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DNA Extraction, Purification and Sequencing of Some Spoilage Fungi of Cocoyam

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ABSTRACT

Polymerase chain reaction (PCR) based assays have been developed to amplify DNA of fungal pathogens as culture-based detection methods show low sensitivity. Fungi are varied in terms of their morphology, ecology, metabolism, and phylogeny. They are known to produce numerous bioactive molecules, which makes them very useful for natural products researchers in their pursuit of discovering new chemical diversity with agricultural, industrial, and pharmaceutical applications. Despite their importance in natural products chemistry, identification of fungi remains a daunting task for chemists, especially those who do not work with a trained mycologist. The purpose of this research is to use DNA to identify some spolage fungi and construction of phylogenetic trees from DNA sequences. Using a standard procedure for Zymo kit for the DNA identification and sequencing. Sequence results of these fungi shows that isolate 1 has 100. % pairwise identity with *Aspergillus niger* and the evalue is 2e-54 isolate 2 has 95.53% pairwise identity with *Pencillium citrinum* strain and the evalue is O. Isolate 3 has 95.65% pairwise identity with Fusarium *solani* isolate and the evalue is 9e-11. Isolate 4 has 100% pairwise identity with *Rhizopus stolonifera* isolate and the e value is 1e-146. Isolate 5 has 100% pairwise identity with *Mucor piriformis* and the e value is 2c-108. . The phylogenetic tree based on the pairwise method with arithmetic average of internal transcribed spacer (ITS) rDNA sequence revealed the variation of the ITS region among fungal strains. We hope that this research provide a set of standardized procedures for the molecular identification of fungi that can be utilized by the natural products research community. We recommend that both morphology and molecular data be used for fungal identification.

Keywords: DNA extraction, Fungi identification, Polymerase chain reaction, Internal transcribed spacer.

INTRODUCTION

Despite having between 2.2 and 3.8 million species, fungi are one of the least well-studied families of multicellular eukaryotes in terms of biodiversity [1]. In reality, only a tiny portion of the predicted species have been formally characterised (about 120,000), and for many of them (around 85,000), no DNA sequences have yet been stored in public DNA databases [2]; [3]. [1], on the other hand, fungal metagenome sequencing is giving researchers access to numerous datasets made up of environmental/uncultured fungus genomes with either no or inadequate taxonomic annotation. In order to bridge the current distance between the various sizes of documented and analysed the variety of fungi, the linking curate DNA sequence data in accurate set of recognised voucher specimens is vital step [4].

Fungal kingdom members have important functions in human existence and are able to fill many different natural and man-made niches. In both fundamental (ecology, taxonomy) and applied (genomics, bioprospecting) applications in scientific study, species-level identification of fungus is essential. This is particularly true for researchers studying natural products that use fungus as a source of bioactive secondary metabolites. [5]. Scientific names are essential for conveying information about fungi because they help researchers to better anticipate, identify other related species groups of chemical genes that have evolved or to prioritise strains that are taxonomically related whenever a productive strain may reduce the amount of important bioactive compounds [6]. More crucially, whether industrial, agrochemical, or medicinal items are to be made from fungi, identification of fungi is important. Fungi are recognised for their potential to create biologically active secondary metabolites that may be utilised for drug development. They produce a variety of natural compounds that are widely employed in industry [7].

To amplify the DNA of fungal infections, Polymerase Chain Reaction (PCR) based assays have been developed since culture-based isolation (detection) approaches are insensitive and unspecific [8]. The current DNA extraction techniques for bacteria or viruses demonstrate inadequate DNA release for fungi. This is because the cell walls of fungus are hard, layered, and complicated, protecting and supporting their cell structures. Chitin [poly-(1-4)-N-acetylglucosamine] is concentrated as discs in the cell walls of yeast cells, whereas filamentous fungi have an inner microcrystalline sleeve of chitin [poly-(1-4)-N-acetylglucosamine] in their cell walls. As a result, the existence of a complex fungal cell wall renders it resistant to lysis by the widely used procedures and hinders the extraction of the fungal DNA [9]. To help in breakdown of the mycelia, several of these techniques suggest mortar and pestle grinding or liquid nitrogen freeze drying. When working with a lot of specimens, this means an increased need for liquid nitrogen as well as a substantial increase in time. Rapid diagnosis and treatment are necessary since invasive fungal infection is a significant source of morbidity and death, especially in immune compromised patients. The turnaround time will be significantly shortened if a trustworthy method for extracting fungal DNA is made available [10]. Prior to the development of Whatman FTA cards, the simplest techniques for the production of fungal DNA were time-consuming, costly, and required many centrifugation processes. The fibre cards used in Whatman FTA filter matrices have been pre-treated with chelators and denaturants, which lyse and inactivate the majority of bacteria upon contact. While cell detritus is quickly eliminated by washing the inoculation card, the big nucleic acids produced following lysis get physically entangled in the FTA matrix. We proved that all 38 yeast and 75 mould species studied could be inactivated by applying aqueous suspensions of yeast cells or hyphal fragments and conidia (in the case of moulds) on dry FTA filters, followed by a quick microwave treatment. Additionally, 218 of the 226 examined fungal isolates (96.5%) liberated amplifiable DNA after application to FTA filters, with a total preparation time of ~15 min [11].

For fungi, several molecular identification strategies have so far been examined. The most uncommon isolates from human illnesses, PCR amplification of genomic DNA followed by sequencing of the amplicons produced has shown the most promise. Target areas for sequence-based methods should ideally develop slowly, exhibit enough conservation among fungi to provide cross-species amplification and be sufficiently variable to permit reliable differentiation between closely related species [12]. sequencing of specific ribosomal RNA nuclear gene areas cassette in investigations utilising a small number of different isolates and more especially the internal transcribed spacer (ITS) sections, were shown to be enough to distinguish between a wide range of species of clinically significant yeasts and suitable for phylogenetic analysis of a number of moulds [13]. In the present study, we use DNA extraction to identify some spolage fungi and construction of phylogenetic trees from DNA sequences. Using a standard procedure for Zymo kit for the DNA identification and sequencing. The efficacy in DNA extraction from various fungal species, was checked by amplification with primers targeting the Interneral Transcribed Spacer (ITS) region of the ribosomal DNA.

MATERIALS AND METHODS

Methodology for isolation and identification of post harvest spoilt cocoyam

In this work, the isolation method from [14] was used. The surfaces were sterilised by dipping completely in a concentration of 40% hypochlorite solution for 60 seconds; the sterilised sections to be inoculated were then removed and rinsed with three changes of sterile distilled water. A small section of infected *Colocasia esculenta* tissues containing the advancing margin of rot and adjoining healthy tissue was cut using a sterilised scalpel and cork borer. In a laminar airflow cabinet, the tuber pieces were dried by blotting with sterile filter paper. Ten pieces of each cut sample were individually infected (90° apart) on solidified Potato Dextrose Agar (PDA) and Sabouard Dextrose Agar (SDA) plates with the help of sterile forceps. The paper tapes were used to secure the inoculation plates, and they were then incubated at 28°–30°C for 72 hours. The inoculation plates were examined for fungi linked to the rotting of the tubers. In order to purify the materials, a sterile inoculating needle was washed in selecting mycelia

threads from the samples and then transferred to freshly prepared PDA and SDA plates. The isolates were subjected to several subcultures for further purification. All of the samples were plated using the pour plate technique [15]; [16]. Using a sterile pipette, 1 ml of dilution (10-1) (the dilution that is neither too murky nor too light) was added to a 9 ml Petri plate. Each dish received a pour of molten potato dextrose agar (10 ml). To make it simple for the sample and the medium to mix together, the plates were turned clockwise. All plates were left on the bench to set up. The plates were all copied. The entire collection of hardened plates was placed in an incubator set at 25 oC for 3-5 days. Mycelia colonies were counted daily based on observation.

