Characterization and phylogeny of fungi isolated from industrial wastewater using multiple genes

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The aim of this study was the isolation and molecular characterization of fungi from untreated refinery effluent by using multiple conserved genes. The Fungi isolated were characterized based on PCR amplification and genomic sequencing of the internal transcribed spacer region (ITS), partial β-tubulin (BenA), calmodulin (CaM), and RNA polymerase second subunit (RPB2) genes, along with morphological characterization. The obtained sequences were subjected to BLAST analysis and the corresponding fungal isolates were assigned species names after comparison with representative sequences available in GenBank. Fifteen (15) Fungi species belonging to four genera of Aspergillus, Penicillium, Fusarium, and Trichoderma with Aspergillus as the predominant genus were identified. Therefore these genes should be used as molecular markers for species level identification of fungi (especially Aspergillus and Penicillium as proven in this study).

Wastewater from various industries are composed of organic and inorganic complex pollutants including heavy metals, xenobiotics, polyaromatic hydrocarbons (PAHs), strong acids and suspended materials which cause major environmental pollution worldwide1,2. The microorganisms present in wastewater are archaea, bacteria, fungi, algae, protozoa and viruses3,4. The abundance and diversity of these organisms are influenced by parameters like temperature, pH, salinity and dissolved oxygen1. A great number of molecular studies have investigated archaea and bacterial diversity of various wastewater5,6, but fewer have addressed fungi1.

Fungi are diverse group of eukaryotic organisms characterized as heterotrophic, saprophytic, symbiotic and parasitic due to their achlorophyllous nature. Their cell walls are made up of β-glucans and chitin7. They are known as the second largest after kingdom Animalia with estimate of over 5 million species8. Fungi have the ability to metabolically utilize various substrates such as carbohydrates, proteins, lipids, aromatic hydrocarbons, and other chemical compounds as sole sources of carbon6,13. They perform several functions in wastewater systems harboring them, which include detoxification, biodegradation and decolorization of pollutants1,9.

Apart from known enormous importance of fungi, the taxonomy of these organisms is still challenging due to a lack of reliable and advanced techniques for their identification and systematic studies. Earlier studies on the composition of fungi in wastewater were either dependent on traditional method of identification based on growth, morphology, metabolism and enzymatic activity10, or the use of one molecular marker gene to identify the fungi to species levels11,12. However, the use of multiple marker genes for identification of fungal communities in wastewater has not been extensively studied. In order to resolve the difficulties of fungal identification to species level, several genetic markers for rapid classification of fungi having conserved sequences, include internal transcribed spacer regions (ITS), Beta-tubulin genes (Ben A), Calmodulin (CaM) and RNA polymerase II gene (RPB2)13,14.
The internal transcribed spacer regions (ITS) are used as official universal DNA barcode for fungi\(^{14,15}\). The ITS1, ITS2, and ITS4 have been proven to be useful for the identification of yeasts and some fungi such Aspergillus, Penicillium, Talaromyces, Cryptococcus, candida, and Trichosporon species among many others\(^{14,15}\). However, ITS sequences cannot be used for phylogenetic analyses of unrelated taxa due to low variability and slow evolution. Also, ITS sequences do not always allow correct species differentiation especially among Aspergillus and Penicillum genera\(^{17,18}\). Hence, additional gene markers are essential for correct species delineation. Secondary molecular markers such as beta tubulin; calmodulin and RP2B have been successfully used in fungal genomics\(^{8,15,18}\). Reports have shown that these protein-encoding genes contained highly variable intron regions which contain highly variable introns that evolve at a faster rate compared to ITS\(^{18,19}\).

