MOLECULAR CHARACTERISATION AND IDENTIFICATION OF THREE MUSHROOMS POTENTIAL FOUND IN THE NIGER DELTA REGION.

ABSTRACT

Mushrooms are a group of fungi that are diverse and in the Niger Delta region, there are various species, some of which share similar morphological features. Over the years, mushrooms have been put to several important uses ranging from food, nutraceuticals, feed for livestock, and more recently bioremediation, hence, the need for an accurate approach of identification is eminent. This study aims to identify three mushroom samples with the potential for crude oil degradation using molecular tools. The molecular identification of the mushrooms was carried out with the ITS (Internal Transcribed Spacer) region to analyze their genetic diversity. The three mushroom samples were identified *as Pleurotus ostreatus, Pleurotus floridanus* and *Lentinus squarrosulus*. The blast results showed 86.0%, 97.9%, and 88.4% for *P. ostreatus, P. floridanus* and *L. squarrosulus* respectively for sequence similarity. The samples were assigned unique accession numbers on GeneBank The use of molecular characterization gives reliable results to the species level. The results from this study have increased the database of *Pleurotus* and *Lentinus* DNA and serve as a basis for the identification of unidentified species by comparing their PCR amplified sequences with ITS primers.

INTRODUCTION

There has been much global suffering due to increased environmental problems resulting from intensified industrialization and the use of chemicals for agricultural practices. This indiscriminate release of chemicals into the environment has caused a high level of pollution. Remediation of oil spill impacted sites in the Niger Delta after initial clean-up has so followed two (2) conventional methods viz: chemical and mechanical. These methods in themselves have had residual effects on the environment and are very expensive to carry-out. Hence, the need for eco-friendly and cost-effective methods of remediating spill sites (Hamman, 2004). Bioremediation uses the biological organisms' metabolic potential to degrade or transform hazardous compounds in the environment into less toxic or harmless forms (Watanabe 2001; Yadav et al. 2019). The use of fungi referred to as mycoremediation, has attained widespread acceptance due to its very low substrate specificity, so they can mineralize a wide range of

highly recalcitrant and persistent organopollutants (Pointing, 2001; Mansur *et al*., 2003, Adenipekun & Lawal, 2012). Prospection for fungi is owing to its ability to secrete high levels of lignin modifying enzymes and other enzymes with desired properties for biotechnological applications (Adebayo et al., 2012). Mushrooms belong to these physiological group known as white rot fungi known to produce lignin modifying enzymes. The need to accurately identify these species is very important as it is the first step to utilize them for various biotechnological applications. This study aims to identify the three mushroom samples using molecular tools.

MATERIALS AND METHODS

SOURCE OF FUNGI

The fungi species used for this work were obtained from (BIODEC) Odi Bayelsa State. The samples were labelled as samples 1, 2, and 3 for the collection of indigenous mushrooms. These were transported to the laboratory immediately in zip lock bags.

MEDIA PREPARATION

Potato dextrose agar (PDA) was used for tissue culture. This was prepared following the manufacturer's instruction using the potato dextrose agar powder (Hi-Media). To prepare 1 Litre of the media 39grams of PDA was dissolved in 1 litre of distilled water and autoclaved at 121ºC for 15 minutes. Chloramphenicol was added as antibacterial agent.

TISSUE CULTURE

This was carried out at the Regional Centre for Biotechnology and Bioresources Research laboratory, University of Port Harcourt, Choba, Rivers State. After surface sterilization, the inner tissue of the stipe was cut with a surgical blade already flamed and cooled around the flame area. The excised tissue was inoculated into the Petri dish with freshly poured media. The Petri dishes

were immediately sealed with masking tape and cling film to avoid external contamination. The plates were then incubated for 14 days in the dark at room temperature 27±2ºC. This was done for each species used. (Bankole and Adekunle 2012 and Chuku et al., 2015). Sub-culturing was done several times to obtain a pure culture.

MOLECULAR CHARACTERISATION

DNA EXTRACTION

A slight modification of the cetyltrimethylammonium bromide (CTAB) Method (Adedokun et al.,2016) fungal mycelium was scraped off and transferred to already sterilized mortar. DNA was extracted with Zymo Quick DNA Fungal /Bacterial miniprep Kit, with the following steps as stated by the manufacturer Zymo Research group CA, USA. For DNA extraction, a five (5) days old culture of the three samples (1, 2, and 3). The extracted DNA was stored in the freezer and maintained on ice to avoid denaturation.