DNA EXTRACTION USING ZR FUNGAL/BACTERIAL DNA MINIPREP (Manufactured by Zymo Research)

Fill a ZR BashingTM Lysis Tube with 2 mL of fungal cells broth. To the tube, add 750ul of the Lysis Solution.
2. Place in a bead equipped with a 2 ml tube holder assembly and process for more than 5 minutes at maximum speed.
3. Centrifuge the ZR BashingBeadTM Lysis Tube at > 10,000 x g for 1 minute in a microcentrifuge.
4. Transfer up to 400 ul of supernatant into a collection tube and spin it at 7,000 x g for one minute using a Zymo-Spin IV Spin Filter (orange top).
5. To the filterate in the collection tube from step 4, add 1,200 ul of fungal/bacterial DNA binding buffer.
6. Centrifuge at 10,000 x g for 1 minute after adding 800 ul of the mixture from Step 5 to a Zymo-SpinTM IIC Column in a Collection Tube.
7. Repeat Step 6 after removing the flow through from the Collection Tube.
8. Centrifuge at 10,000 x g for 1 minute after adding 200 ul of DNA Pre-Wash Buffer to the Zymo-Spin TM IIC Column in a fresh Collection Tube.
9. Centrifuge at 10,000 x g for 1 minute after adding 500 ul of fungal/bacterial DNA wash buffer to the Zymo-SpinTM IIC Column.
10. Place the Zymo-SpinTM IIC Column in a clean 1.5 ml microcentrifuge tube, and add 100ul (at least 35ul) of DNA Elution Buffer directly to the matrix of the column. Centrifuge for 30 seconds at 10,000 x g to elute the DNA.

Electrophoresis for DNA and PCR

1. Measure 1 grammes of agarose for DNA and 2 grammes for PCR
2. In a microwave-safe flask, combine 100 mL of 1xTAE and agarose powder.
3. Microwave for 1 to 3 minutes to thoroughly dissolve the agarose; do not overheat the mixture as this may cause part of the buffer to evaporate, which will change the final concentration of agarose in that gel.
4. Allow the agarose solution to cool for five minutes, to a temperature of around 50 °C (about the point at which you may comfortably rest your hand on the flask).
5. Include 10L of the EZ Vision DNA stain. When exposed to ultraviolet (UV) light, EZ vision bonds into DNA and makes it visible.
6. Insert the well comb before pouring the agarose onto a gel tray.
7. To thoroughly solidify freshly poured gel, either place it at 4°C for about 10-15 minutes OR allow it to stay at room temperature for 20–30 minutes.

Loading of Samples and Running of an Agarose Gel

1. Include loading buffer with each of your PCR results or DNA samples.
2. After it has set, put the agarose gel in the gel container (electrophoresis unit).
3. Pour 1xTAE (or TBE) into the gel box till the gel is completely coated.
4. Gently insert molecular weight ladder into the gel's first lane.
5. Carefully place your samples into the extra gel wells.
6. Run the gel at 80 to 150 V for about one and a half hours.
7. Remove the electrodes from the source of power., turn the power OFF, and then gently take the gel out of the gel box.
8. Use a UV transilluminator to see DNA fragments or PCR products in action.

PCR Mix Components

The PCR mix is made up of 12.5µL of Taq 2X Master Mix from New England Biolabs (M0270); 1µL each of 10µM forward and reverse primer; 2µL of DNA template and then made up with 8.5µL Nuclease free water.

Sequencing of the amplified Internal Transcribed Spacer (ITS)

The amplified fragments were sequenced using an Applied Biosystems 3500 Genetic Analyzer (USA). The manufacturer's instructions for the BigDye terminator V3.1 cycle sequencing kit (Applied BiosystemsTM 4337455) were followed. The sequences were aligned using MEGA 6 and modified with Bio-edit Bio- Edit software (version

7.2.5). Using Basic Local Alignment Search Tools (BLAST), the cleaned sequences were aligned with sequences in the database of the NCBI to look for homologous sequences for identification.

Primer Sequences

ITS 1: 5' TCC GTA GGT GAA CCT GCG G 3'

ITS4 5' TCCTCCGCTTATTGACATGS 3'

Cycling Conditions

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 and hold temperature at 10 °C forever.

RESULTS

Molecular characterization

Isolate 1 has 100.00% pairwise identity with Aspergillus niger isolate WCK471 which has NCBI accession number OK091646.Isolate 2 has 95.53% pairwise identity with Penicillium citrinum strain FC24773 which has NCBI accession number MK578179.Isolate 3 has 95.65% pairwise identity with *Fusarium solani* isolate ACSIKS_2101973 which has NCBI accession number MN583468Isolate 4 has 100.00% pairwise identity with *Rhizopus stolonifer* isolate MJU-5 which has NCBI accession number MN413688Isolate 5 has 100.00% pairwise identity with *Mucor piriformis* which has NCBI accession number AJ269842.