Beta-tubulin genes are found in all eukaryotes encoding for polypeptide proteins. They have been used for phylogenetic analysis in fungi from the entire kingdom to the species level. Four Beta tubulin genes are found in all fungi; two α-tubulin (tub A) and two β-tubulin (tub B) genes. Tub A is responsible for the production of two alpha tubulin polypeptides (alpha 1 and alpha 2) while Tub B produces one alpha polypeptide (alpha 2)\(^{13}\). Reports have it that beta-tubulin gene sequences contain 3.5-fold more phylogenetic information than the small sub-unit (SSU) rRNA gene, thus it has been reported that it is an ideal marker for analysis of deep-level phylogenies and for complex species groups\(^{8,15}\).

Calmodulin (CaM) is a small acidic protein present in all eukaryotic cells and shown to be highly conserved both functionally and structurally\(^{20,21}\). Its primary role is to serve as an intracellular Ca\(^{2+}\) receptor which signal proliferation, motility, and cell cyclic progression. Ca\(^{2+}\)-CaM complexes act by controlling the activity of numerous intracellular proteins such as phosphodiesterase, Ca\(^{2+}\)-ATPase, serine protein kinases, and protein phosphatases. It also acts on several metabolic pathways and gene expression regulation in many eukaryotic organisms including fungi\(^{22}\).

RNA polymerase II gene (RPB2) encodes for second largest protein subunit in eukaryotes which synthesizes mRNA precursors and functional non-coding RNAs\(^{22,23}\). A study\(^{24}\) reported that RPB2 gene is a viable alternative molecular marker for the analysis of environmental fungal communities due its discriminative power, quantitative representation of community composition and suitability for phylogenetic analyses. Therefore this study was aimed at isolation and molecular identification of indigenous fungi from untreated refinery wastewater using multiple genes.

### Materials and methods

#### Collection of untreated refinery effluents.

Samples of untreated effluent were collected from waste water channel of Kaduna Refinery and Petrochemical Company (KRPC), Kaduna State, Nigeria. The samples were collected in sterile sample bottles. The bodies of the bottles were rinsed thoroughly with sterile distilled water before transporting them in ice box to the laboratory for fungal isolation\(^{35}\).

#### Isolation and molecular characterization of test fungi from untreated refinery effluent.

**Isolation of fungi from untreated waste water.** The effluent samples were removed from the ice box and kept to stand on a sterile laboratory work bench. 10 ml of the samples in duplicates were aseptically dispensed in sterile centrifuge tubes and centrifuged at a speed of 250 rpm for 10 min to concentrate the samples. A portion (0.1 ml) of the residue of each sample was spread-plated on sterile potato dextrose agar (PDA) (Oxoid ltd, Basinstoke, United Kingdom) and Malt Extract Agar (MEA) (Oxoid ltd, Basinstoke, United Kingdom) plates in duplicate (containing 50 µg/L of chloramphenicol), using sterile bent glass rod. The plates were incubated at room temperature (30 °C) for 7 days\(^{26}\).

**Colony morphology and microscopic characterization of fungal isolates.** Colonies grown on each medium were distinguished on the basis of their surface characteristics such as texture, colour, zonation, sporulation and diameters\(^{25}\). The distinguishable colonies were sub-cultured on PDA slant and incubated at room temperature (30 °C) for 7 days to obtain pure isolates. The microscopic characteristics were carried out by mounting small portion of the growing region of the fungi on a clean grease free slide with a drop of lacto phenol cotton blue, covered with a cover slip and examined under electron microscope using x 40 objective lens. The isolates were characterized and identified using taxonomic guide\(^{19,27,28}\). The pure isolates were maintained in PDA slants and stored in refrigerator for further identification.

**Molecular identification of fungal isolates.** Extraction of fungal genomic DNA. Each of the isolates was grown on potato dextrose agar at room temperature for 5 days. This was followed by sub-culturing each isolates into a 250 mL Erlenmeyer flask containing 100 mL potato dextrose broth (Oxoid ltd, Basinstoke, United Kingdom) and incubated for 5 days. The mycelial mass produced by each isolate was separated from the broth by filtration through sterile No 5 Whatman filter paper. The mycelial mass was crushed using porcelain mortar and transferred to Eppendorf tubes for extraction.

