) and in human nails, causing onychomycoses (Tsang *et al*., 2016).

CONCLUSION

The cultural method used in this study was only able to suggest the suspected organisms isolated based on the morphological characteristics observed; but this method cannot be used to successfully characterize fungal organisms to the species level. Molecular techniques have been used in the study of fungal ecology as it provides a comprehensive data on the organisms under study. Solving a problem begins with the correct identification of the cause of the problem. Therefore, correct identification of microorganisms is pertinent in every field of life sciences in order to provide accurate information. Polymerase chain reaction amplification of the internal transcribed spacer (ITS) region, sequencing of the PCR products and bioinformatics analysis led to the successful identification of the isolates obtained from this study as: *Trichoderma harzanium* and *Aspergillus welwitschiae.* This study has promoted the knowledge of the fungal organisms associated with sewage-impacted soil which will make individuals aware of the dangers of building septic tanks close to ground-water reservoirs.

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