DNA QUANTIFICATION

This step was done to determine the concentration and purity of the extracted DNA using the NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, Delaware, USA). The sample was vortexed briefly to homogenize. 1.7µL of the extracted DNA was used. Then sample concentration (µg/µl) was measured absorbance at 260/280nm wavelength appropriate for nucleic acid sample. This reading was taken in triplicates. (Adedokun et al.,2016; Khan et al., 2018).

DNA QUALITY CHECK

To determine the quality of extracted DNA, Agarose gel electrophoresis method was adopted to determine the quality of nucleic acid extracted (Lee et al., 2012). The gel was viewed in a gel documentation system by exposing it to UV light and pictures were taken to show the DNA Bands.

POLYMERASE CHAIN REACTION (PCR)

PCR Amplification of DNA samples was done using universal primers in a thermal cycler (GeneAmp PCR system 9700). ITS4: Reverse (5'- TCCTCCGCTTATTGATATGC-3') and ITS5: Forward (5'- GGAAGTAAAAGTCGTAACAAGG -3') (White et al., 1990; Bellemain et al., 2010 and Gao et al., 2011). The total reaction volume of 25µl made up of 2.5µl of 10x PCR buffer, 1µl of 25mM $MgCl_2$, 1µl each of the forward primer and reverse primer, 1µl of DMSO, 2µl of 2.5mMDNTPs, 0.1µl of 5µ/µl Taq DNA polymerase, and 3µl of 10ng/µl DNA and 13.4µl Nuclease free water. The PCR cycling parameters: Initial denaturation at 94˚C for 5mins, followed by 36 cycles of denaturation at 94˚C for 30sec, annealing at 54˚C for 30secs and elongation at 72˚C for 45sec. Followed by a final elongation step at 72˚C for 7 minutes. Amplified fragments were visualized on Safe view- stained 1.5% agarose electrophoresis gels and photographed under UV light in a gel documentation system. A 1kb ladder (Bioline) used as a marker for the gel run served as control.

SEQUENCING and BIOINFORMATIC ANALYSIS

The products of PCR were subjected to Sanger dideoxy sequencing at IITA, Ibadan, Nigeria. The sequences obtained were deposited to the GenBank database to compare with the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool

(BLAST) algorithm (Chang and Coppola, 2009). Phylogenetic trees were constructed for each organism using the best Basic Local Alignment Search Tool BLAST hits Multiple sequence alignment with the Clustal W algorithm and the phylogenetic trees showing maximum likelihood algorithm (Juke-Cantor) was obtained on (MEGA) version 8 (Tamura et al., 2011).

RESULTS and DISCUSSION

DNA concentration range from 26.6 ng/µL to 30.53 ng/µL for the samples while DNA purity range from 1.66 to 1.79 respectively. Quality check was then conducted on agarose gel which produced single bands of intact DNA when viewed on the gel with a gel documentation system, the result is as shown in plate 1. The results obtained indicated that the extracted DNA were qualified and appropriate for further molecular analysis. The results indicate contaminants like proteins or phenols are absent with the lower and higher range respectively (Couto et al., 2013; Lucena-Aguilar et al., 2016).

Concentration and Purity of Extracted Nucleic Acid (DNA, RNA and cDNA)

Table 1: Concentration and Purity of DNA extracted from the samples used

Sample ID	DNA concentration (ng/µL)	DNA Purity (260/280nm)

1	29.97 ±2.73	1.79 ±0.05
2	30.53 ±0.40	1.66 ±0.01
3	26.60 ±1.41	1.66 ±0.01

Each value is the mean of three replicates ± standard deviation.

1 2 3

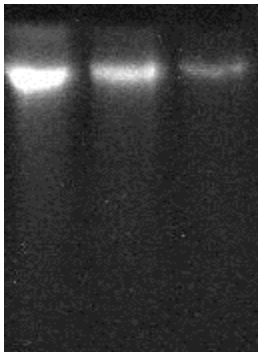

PLATE 1: Agarose gel photograph of DNA extracted of the three samples used lane 1-3 representing sample 1-3 respectively.