The genomic DNA extraction was carried out using ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) according to manufacturer’s manual instructions\(^{14,16,29,30}\).

**PCR amplification of the target genes.** Primers specific for internal transcribed spacer region (ITS), beta-tubulin gene (benA), calmodulin gene (CaM) and RNA polymerase II second largest subunit (RPB2) loci are presented in Table 1.

**PCR amplification of the extracted DNA was performed in a 20 µL reaction mixture as follow:** 1 µL gDNA template, 0.2 µL DNA polymerase, 0.5 µL each forward and reverse primers, 1µL dNTPs and sterile double distilled water to a final volume of 20 µL. The thermocycler was programed for the following PCR conditions:

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**References:**

1. Aspergillus, Penicillium, Talaromyces, Cryptococcus, candida, and Trichosporon species.
2. Beta-tubulin genes.
3. Calmodulin (CaM).
4. RNA polymerase II gene (RPB2).
5. ITS sequences.
6. Phylogenetic analyses.
7. Beta-tubulin gene sequences.
8. Calmodulin (CaM).
9. RNA polymerase II gene (RPB2).
10. Internal transcribed spacer regions (ITS).
11. Phylogenetic analysis in fungi.
12. Beta-tubulin genes.
13. Calmodulin (CaM).
14. RNA polymerase II gene (RPB2).
15. ITS sequences.
16. Phylogenetic analyses.
17. Beta-tubulin gene sequences.
18. Calmodulin (CaM).
19. RNA polymerase II gene (RPB2).
20. Internal transcribed spacer regions (ITS).
21. Phylogenetic analysis in fungi.
22. Beta-tubulin genes.
23. Calmodulin (CaM).
24. RNA polymerase II gene (RPB2).
25. ITS sequences.
26. Phylogenetic analyses.
27. Beta-tubulin gene sequences.
28. Calmodulin (CaM).
29. RNA polymerase II gene (RPB2).
30. Internal transcribed spacer regions (ITS).
31. Phylogenetic analysis in fungi.
initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. For the amplification of RPB2 gene region, touch-up PCR conditions of 5 cycles with annealing temperature 48 °C followed by 5 cycles at 50 °C and final 25 cycles at 52 °C were used. After complete amplification, the PCR products were analyzed for gel electrophoresis by using 1% agarose gel (1 g of agarose in 100 ml of Tris buffer) with ethidium bromide as the staining agent31.

Sequencing and phylogenetic analysis. The fungal isolates were identified by DNA sequencing according to standard protocols. Sequencing was carried out in a 28 μl reaction mixture as follows: 4 μl of each primer, 8 μl of purified DNA and 16 μl of PCR water and the sample was sequenced with the Di-Deoxy Terminator sequencer. The contigs (formed from forward and reverse sequences) obtained were analyzed using BioEdit 7.2.5 software and aligned using Clustal W of MEGA 7.0 software14,32,33. The fungal isolates were assigned species names after comparison with representative sequences available in NCBI (National Center for Biotechnology Information). The obtained sequences were deposited in GenBank and accession number assigned.

The evolutionary history of the fungi was analyzed using the Maximum Likelihood (ML) method based on the Tamura-Nei model of MEGA 733,34. The bootstrap tree formed from 1000 replicates represents the evolutionary history of the taxa analyzed. The percentage taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches31.