DNA GEL IMAGE

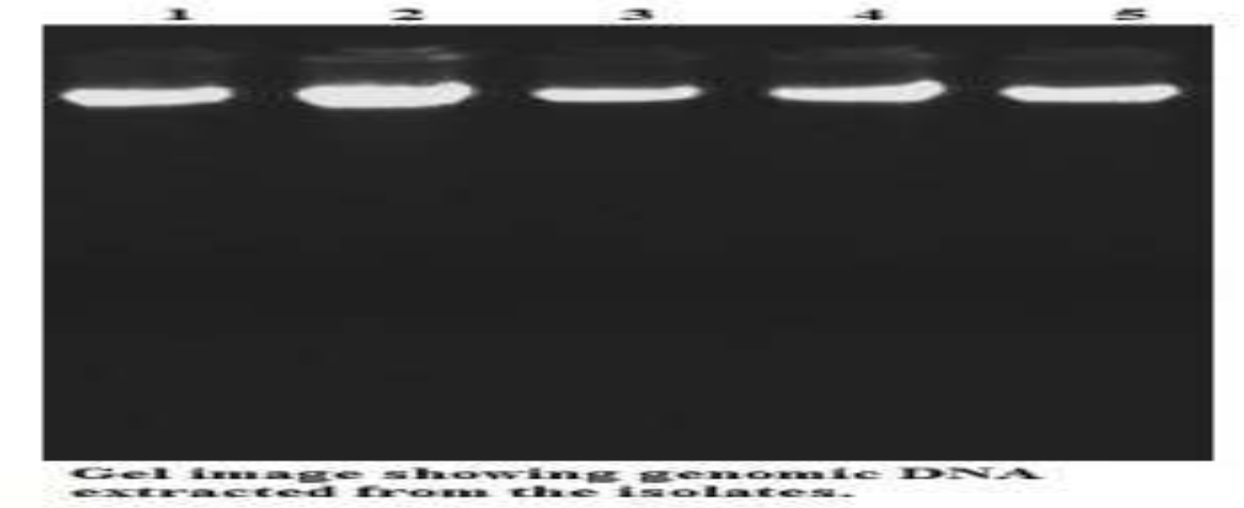
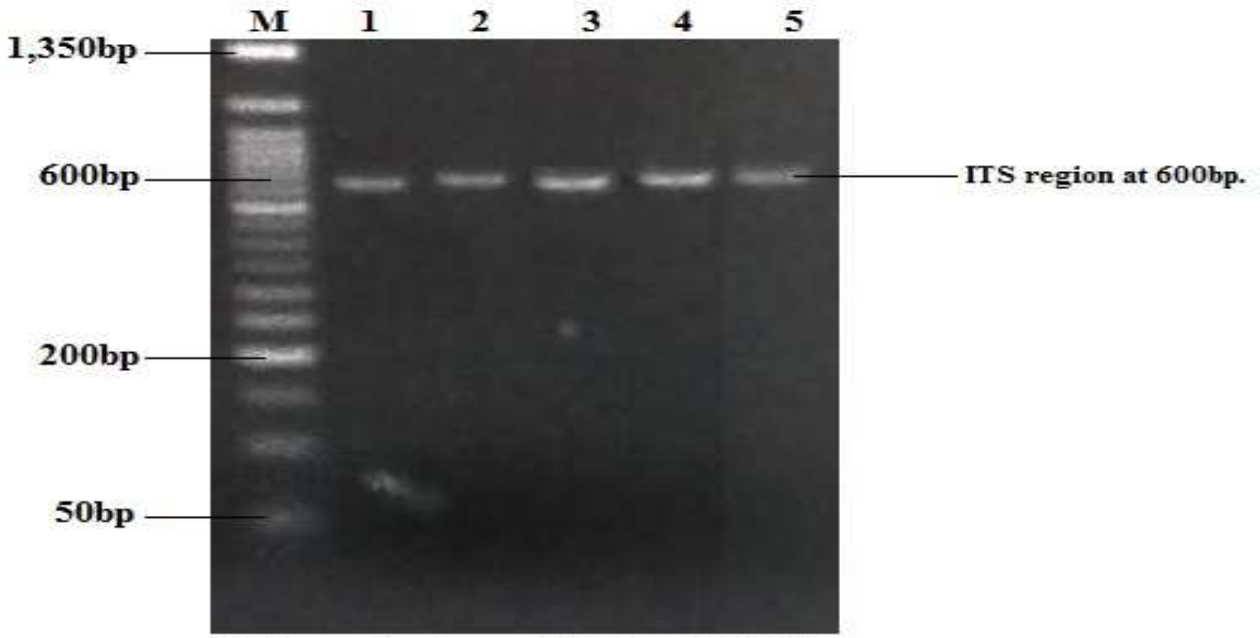


Figure 17: DNA gel image

PCR GEL Image



Gel image showing amplification of the ITS region at 600bp.
M is 50bp Ladder NEB.

Figure 18: PCR gel image

Names of Identified Organisms

Identified Organism	Strain	Pairwise identity (%)	E Value	Accession number
Aspergillus niger	WCK471	100.00	2e-54	OK091646
Penicillium citrinum	FC24773	95.53	0	MK578179.
Fusarium solani	ACSIKS_2101973	95.65	9e-11	MN583468.
Rhizopus stolonifer	MJU-5	100	1e-146	MN413688.
Mucor piriformis	-	100	2e-108	AJ269842.

Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA X were used for all genetic analysis.

Sequence Results

Isolate 1 has 100.00% pairwise identity with *Aspergillus niger* isolate WCK471 which has NCBI accession number OK091646.1. The e value is 2e-54 while your isolate sequences are as shown below:

Isolate 1 sequences

GGGGTGGCGGCAAGGGGAGTTGGGGGGTAGTTGGAAGGTGAGGGATTGCTTCTACGCTGTTGA
AATTTGGCTGAGAGACTCAGACTGGTCATGGGTAGACCTATCTGGGGTTTGATCGACTTTGGNCC
NACCTCCCATCCGTGTCTATTATACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTCGGCCGCCGGGG
GGGCGCCTTTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACACGTGCCACTCCTGGTTTCAGG
AGCACCCTTCATAATAAACCTAGAAATTCAGTATTATAAAGTTTAATAAAAAACAACTTTTAACAAT
GGATCTCTTGGATCTCGCATCGATGAAGAACGTAGCAAAGTGCGATAACTAGTGTGAATTGCATA
TTCAGTGAATCATCGAGTCTTTGAACGCAGCTTGCACTCTATGGTTTTTCTATAGAGTACGCCTGC
TTCAGTATCATCACAAACCCACACATAACATTTGTTTATGTATAATGGGTCGCATCGCTGTTTTAT
TACAGTGAGCACCTAAGAGGTGTGTGATTTTCTGTCTGGCTTGCTAGGCAGGAATATTACGCTGC
GCTCAGGATCTTTTTCTTTGGTCGCCCAGGAAGTAAAGTACAAGAGTATAATCCAGCAACTTTCAA
ACTATGATCTGAAGTCAGCTGGGATTACCCGCTGAACTTAAGCATGTCACAAGCGGAGGAA

Isolate 2 has 95.53% pairwise identity with *Penicillium citrinum* strain FC24773 which has NCBI accession number MK578179.1. The e value is 0 while your isolate sequences are as shown below:

Isolate 2 sequences

TTGGCTTAAGTCGGCAATACCTCTTAGGGTTCCTCTGAGGTGATGGATTATACTCTACGCACTTT
ACTTCCTGGGCGAACCACAGAAAAAGATCCTGAGACCAGCGATATATTCCTGCGAAGCAAGCCACA
CAGAAAATCACACACATTTTAGGTGCTCACTGTAATAAAACAGCGATGTAACCCATTACCACATAAA
CAAATGTTATGTGTGGCTTTGTGATGATACTGAAGCAGGCGTACTCTATAGAAAAACCATAGAGT
GCAAGCTGCGTTCAAAGACTCGATGATTCACTGAATATGCAATTCACACTAGTTATCGCACTTTGC
TACGTTCTTCATCGATGCGAGAACCAAGAGATCCATTGTTAAAAGTTGTTTTTTATTAAACTTTAT
AATACTGAATTTCTAGGTTTATTATGAAGGGTGCTCCGGAAACCAGGAGTGGCATCGATCAAACC
CCAGATAGGTCTACCCATGACCAGTCTGAGTCTCTCAGCCAAATTTTCACAGTGTAGAGGCAATCA
CTTACCCCCGAGGAAACCCTAAGAGGTAAGGCGCTTTAACATAATTAATGAACCTTCCGCAGGTCA
CCCTTACGGGAAAATTTTTTATTTATGTTAAAGCGCCTTACCTCTTAGGGTTTCCTCTGGGGGAA
GTGATTGCTTCTACACTGTGAAAATTTGGCTGAAAACTCAGACGGGCCGGGGTGGCCTATTTGGG
GTTTGACCAAGCCCCTCCGGGTTCGGGACCCCCTTCAAAAAAACCTGGGATTCGGTTTAAAAAGTT
TAAAAAAAAAAATTTTAAAAAGGGCTTTTGGTTCCGCTCGAGAAAAAGGGAAAAGGGGGACCGGG
GGGGGGGAATTCGGGAATTCCGGCTTTTACCGCGTGGCTCCCGGGTTTTTTTTGAGGACCCCGTT
TGTTTCCCCCAACCCCACAAAAAATTTTTTTTTTGGAAGGGGCCCCGCGTTTTTTTTACTATAGAG
CCCAAAAAGGGGGTGTTTTTCTGCTCTGGGGTGGGGGAGTATATGCCGTCCTCGACCTTTTTTTT
TTGTTCCCCCGCGCGAGGAGCGAGAATAGATACGACACGTCTCTACATGATATATGAGCGAGAGG
GTGTCTGCTAGTTTAGCCTTGGAGTGGGGTTGAGAATAAGAGATC

Isolate 3 has 95.65% pairwise identity with *Fusarium solani* isolate ACSIKS_2101973 which has NCBI accession number MN583468.1. The e value is 9e-11 while your isolate sequences are as shown below:

Isolate 3 sequences

CAGGTTTGGGGCTCAAAGCTCGCCGACCTCTGCCCGTGTGTACTGTTTTGTTACGGGCTTCGCCG
GCCCGGTTACAGAGCGGGTGACAAAGCCCCATACGCTCGAGGATCGGACGTGGTGCGCCAGATACA
TTTCACGCACGTTTTACCCGAAGAGTGTCATCGATCAACCACGCAACCCGTGACCACGGGGCAAAT
GAATGTGTGCATTGAGATGAACTGAAGCAGGGTACTCTCGGAAGACTATAGAGTGTAGCTGAGTT
AAGTTCGATGCTCAAGTTATGCATTGATGTAGAAGCGGCCTGGGCGACTTATCATGTAGACGAGT
ACGTTGTGTAGGCTCTTAAAAGCTTAAAGATGATTTAGTTATCTGGGGGGCTGGAGTGGGCGAT
GTGACCCTAGGCTGCCTGCAACTTTCCTTCCCGCTTTCGAGGAAACATATTACCAATAAACTAAGA
GGGTTTAAAATTCGACTTCCGCCCCTAGGAATCTGTGTTTTGGCCTTGCTTTGGTCCTCTTGAAGC
AGGGTTCTCCCGTGAATTTGCTGAAAACTAAACTGTTATGGTGGACGATGTGGGTTTATCTTGCC
CTCTGGTTCAGTTCACCTGGAAATGACTTGTAATTAGATTAAAAGATAGAAAAAAAGTTTAAGCAT
TGTTTTGTGTTCCGTAAAGGCAGGGGAAAAGAGGGCCGGATGTGTTGGCGGGGGCCTGGGGCTT
GGCCGGCGGCCCGGGGTTTTTGAGGGGGGCCCCCCTGCCCGCCAAACCCCCCCTAATTTGTTTTCG
G

Isolate 4 has 100.00% pairwise identity with *Rhizopus stolonifer* isolate MJU-5 which has NCBI accession number MN413688.1. The e value is 1e-146 while your isolate sequences are as shown below:

Isolate 4 sequences

GGGGTTCATACCGATCCGAGCTAACCTGCGTGGATTGGTACCATGTTGGTTCGGCGGGCCGCTGT
AGTGACTGAGGAGGACATTACCGAGTGCGGGTCCTTTGGGCCCAACCTCCCATCCGTGTCTATTAT
ACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTCGGCCGCCGGGGGGGCGCCTTTGCCCCCCGGGCCC
GTGCCCGCCGGAGACCCCAACACGAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAA
TCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGA
TAACTAATGTGGCCTCCTGCGAGCATGTGAGGAGCCCTCAGGCTCGAGGATCGGACGCGGTGCAA
ACCTGACTTTCGGACTGTCCAAACGGTGAGGCCTGGGTGATGAATGCAAATCGGTAAATTTTTTA
AAAAATGAATCTTTGGTTTCGGTTTCCAATGAAAACCCAGGGAAGTGAATAACCTACGGGAAATT
GAGAATTTACGTGAACCTCGATTTCTTTAAGCTCAATTCTTCCGCTGGTATTCCGGGGGATGCTG
CTCAGAAGTCAATTGTGGCCCTATGCCCGCTTGTGTGTGGCAGCGTCCCCCTCTCCGGCGGACGTC
TCAAAGTGAGTGTCGACCGGTCGATCTGCGAGCGAAGAGGCTTCGCAACTCCTCATAGATGGCGG
CCATGCACGTTTCAACATCGTCAGATGACTCCAAAGACGCACCGCTGACTTAGAGTCAAGCGGAAG

Isolate 5 has 100.00% pairwise identity with *Mucor piriformis* which has NCBI accession number AJ269842.1. The e value is 2e-108 while your isolate sequences are as shown below:

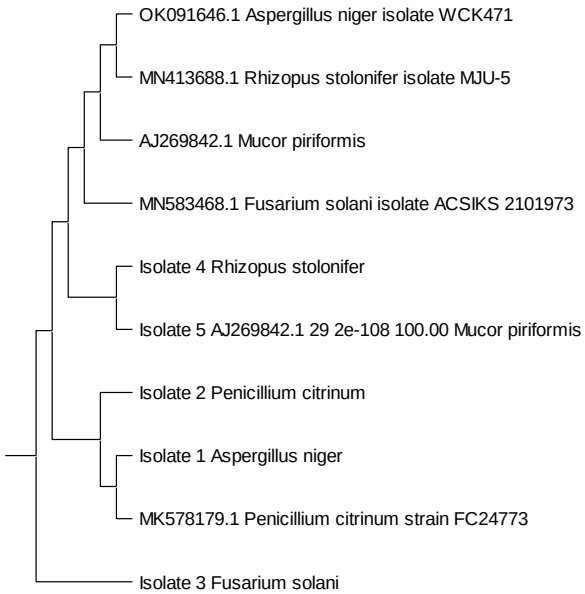
Isolate 5 sequences

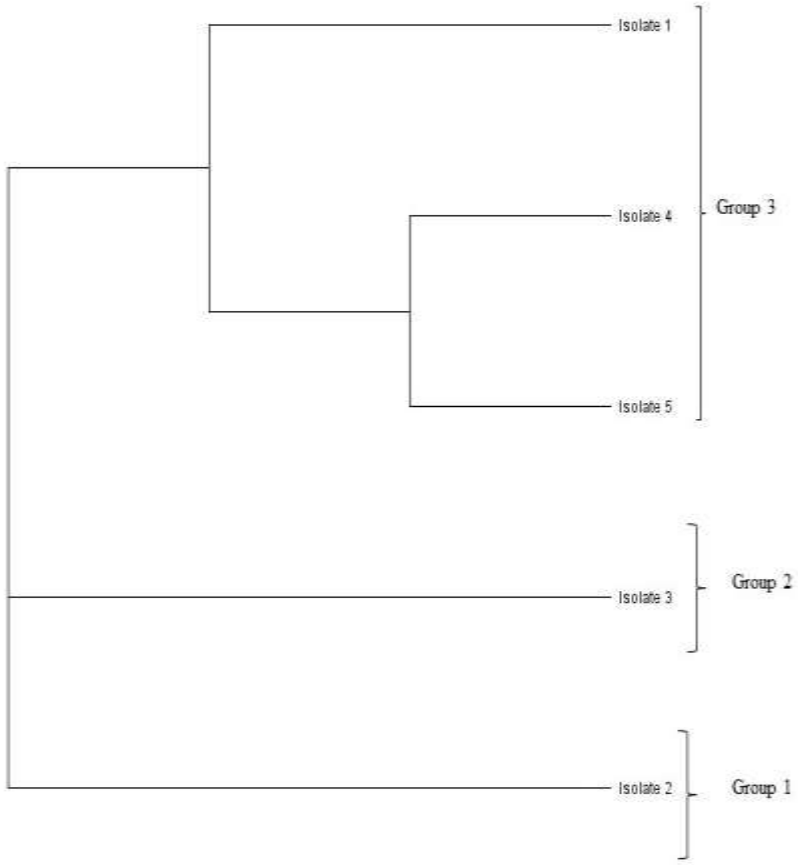
ACGTGGGATCCCATAACGGATCAGAGGTAACCTGCGAAAGCATGTGTGCAATACGTGCGTTCGGC
GGGCCCGCCTCCTACGATTAGCGAGTGGGGGTCCTTTGGGCCCAACCTCCCAGCAGTGTCTATTGT
ACCCTGTTGCTACGGCGGGCCCGCCACTTGTCCGACGCCGGGGGGGCGCCTCTGCCCCCCGGTCCC
GTGCCCGTCGGAGACGCCAAGAGGAACACTGTCTAAAAGCGTGCAGTCTGAGTTGGATTAGAATG
CAATCAGTTAAAACTTTCAACAATGGATCAGCGTGTGACAGAGCTCTCTGCCTCGGCCCGGACGCG
GTGAAACCCTAAATTTAGACTGGTTGAAACGTGAGGCGGGTTGATTAATCAAATGGGAAAATTTT
TAAAAATGGACCTTTGGATCCGGCTCCCATGAAAAACCAGGGAACTGAAAAACTAAGGGATTGAAG
ATTACGGAATCTCAATTCTTGACGAACTGGGTCCCCGGATATCCGGGGGATGCTTGTCGAGTTCA
TTGCTGCCTTAGGACCGATTGGTGTTGGCACTCCAGCCCCCTCTCTGCGGAAGGGCTCGAATGGA
GGCGCGAGACGGCCGGACCTGGGAGAGAGAGGCTTCTCAATCCTATAGGTTGGCCGGCCTCAAAT
TTCAACATCGTCAGATGAGCTCGTTAGTCGCATCGCTGACTATCAGTATAGTGGGAAT

Evolutionary relationships of taxa

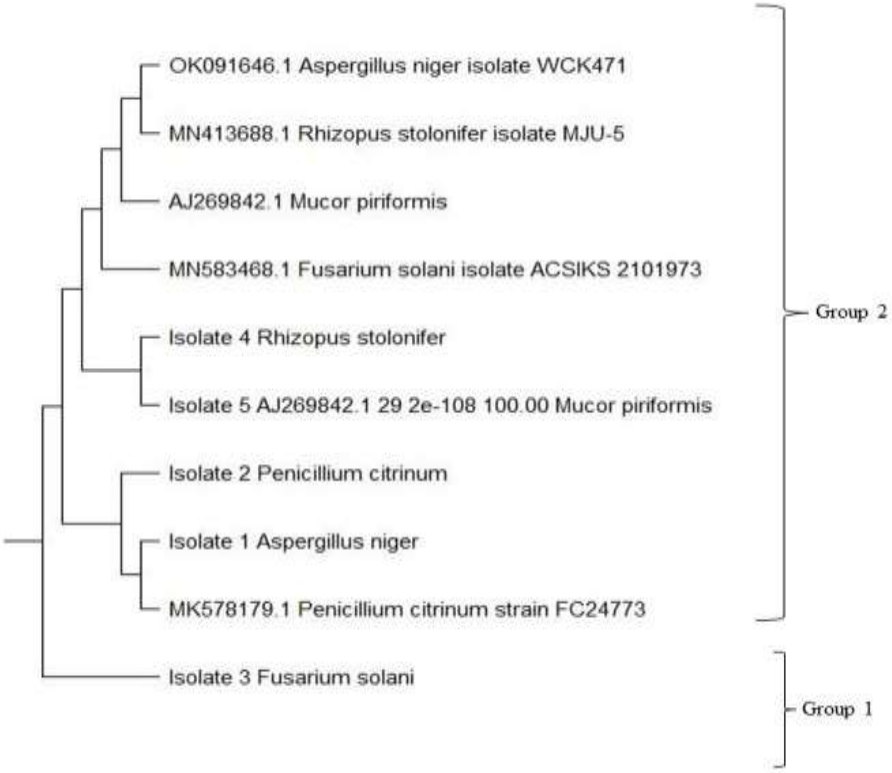
The evolutionary history was inferred using the UPGMA method [17]. The optimal tree is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method [18] and are in the units of the number of base substitutions per site. This analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1365 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [18].

PHYLOGENETIC TREE





Phylogenetic relationship between the 5 isolates shows that there are three distinct groups. Isolate 2 and 3 belongs to group 2 and 1 respectively and are closely related sequence wise while isolate 1, 4 and 5 belongs to group 3. Group three is further sub grouped into 2 other groups where isolate 4 and 5 are more closely related than to isolate 1.



The 5 isolate sequences were aligned to homologous sequences on the NCBI data base, and the phylogenetic tree grouped the organisms into two main groups. Isolate 3 identified as *Fusariumsolani* showed clear distance with other isolates and identified organisms.