PCR Amplification

Isolated DNA from the samples used was amplified with ITS primers (ITS-4 and ITS-5). The amplified products obtained were visualized on Safe view- stained 1.5% agarose electrophoresis gel. They appeared as single intact bands on the agarose gel with safe view-stain as shown in the plate below. The size of the PCR fragment for all the samples was deduced with the ladder used. Products of PCR migrated between 654bp and 727bp as shown in Plate2. The result of all the

mushrooms was within the range (350 and 880bp) for fungi species using ITS1 and ITS4 (Fujita et al 2001) and the range of (651 and 800bp) (Imtiaj et al., 2011).

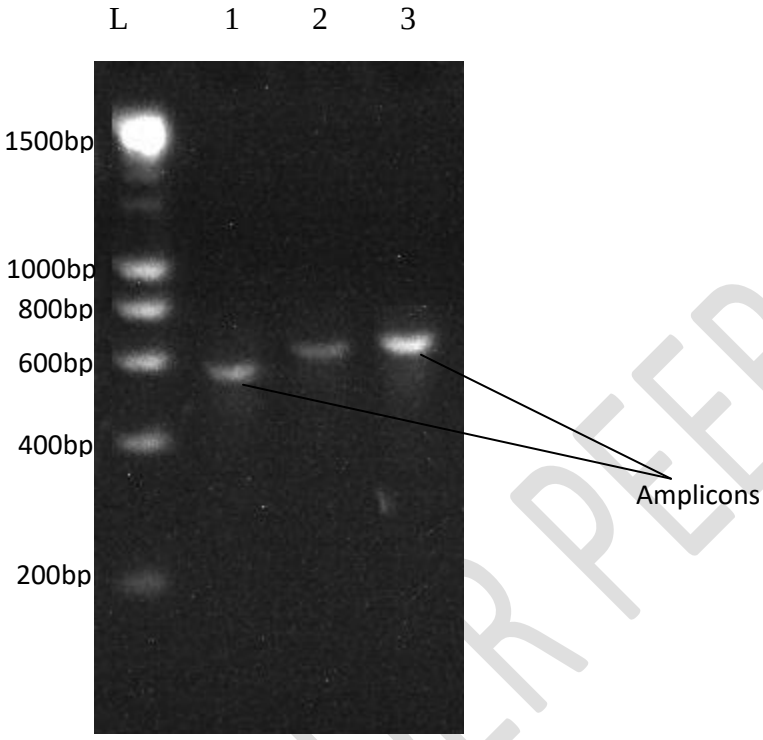

PLATE 2: Amplicons obtained from a polymerase chain reaction of the internal transcribed spacer (ITS) region of the fungi understudy

L – 1Kb DNA Ladder (Bioline)

The numbers represent the samples used.

Amplicons of PCR using Internal transcribed spacer primers of the test samples were sequenced and BLAST analysis was done to obtain phylogenetic trees (Figure 1-3). The result of the full

length ITS sequences obtained for the samples were deposited to the GenBank Nucleotide Database NCBI (Nucleotide Center for Biotechnology Information) with the strain numbers. The submission was subsequently assigned unique accession numbers as shown in Table 2. The blast results showed significant sequences similar to the query sequences used to identify the organisms as given in the Table3 with their respective percentage similarities. Blast results have identified the samples used as three different species belonging to the basidiomycetes. The sequences of strains obtained after the sequencing of ITS PCR products were used to generate phylogenetic trees generated by Maximum composite likelihood analysis showing sample 1 as *Pleurotus ostreatus*, sample 2 as *Pleurotus floridanus* and sample 3 as *Lentinus squarrosulus* respectively.

TABLE 2: Taxonomic affinities of samples with blast searches of ITS sequences on GenBank.

SAMPLE ID	TAXONOMIC AFFINITY (Gene Bank No.)	PERCENTAGE SIMILARITY (%)	ACCESSION NUMBER
1	*Pleurotus ostreatus* (MN049516.1)	86.00	MN968774
2	*Pleurotus floridanus* (MN173381.1)	97.88	MN968775
3	*Lentinus squarrosulus* (KT273380.1)	88.44	MN968776