Results and discussions

Cultural and microscopic characteristics of fungal isolates. Fifteen (15) fungal isolates consisting of four genera; Aspergillus, Penicillium, Fusarium, and Trichoderma were obtained in this study with Aspergillus as the predominant genus (Table 2). The results in Table 2 also revealed the cultural features of the isolates (F1

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**Table 1.** Primers used for the amplification of specific genes in the fungal isolates.

| Locus                        | Primer  | Direction | Oligonucleotide sequence (5′-3′)                          | Length (bp) | References |
|------------------------------|---------|-----------|-----------------------------------------------------------|-------------|------------|
| Internal Transcribed Spacer (ITS) | ITS1    | Forward   | TCC GTA GGT GAA CCT GCC G                                  | 600         | 14,17      |
|                              | ITS4    | Reverse   | TCC TCC GCT TAT TGA TAT GC                                 |             |            |
| β-tubulin (BenA)             | Bt2a    | Forward   | GGT AAC CAA ATC GGT GCT GCT TTC                            | 550         | 14,17,31   |
|                              | Bt2b    | Reverse   | ACC CTC GTC GTA GTG ACC CTT GGC                           |             |            |
| Calmodulin (CaM)            | CMD5    | Forward   | CGG AGT ACA AGG ARG GCT TC                                 | 580         | 14         |
|                              | CMD6    | Reverse   | CGG ATR GAG TGT ATR AGC TGG                               |             | 14         |
| RNA polymerase II second largest subunit (RPB2-1) | 5F      | Forward   | GAY GAY MGW GAT CTT TYT GG                                 | 700         | 14,17,31   |
|                              | 7CR     | Reverse   | CCC ATR GCT TGY TTR CCC AT                                 |             |            |

**Table 2.** Cultural characteristics of fungal isolates from untreated refinery effluent.

| Isolate code | Colour            | Surface characteristics | Edge         | Reverse colour | Colony diameter (mm) (mean ± SD) | Identity of isolates          |
|--------------|-------------------|-------------------------|--------------|----------------|---------------------------------|------------------------------|
| F1           | Mint green        | Powdery                 | White, circular | Cream          | 2.70 ± 0.00                     | Aspergillus flavus           |
| F2           | Black             | Granular                | White, irregular | Cream          | 2.50 ± 1.02                     | Aspergillus japonicus        |
| F3           | Brownish-black    | Black                   | Grey, irregular | Black          | 2.70 ± 0.14                     | Aspergillus niger            |
| F5           | Black             | Granular                | Black, irregular | Cream          | 1.90 ± 1.02                     | Aspergillus niger            |
| F6           | Pale pink         | Granular                | Light pink, irregular | White          | 2.35 ± 0.07                     | Aspergillus melleus           |
| F7           | Dark-green        | Cotty                   | White, irregular | White          | 1.35 ± 0.14                     | Aspergillus sydowii          |
| F8           | White             | Smooth                  | White, circular | Cream          | 1.20 ± 0.28                     | Fusarium incarnatum         |
| F10          | Black             | Granular                | White, irregular | Cream          | 2.80 ± 0.97                     | Aspergillus niger            |
| F12          | Whitish-gray      | Smooth                  | White, circular | Cream          | 0.90 ± 0.00                     | Penicillium shearii          |
| F13          | Whitish-green     | Granular                | Circular      | White          | 8.00 ± 0.00                     | Trichoderma erinaceum        |
| F14          | White             | Smooth                  | White, irregular | Cream          | 1.80 ± 0.00                     | Aspergillus quadri-lineatus  |
| F16          | Greenish blue     | Smooth                  | White, circular | White          | 1.15 ± 0.07                     | Aspergillus fumigatus        |
| F18          | White             | Cotty                   | White, irregular | Cream          | 1.85 ± 0.07                     | Aspergillus sydowii          |
| F19          | Bluish-green      | Cotty                   | White, irregular | White          | 1.30 ± 0.00                     | Penicillium citrinum         |
| F23          | Dark green        | Cotty                   | White, irregular | White          | 1.25 ± 0.07                     | Penicillium simplicissimum   |
to F23) in terms of colour, surface characteristics, reverse, edge and diameter. Pictorial representations of the surface and reverse characteristics of the fungal isolates are shown in Fig. 1(a) and (b).