DISCUSSION

Obtaining DNA from fungi is more difficult than from bacteria or from mammalian cells as the fungal cell wall is tough to break using the conventional extraction methods employed for bacteria or viruses. This is attributed to fungi possessing a thick and complex cell wall resulting in poor release of DNA. Poor efficiency of DNA extraction, low numbers of fungal cells in clinical material may be the reasons for obtaining false negative results from clinical specimen [19], [20]. Griffiths *et al.* 2016 have reported extraction with a Qiagen kit, Norgen kits, Zymo kits respectively utilizing bead beating technique along with a lysis buffer where they found the yield of DNA better than other methods. Whit et al. 2019 evaluated the use of glass beads with vortexing for extended periods and found it better for extraction of DNA from filamentous fungi. Most fungal DNA extraction methods disrupt the fungal cell wall. Using buffers like the Zymo Fungal/Bacterial DNA Binding Buffer and forward and reverse primer. The sonication technique described here can be used for fast fungal DNA extraction and PCR screening. The fungi samples for extraction of fungal genomic DNA using commercial kits that have expiration date of 6–12 months once opened was used. Fungal isolates in this study include: *Aspergillus niger*, *Penicillium citrinum*, *Fusarium solani*, *Rhizopus stolonifer* and *Mucor piriformis*

From the result of DNA extraction, purification and sequencing of fungal isolated from spoilt cocoyam, the molecular characterization done in this study reported that isolate 1 has 100.00% pair wise identity with *Aspergillus niger* isolate WCK 471 which has NCBI accession number OKO91646. This result confirms the *Aspergillus niger* in postharvest cocoyam which causes the spoilage. This finding has a closely resemblance with that of [21] and also with [22] who did a study of simple chemical extradition method for DNA isolation from *Aspergillius fumigates* and other *Asperigellus* species. Previous reports of using sonication for fungal DNA extraction (from the Ascomycete *Aspergillus fumigatus*) were performed using CTAB (cetyltrimethylammonium bromide) buffer with and without glass beads at 55°C [23]. The percentage yield for this study was much lower (0.028%) than the average yield percentage we obtained using the sonication method described here (100%). The same study compared different

methods of DNA extraction using glass beads and vortexing which could extract DNA in 2-3 hrs which yielded a similar pairwise result as this study. Commercial kits take around an hour for DNA extraction [23].
Isolate 2 also has 95.53% pairwise identity with *Penicillium citrinum* strain in FC24773 which has NCBI access ion number MK578179. This correlate with the findings done in the laboratory using cultural and isolation method. It also confirms the presence of *Penicilium ctrinum* strain in postharvest spoilt cocoyam corms. Isolate 2 also has 95.53% pairwise identity with *Pencicillium citrinum* strain based on PCR Genentic Analyzer and sequencing method which was found in isolated spoilt ccoyam. [24], used the sequencing approach to detect *Pencillum citrinum* in a soil plant isolate that can harm the majority of soil crops, including cocoyam and others. Additionally, this outcome is consistent with a work by [25] that used PCR-based finger printing to identify *Penicillum citrinum* in an isolate from rye bread. The results from the six combinations of DNA extraction and purification methods on the three species of *Penicillium* (*Penicillium expansum*, *Penicillium chrysogenum*, *Penicillium citrium*) obtained from potato nearly all yielded acceptable A260/A280 ratios and similarities of 95% and 100% pairwise which supports the findings of this work the exception being with DNA extracted and purified with phenol- chloroform, yielded a very low similarities and ratio. The very low ratio of this sample is probably caused by the presence of visible insoluble material, likely a carbohydrate polymer. In general, the combination of bead beating and DNeasy PowerSoil Kit yielded the highest amounts of extracted DNA compared to the phenol-chloroform method, but increased fragmentation of the DNA was observed [26], [20]. Sequencing of the fungi isolated from maize with universal primers ITS-1 and ITS-4 confirmed the morphological results (GenBank accession number KJ467353.1; amplicon size: 559 bp), showing 99% identity with the sequences of *P. citirum* (accession numbers GU566212.1 and GU566224.1) [27]. In the study of Nilsson *et al*., 2019, the genomes of two spoilage fungi strains were successively sequenced using the Pacific Biosciences RS II and Illumina MiSeq platforms with accession numbers CBS134186 and ATCC 48735, they demonstrated the high integrity of the genome assembly. The size of the genome is similar to reported *Penicillium* genome. The combined application of these second and third generation sequencing technologies shows good potential for the development of genome sequence assemblies without reference to genomic data, thus resulting in fewer unresolved gaps or ambiguities and a smaller number.
Isolate 3 has 95.65% pairwise identity with *Fusarium solani* isolate ACSIKS 2101973 which has NCBI accession number MN58348. This identity is in line with the result found in the laboratory by cultural and isolation method. This also shows a close identity with that work of [28]. This result is in line with the works of Chen *et al*., 2015 using multiplex PCR have been used to detect fungal pathogens of wheat *Puccinia graminis*, *Puccinia triticina*, *Blumeria graminis*, *Fusarium graminearum* and *Fusarium solani*, which causes wheat stem, leaf rust and wheat powdery mildew respectively. [29] Confirmed the identity of Fusarium spp in taro specimens. The phylogenetic study presented in Pyronemataceae tree, where the sequences grouped with the *Aleuria aurantia* sequence in well supported pattern, also supported the conclusion pairwise of 93%. Being a soil borne fungi Fisher *et al*., 2015 in his work extracted DNA from 47 field soil samples from 20 counties in Illinois was subjected to the PCR using universal fungal primers NMS1 and NMS2. *F. solani* was detected (100% identical pair wise) in soil samples from all Illinois Agricultural Statistic Districts in both. [30] in his work on sweetpotatoes had a 97% pair wise smiliarities from the DNA extracted from *Fusaruim solani*. Osmundson *et al*., 2016 chose to use the DNA extracted using Genomic Buffer Set for sequencing of Fusarium spp and phenol- chloroform extraction for *Fusarium sp*. All purifications were conducted using the Genomic- Tips 20 G−1. Before sequencing, The DNA smiliarity for *Fusarium solani* was detected to be 96%.
Isolate 4 has 100.00% pairwise identity with *Rhizopus stolonifer* isolate MJU-5 which has NCBI accession number MN413688 isolate which also confirms the result gotten from the isolate in the laboratory using culture and isolation method. On the contrary, this study disagreed with the Unizik Awka study (research repository). The results of the analysis point to several species of Rhizopus as the root cause of spoiled tuber harvests. This can be because of the kind of soil crop that was employed, the sequencing technique, or the surroundings where the spoil crop was discovered. This result is consistent with [32] study, which used PCR-RFLP to detect *R. stolonifer* in an isolation from biological environment. The presence of *R. stolonifer* in the environment may have an impact on crops that were grown there. *Rhizopus* species have been described as an important agent of postharvest fungal infections [33] causing decay in highwater content. [34] analyzed the ITS sequences of 6 different isolates from cocoyam and *Rhizopus stolonifer* was among the *Rhizopus* fungi identifed with pairwise similarities of 96% observed, allowing a good identification, Overall, ITS sequences were very different between species. This shows the interest of ITS sequencing for identification of Zygomycetes to the species level and will be useful for epidemiological studies. The two most common species of *Rhizopus*, *R. oryzae* and *R. microsporus*, could be clearly differentiated from each other, as their sequences showed only 70% similarity. Out of thirty-nine isolates [35] obtained from sweet potatoes, cassava and cocoyam *Rhizopus stolonifer* was among epidemiologically related isolates from the three plant samples