Sample 1

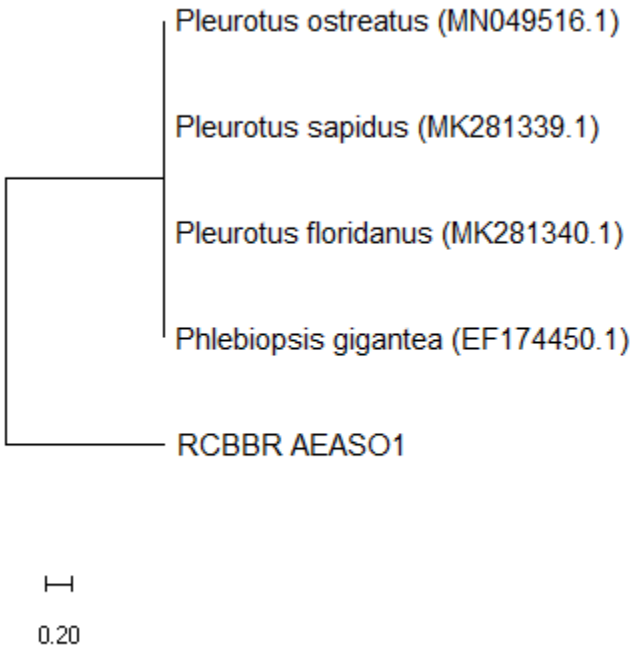

Figure 1: Phylogenetic tree generated by Maximum composite likelihood analysis based on the ITS 1-2 gene sequences.

Sample 2

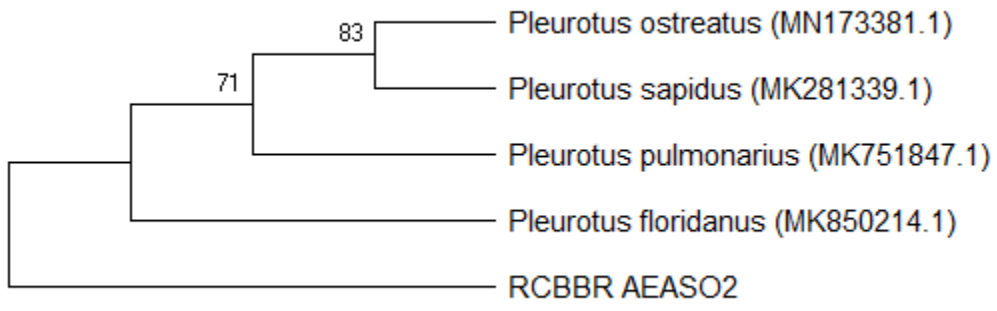

Figure 2: Phylogenetic tree generated by Maximum composite likelihood analysis based on the ITS 1-2 gene sequences.

Sample 3

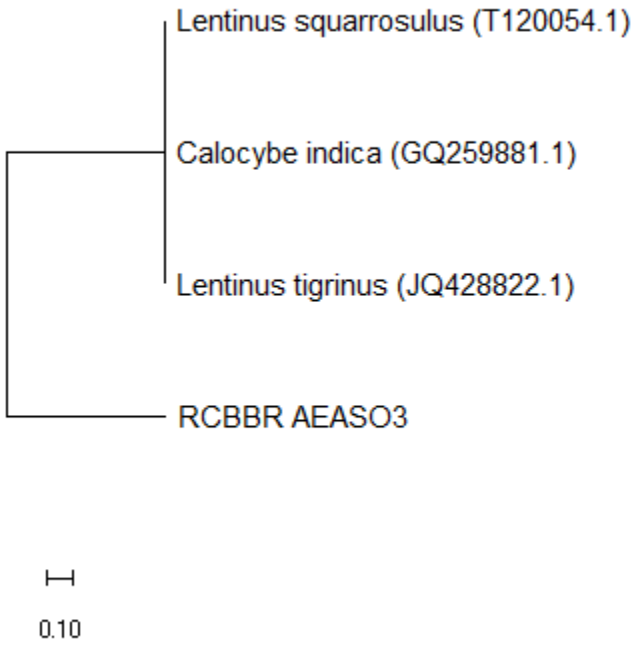

Figure 3: Phylogenetic tree generated by Maximum composite likelihood analysis based on the ITS 1-2 gene sequences.

CONCLUSION

The mushrooms in the Niger Delta region are diverse. Most of them share identical features which could lead to conflicting results making it inappropriate to rely on morphological characterization for identification. The use of molecular characterization gives reliable results to the species level. The results from this study have increased the database of Pleurotus and Lentinus DNA and serve as a basis for the identification of unidentified species by comparing their PCR amplified sequences with ITS primers.

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