The microscopic features of the isolates are presented in Fig. 2(a) and (b) showing the conidia, spores and conidiophores. *Aspergillus* species had septate hyphae, hyaline conidiophores and radial conidial head bearing...
Figure 2. (a) Microscopic features of *Aspergillus* species (×40 magnification). (b) Microscopic features of *Penicillium*, *Fusarium* and *Trichoderma* species (×40 magnification).

**Keys:**

- **a**: *Aspergillus flavus*
- **b**: *Aspergillus japonicus*
- **c**: *Aspergillus niger*
- **d**: *Aspergillus melleus*
- **e**: *Aspergillus fumigatus*
- **f**: *Aspergillus sydowii*
- **g**: *Aspergillus quadrilineatus*

**Keys:**

- **1**: *Penicillium citrinum*
- **2**: *Penicillium shearii*
- **3**: *Penicillium simplicissimum*
- **4**: *Fusarium incarnatum*
- **5**: *Trichoderma erinaceum*
the spores (Fig. 2a). *Penicillium* species appeared as septate hyphae with conidiophores and secondary branches (metulae). The metulae bear flasked shaped phialides with unbranched chains of round conidia (Fig. 2b). *Fusarium* species showed septate hyphae, multiseptate canoe shaped macroconidia attached to the conidiophores (Fig. 2b). *Trichoderma* species appeared as septate hyphae, short conidiophores which are flask shaped clustering together at the end of each phialides (Fig. 2b).

The genus *Aspergillus* is one of the most well researched fungi genera with over 200 officially recognized species. The ubiquitous nature of *Aspergillus* may be due to their saprophytic feeding habit as well as their ability to grow in a wide range of environment. This observation sturdily indicates that members of these fungal genera isolated, have the capacity to survive and withstand toxic effects of polycyclic aromatic hydrocarbons.

**Polymerase Chain Reaction (PCR) of fungal isolates obtained.** PCR amplification of internal transcribed spacer (ITS), beta tubulin gene (Ben A), calmodulin gene (CMD) and RNA Polymerase II Second Largest Subunit (RPB2) genes of the fungal isolates are discussed below.

**Amplification of internal transcribed spacer.** Thirteen out of the fifteen fungal isolates were positive to PCR amplification of ITS regions, with amplicon sizes of 600 base pairs. Although, the ITS region is widely used as universal primers for fungi, it is not sufficient for identifying most fungi to specie level due to their low variability and slow evolution. Visagie et al. however suggested the use of other molecular markers for accurate identification of fungal species and phylogenetic relationships. Other secondary identification markers for *Aspergillus* and *Penicillium* species (and other ascomycetes) used in this study were beta tubulin; calmodulin and RPB2. These protein-encoding genes contained highly variable intron regions.

| Fungi           | Isolate | ITS Identity (%) | Accession No. | Beta-tubulin Identity (%) | Accession No. | Calmodulin Identity (%) | Accession No. | RPB2 Identity (%) | Accession No. |
|-----------------|---------|------------------|---------------|---------------------------|---------------|-------------------------|---------------|-------------------|---------------|
| *Aspergillus flavus* | F1      | 96.89            | MK828704      | 100                       | MH180047      | –                       | –             | –                 | –             |
| *A. flavus*     | F1D     | –                | –             | –                         | –             | –                       | –             | –                 | –             |
| *A. japonicus*  | F2      | 99.11            | MK840963      | 100                       | MH208743      | –                       | –             | –                 | –             |
| *A. japonicus*  | F2D     | 97.53            | MK840964      | –                         | –             | –                       | –             | –                 | –             |
| *A. niger*      | F3      | 100              | MK828713      | 99.79                     | HQ632731      | –                       | –             | –                 | –             |
| *A. niger*      | F3D     | –                | –             | –                         | –             | –                       | –             | –                 | –             |
| *A. niger*      | F5      | 98.99            | MK840965      | 100                       | MH781323      | 98.48                   | JX500080      | –                 | –             |
| *A. niger*      | F5D     | 97.75            | MK840966      | 99.59                     | LC389053      | 98.87                   | MG991517      | –                 | –             |
| *A. niger*      | F6      | 96.71            | MK840967      | –                         | –             | –                       | –             | –                 | –             |
| *A. sydowii*    | F7      | 98.85            | MK82705       | 100                       | MH426599      | 98.66                   | LN898812      | –                 | –             |
| *A. sydowii*    | F7D     | 99.45            | MK828710      | 100                       | MH644075      | 96.93                   | LN898808      | –                 | –             |