with a relative genetic sequence similarity of 95%, 96% and 98% respectively, but in general, different groupings were obtained, in other words his works are in line with the results obtained from this study. Isolation 5 has 100% pairwise identity with *Mucor piriformis* which has NCBI accession number AJ269842. This result also agrees with the finding done with pure culture and isolation method in laboratory. The present study shows the identity of *Mucor piriformis* from isolation gotten from the spoilt cocoyam using a Genetic Analyzer. This correspond with the study done by [36] as most *Mucor* species have been isolated from compositing plant material as this confirms the presence of *Mucor piriformis* in an isolation from spoilt cocoyam as the environment may be brought about the contaminations of cocoyam. Using two different primers, [37] recorded considerable numbers of DNA patterns of *Mucor piriformis* each collected from infected fruit indicating a much higher degree of variability. Their results demonstrated that RAPD analysis can identify isolates and subspecific populations of *M. piriformis*. A similar observation has been made with *Mucor piriformis* isolates from infected peaches and nectarines; in this case, reproducible RAPD markers were obtained which correlated with the mating types (plus or minus) of the isolates [38]. Identification made using the ITS sequencing was further confirmed by the morphological characteristics of the five *Mucor* spp. that were examined under the same culture growth conditions as those described by [39]. DNA sequencing provides a fast approach for identification of fungi. However, because the ITS region is variable between members in the order Mucorales, ITS sequences of some species may differ to such an extent that they could not be aligned confidently with their putative sibling species [40]. [41], found that *M. piriformis* was the primary species responsible for Mucor rot in mandarin fruit. This finding is similar to Mucor rot in pome and stone fruit in California and the U.S. Pacific Northwest. In apple and pear, *M. piriformis* is the primary cause of Mucor rot, though *M. mucedo*, *M. racemosus*, and *M. strictus* have also been reported to cause decay [42]. In stone fruit in California, *Mucor* spp. causing *Mucor* rot were recovered from orchard soils and identified as *M. piriformis*, *M. racemosus*, *M. circinelloides*, *M. hiemalis*, *M. plumbeus*, and *M. genevensis* and other *Mucor* spp. are soilborne pathogens.Sequence results of these fungi shows that isolate 1 has 100. % pairwise identity with *Aspergillus niger* and the evalue is 2e-54 isolate 2 has 95.53% pairwise identity with *Pencillium citrinum* strain and the evalue is O. Isolate 3 has 95.65% pairwise identity with Fusarium *solani* isolate and the evalue is 9e-11. Isolate 4 has 100. % pairwise identity with *Rhizopus stolonifera* isolate and the e value is 1e-146. Isolate 5 has 100% pairwise identity with *Mucor piriformis* and the e value is 2c-108.

CONCLUSION

The findings of this study highlight two important points. First, successful fungal PCR experiments may be predicted well based on using the DNA from fungus. Despite the fact that there are other commercial PCR kits available, the Zymo kit is still among the best. Second, the phylogenetic tree with five isolate sequences was sorted into two major groups after being matched to homologous sequences in the database of the NCBI. When compared to other isolates and known organisms, Isolate 3 identified as Fusarium solani showed a definite difference. PCR is a potential tool for fungal diagnosis and has certain benefits over culture-based techniques, such as quick analysis and the capacity to analyse specimens far from the site of collection. PCR tests have a number of properties that might help diagnose fungal infections more effectively than they currently can. When targeting genes that are present in several copies per fungal genome, these tests can have detection limits of a few gene copies per response, giving them the potential to detect a portion of an organism. Using a consensus sequence PCR technique, the target can be extended to encompass the majority of fungus or narrowed to a particular phylogenetic/taxonomic level, such as species or genus. Additionally, the Zymo kits product's sequence variation can provide precise species-level identification. Our innovative technique enhances clinical applicability by delivering a quick species-level identification the same dayshift (8hr), while rapid panfungal FBR-PCR/S testing has equal diagnostic efficiency compared to established procedures with increased specificity.

REFERENCES

1. Hawksworth, D. L., and Lücking, R. (2017). "Fungal diversity revisited: 2.2 to 3.8 million species," in The Fungal Kingdom. *Molecular Ecology*. **11**: 79–95.
2. Prosser, S. W. J., Dewaard, J. R., Miller, S. E., and Hebert, P. D. N. (2016). DNA barcodes from century-old type specimens using next generation sequencing. *Molecular Ecology and Resource*. **16**: 487–497.
3. Martínez-García, L. B., De Deyn, G. B., Pugnaire, F. I., Kothamasi, D., and van der Heijden, M. G. A. (2017). Symbiotic soil fungi enhance ecosystem resilience to climate change. *Global Change Biology*. **23**:5228–5236.
4. Okigbo, R.N and Anyagebu, C.F. (2021). Underutilized plants of Africa. *Journalof Biology and Nature*. 13(**2**):34-49.
5. Harrington, F. A., and Potter, D. (2017). Phylogenetic relationships within Sarcoscypha based upon nucleotide sequences of the internal transcribed spacer of nuclear ribosomal DNA. *Mycologia*. **89**:258–267.

6. Koetschan,C.,Förster,F., Keller,A.,Schleicher, T.,Ruderisch,B.,Schwarz, R.,etal. (2015). The ITS2 Database III-sequences and structures for phylogeny. *Nucleic Acids Research.* **38**:275–279.
7. Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology Evolution.* **33**, 1870–1874.
8. Skrede, I., Carlsen, T., and Schumacher, T. (2017). A synopsis of the saddle fungi (Helvella: Ascomycota) in Europe – species delimitation, taxonomy and typification. *Persoonia.* **39**:201–253.
9. Lindahl, B. D., Nilsson, R. H., Tedersoo, L., Abarenkov, K., Carlsen, T., Kjøller, R.,. (2016). Fungal community analysis by high-throughput sequencing of amplified markers - a user's guide. *New Phytopathology.* **199**: 288–299.
10. RFlouri, T., Nichols, B., Quince, C., and Mah,é, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *Nature.* **22**: 584–592.
11. Fallahi, S.; Babaei, M.; Rostami, A.; Mirahmadi, H.; Arab-Mazar, Z. and Sepahvand, A. (2020). Diagnosis of *Candida albicans*: Conventional diagnostic methods compared to the loop-mediated isothermal amplification (LAMP) assay. *Archieves of Microbiology.* **202**: 275–282.
12. Okigbo, R.N, Ezebo, R.O and Ugwu, S.C. (2018). Antifungal attributes of extracts of *Ocimum gratisimum*, *Zingiber officinale* and *Cymbopogon citrates* on rot fungi of soursop fruit. *Clinical Journal of Nutrition and Dietetics.* 1(**1**): 1-7.
13. Kullberg, B.J.; Arendrup, M.C. (2015). Invasive Candidiasis. *England Journal of Medicine.* **373**: 1445–1456.
14. Onuh, J.O., Shiriki, D., Ubwa, S.T. and Shambe, T. (2015). Isolation of Six Microorganisms from Rotten *Dioscorea alata* (Water Yam), and Antimicrobial Sensitivity Test with Nine Plant Extracts. *Food and Nutrition Sciences*, **6**: 1381-1394.
15. Dhawale, S. and LaMaster,A. (2003).*Microbiology laboratorymanual.* The mcHillcompany.inc.USA (5):209-229.
16. Chinwendu, S., Ejike, E.N., Ejike, Bu., Oti, W. & Nwachukwu, I. (2016) phytochemical properties of uziza *(piper guineese)*.
17. Nilsson, R. H., Kristiansson, E., Ryberg, M., Hallenberg, N., and Larsson, K. H. (2018). Intraspecific ITS variability in the kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. *Evolution and Bioformatics.* **8**:193–201.
18. Tedersoo, L., Anslan, S., Bahram, M., Põlme, S., Riit, T., Liiv, I. (2015). Shotgun metagenomes and multiple primer pair-barcode combinations of amplicons revealbiasesinmetabarcoding analysesoffungi. *MycologyKeys:* **10**,1–43.
19. Muñoz-Cadavid C., Rudd S., Zaki S.R. (2018). Improving molecular detection of fungal DNA in formalin-fixed paraffin-embedded tissues: comparison of five tissue DNA extraction methods using panfungal PCR. *Journal of Clinical Microbiology.* **48**:2147–2153.
20. Okigbo, R. N., Anyaegbu, C. F and Dalafi, T. P. (2020). Pollution from Spent Engine Oil with their Bioremediation Potentials In Auto-Mobile Workshops in Awka, Anambra State, South Eastern Nigeria. *Nigerian Journal of Biomedical Engineering.* **13**:39 - 44
21. Schoch, C.L, Seifert, K.A, Huhndorf, S.N, Robert, V.V, Spouge, J.L, Levesque, C.A and Chen W. (2015). Fungal Barcoding Consortium. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences of the United States of America.* **109**: 6241–6246.
22. Mishra, I.G, Tripathi, N and Tiwari S. (2014). A simple and rapid DNA extraction protocol for filamentous fungi efficient for molecular studies. *Indian Journal of Biotechnology.* **13**: 536– 539.
23. Paola Piloa, Anna M.M. Tileya,b, Colleen Lawlessa,c, Sujit J. Karkia, James Burkea, Angela Feechana, (2022). A rapid fungal DNA extraction method suitable for PCR screening fungal mutants, infected plant tissue and spore trap samples. *Physiological and Molecular Plant Pathology.* **117**:101-108
24. Erkens, R. H. J., Cross, H., Maas, J. W., Hoenselaar, K., and Chatrou, L. W. (2018). Assessment of age and greenness of herbarium specimens as predictors for successful extraction and amplification of DNA. *Blumea* **53**:407–428.
25. Nawrot U, Wlodarczyk K, Wrobel M, Wasik A and Dobosz T. (2018). Comparison of the utility of five commercial kits for extraction of DNA from *Aspergillus fumigatus* spores. *Acta ABP Biochimica Polonica.* **57**: 567–571.
26. Martin, K. J., and Rygiewicz, P. T. (2015). Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microbiology.* **5**:28-33.

27. Nilsson, R. H., Ryberg, M., Abarenkov, K., Sjökvist, E., and Kristiansson, E. (2019). The ITS region as a target for characterization of fungal communities using emerging sequencing technologies. *FEMS Microbiology Letter*. **296**:97–101.
28. Wang, K.; Luo, Y.; Zhang, W.; Xie, S.; Yan, P.; Liu, Y.; Li, Y.; Ma, X.; Xiao, K. and Fu, H. (2020). Diagnostic value of Candida mannan antigen and anti-mannan IgG and IgM antibodies for Candida infection. *Mycoses*. **63**: 181–188.
29. Prabha, T.R, Revathi, K, Vinod, M.S, Shanthakumar, S.P and Bernard P. (2015). A simple method for total genomic DNA extraction from taro and water moulds. *Current Science*. **104**: 345–347.
30. Fisher, M. C., Henk, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L., (2015). Emerging fungal threats to animal, plant and ecosystem health. *Nature*. **484**:186–194.
31. Osmundson, X, T. W., Robert, V. A., Schoch, C. L., Baker, L. J., Smith, A., Robich, G., (2016). Filling gaps in biodiversity knowledge for macrofungi: contributions and assessment of an herbarium collection DNA barcode sequencing project. *Global Change Biology*. **8**: 5228–5268.
32. Panda, B.B.; Meher, A.S. and Hazra, R.K. (2019). Comparison between different methods of DNA isolation from dried blood spots for determination of malaria to determine specificity and cost effectiveness. *Journal of Parasitic Diseases*. **43**: 337–342.
33. Anyaegbu, C. F, Oledibe, O.J and Amadi, J.E. (2019). Proximate and phytochemical analysis of healthy and infected maize grains in Anambra State. *European Journal of Biology*. 4(**1**):15-40.
34. Edgar, R. C. (2016). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*. **32**, 1792–1797.
35. Zheng, J.; Zhou, Y.; Li, Y.; Xu, D.P.; Li, S. and Li, H.B. (2019). Spices for prevention and treatment of cancers. *Nutrients*. **8**:49-51.
36. Voyron, S., Ercole, E., Ghignone, S., Perotto, S., and Girlanda, M. (2016). Fine-scale spatial distribution of orchid mycorrhizal fungi in the soil of host-rich grasslands. *New Phytopathology*. **213**: 1428–1439.
37. Bani, A., Pioli, S., Ventura, M., Panzacchi, P., Borruso, L., Tognetti, R. (2018). the part the microbial population plays in the decay of deadwood and leaf litter. *Applied Soil Ecology*. **126**, 75–84.
38. Abarenkov, K., Nilsson, R. H., Larsson, K. H., Alexander, I. J., Eberhardt, U., Erland, S. (2016). The UNITE database for molecular identification of fungi recent updates and future perspectives. *New Phytopathology*. **186**: 281–285.
39. Blaalid, R., Kumar, S., Nilsson, R. H., Abarenkov, K., Kirk, P. M., and Kauserud, H. (2015). ITS1 versus ITS2 as DNA metabarcodes for fungi. *Molecular Ecolology and Resources*. **13**, 218–224.
40. Kigawa, R., Nochide, H., Kimura, H., and Miura, S. (2003). Effects of various fumigants, thermal methods and carbon dioxide treatment on DNA extraction and amplification: a case study on freeze-dried mushroom and freeze-dried muscle specimens. *Collection Forum* **18**: 74–85.
41. Bazzicalupo, A.L., Bálint,M., and Schmitt, I. (2015).Comparison of ITS1 and ITS2 rDNA in 454 sequencing of hyperdiverse fungal communities. *Fungal Ecology*. **6**:102–109.
42. Bengtsson-Palme, J., Ryberg, M., Hartmann, M., Branco, S., Wang, Z., Godhe, A. (2015). The examination of environmental sequencing data, methods for improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungus and other eukaryotes. *Ecology Evolution*. **25**:914–919.

Ejimofor, C.F and Okigbo, R. N. (2023). DNA Extraction, Purification and Sequencing of Some Spoilage Fungi of Cocoyam. NEWPORT INTERNATIONAL JOURNAL OF SCIENTIFIC AND EXPERIMENTAL SCIENCES (NIJSES) 3 (1): 57-70.