Fusarium carnatum F8 – – 98.83 KIT374271 – – – –

F. incarnatum F8D – – 98.90 KJ0280856 – – – –

*A. niger* F10 99.41 MK828708 100 MH781319 – – – –

*A. niger* F10D – – 100 MH208814 – – – –

*Aspergillus fumigatus* F11 99.27 MK816855 – – – – – –

*Penicillium shearii* F12 98.96 MK840968 – – – – – –

*P. shearii* F12D 95.83 MK828709 – – – – – –

*T. erinaceum* F13 98.53 MK840969 – – – – – –

*Aspergillus quadrilineatus* F14 97.98 MK840970 – – – – – –

*Aspergillus fumigatus* F16 – – 100 MH781343 – – – –

*Aspergillus fumigatus* F16D – – 100 MH781334 – – – –

*Aspergillus sydowii* F18 99.41 MK828707 100 LC367596 – – – –

*Aspergillus sydowii* F18D 97.34 MK828706 97.34 MK828706 – – – –

*P. citrinum* F19 99.27 MK828711 99.63 MG991339 99.27 MK828711

*P. citrinum* F19D 99.54 MK840969 – – – – – –

*P. simplicissimum* F23 98.93 MK840973 99.02 GU981631 – – – –

*P. simplicissimum* F23D 99.27 MK828712 99.32 GU981632 – – – –

**Table 3.** Accession numbers of amplified nucleotide sequences from fungal isolates. “–” denotes no clear PCR products were obtained using primers from Table 1.
Amplification of beta-tubulin gene. Thirteen isolates (13) out of the fifteen (15) isolates were positive with amplicon sizes of 480-600 bp. This result was similar to those obtained in previous studies\textsuperscript{38–40}. Eulalia et al.\textsuperscript{39} and Kamarudin and Zakaria\textsuperscript{40} amplified _Aspergillus_ fragments of beta tubulin genes with amplicon sizes in the range of 550 to 600 bp. Samson et al.\textsuperscript{41} and Erika et al.\textsuperscript{42} obtained beta tubulin gene amplicon sizes ranging from 432 to 550 bp for _Aspergillus_, _Penicillium_ and other fungal species. Beta-tubulin genes are found in all eukaryotes and have been used for phylogenetic analysis in fungi from kingdom to the species level. Reports have shown that beta tubulin genes have more variability compared to the ITS region\textsuperscript{18}. This amount of variation is suitable for determining phylogenetic relationship of closely related species of _Penicillium_ and _Aspergillus_ genera\textsuperscript{19}.

Amplification of RPB2 gene. The amplified partial RPB2 genes of the isolates revealed that only two isolates, _P. citrinum_ (F19) and _P. citrinum_ (F19D) were positive with amplicon sizes of approximately 650 and 600 base pairs respectively. This result is in agreement with the studies of Houbraken & Samson\textsuperscript{17} who identified _Penicillium citrinum_ using RPB2 genes.

Amplification of Calmodulin gene. The result of the amplified calmodulin genes of the isolates showed that _A. niger_ (F5), _A. niger_ (F10) and _P. citrinum_ (F19) had sizes of 500 bp, 550 bp and 500 bp respectively. Calmodulin gene has been considered important for the identification of _Aspergillus_ species, and some reports have even stated it should be used as the primary gene for identification of _Aspergillus_ species\textsuperscript{38,41}. 

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**Figure 3.** Phylogenetic tree of partial ITS gene sequences by maximum likelihood. Note: Sequences from this study are shown in red.
Gene Sequences of Fungal Isolates. Identifications based on cultural features were confirmed by sequence analysis of the isolates. Basic Logical Alignment Search Tool (BLAST) results of ITS region, Beta-tubulin, RPB2 gene and calmodulin gene sequences of this study in National Center for Biotechnology Information (NCBI) provided relationships and similarities with reference sequences in GenBank. The amplified sequences of each gene were submitted to GenBank and their accession numbers were assigned (Table 3). The results in Table 3 revealed that most isolates had above 96% similar identity to reference sequences of GenBank.

There has been little or no extensive research on identification of the Fungi using different molecular marker approach in Nigeria. Focus has been on macroscopic and microscopic features.

Phylogenetic Tree. Phylogenetic trees of the fungal isolates revealed that the isolates were clustered in grouping patterns of close resemblance. Sequences from this study are shown in red colours while sequences from GenBank are shown in black. Test of phylogeny was bootstrap of 1000 replications. Phylogenetic tree based on ITS gene revealed that the alignment matrix contained 54 nucleotide sequences with 209 positions in the final dataset. All isolates of Aspergillus and Penicillium species were clustered had cluster identity of above 95% with those from GenBank. The tree was out grouped by T. erinaceum (Fig. 3).

Beta-tubulin gene alignment matrix contained 52 nucleotide sequences with 19 positions in the final dataset. All the fungal species had above 85% cluster similarity with fungal species from GenBank while P. simplicissimum was placed in the out group (Fig. 4).

Phylogenetic tree based on partial RPB2 gene revealed that the alignment matrix involved 19 nucleotide sequences with a total of 404 positions in the final dataset. The two positive isolates of Penicillium citrinum shared 90% cluster similarities with sequences from GenBank (Fig. 5).
Figure 5. Phylogenetic tree of partial RPB2 gene sequences by maximum likelihood. Note: Sequences from this study are shown in red.

Figure 6. Phylogenetic tree of calmodulin gene sequences by maximum likelihood.
Phylogenetic tree based on calmodulin gene had an alignment matrix of 14 sequences. A. niger (F5D) shared 89% cluster similarity while the two isolates of A. sydowii (F7 and F7D) had equal (98%) cluster similarities with sequences from GenBank. A. niger (F5) falls in the outgroup (Fig. 6).

The phylogenetic trees revealed that related species are clustered together which indicates a clear and well resolved classification and evolutionary history of the isolates.

**Conclusion**

There has been little or no extensive research on identification of the Fungi using different molecular marker approach in Nigeria. Focus has been on cultural and microscopic features. The fungal isolates from this study were further subjected to PCR amplification coupled with DNA sequencing of four molecular genes markers. The fungal species isolated from untreated refinery effluent consist of the following genera: Aspergillus, Penicillium, Fusarium, and Trichoderma with Aspergillus being the predominant genus. Sequence results obtained revealed above 95% similarities between the isolates in this study and those found in GenBank. The identification and molecular characterization of the fungal isolates to specie level gave a better result by PCR amplification and sequencing of ITS region, partial beta tubulin, calmodulin and RPB2 genes. Therefore should be used as molecular markers for species level identification of fungi (especially Aspergillus and Penicillium as proved in this study).

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Conceptualization, B.A.E., M.D.A., W.C.M.Z. and J.W.S.; methodology, B.A.E., M.D.A., W.C.M.Z. and J.W.S.; formal analysis, B.A.E., M.D.A., W.C.M.Z. and J.W.S.; writing original draft, B.A.E., M.D.A., W.C.M.Z. and J.W.S.; resources, review and editing, B.A.E., M.D.A., W.C.M.Z. and J.W.S.; project administration, B.A.E., M.D.A., W.C.M.Z., G.M.-H. and G.D.Z.

Competing interests
The authors declare no competing interests.

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