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# MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF PREVALENT PLANT PATHOGENIC FUNGI ASSOCIATED WITH ROT IN COCONUTS (*Cocos nucifera* L.)

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**Abstract**

The aim of this study is to isolate and identify fungi associated with the rot of coconut (*Cocos nucifera* L.). Samples of the deteriorating coconut were collected from Rumuodomaya Market in Obio/Akpor Local Government Area of Rivers State. Fungal isolates were collected at the Regional Centre for Biotechnology and Biofuel Research Laboratory and morphologically characterized. The DNA of the most common fungal isolates, sample 12 and 13 was molecularly characterized using Internal Transcribed Spacer 4 and 5 (ITS-4 and 5) molecular markers. The DNA of the isolate sequence was aligned using Basic Local Alignment Search Tool for Nucleotide (BLASTN) 2.8.0 version of National Center for Biotechnology Information (NCBI) database. Based on sequence similarity, it was observed that the diseased isolate sample 12 was 99.83% identical to *Rhizopus delemar* and sample 13 was 69.06% identical to *Aspergillus flavus*. The molecular weight of the DNA of the isolates were 657 base pairs for *Rhizopus delemar* and 595 base pairs for *Aspergillus flavus*. These findings showed that *Rhizopus delemar* and *Aspergillus flavus* are the major causal fungal pathogens infecting coconut. A Phylogenetic tree was constructed showing the relationship between the isolates from the study and other isolates on the GenBank. This study revealed vital information on the molecular characterization of fungi associated with coconut. It is hoped that the obtained results will serve as a handy information for individuals that wishes to do further studies.

**Keywords**: Fungi, *Cocos nucifera,* molecular identification, Economic Cash Crops. extracts.

## Introduction

The Coconut plant (*Cocos nucifera* L.) is a multipurpose, monoecious perennial palm according to Niral (2019), that belongs to the family Arecaceae. It is one of the most useful trees globally and is often referred to as the "tree of life" since every part of the plant is useful (Samosir, *et al.,* 2005). Coconut is an important seed with economical, medicinal, traditional as well as nutritional values. Economically Coconut is a significant cash crop in Nigeria. Depending on the -variety planted, it can earn revenue - between N16 million – N20 million yearly (Baje, 2022) which is $34,727.406 to $43,409.258.

They provide nutritious products, edible coconut oil, fiber for commercial value and coconut shell for fuel and industrial uses. Their husk and shells can be used for fuel since they are a source of charcoal

(Sulistyania *et al.,* 2015). Coconut water, is consumed as a refreshing drink and - it's currently gaining popularity as a sports drink. -Coconut -water also has important clinical applications such as treating renal disorders of the kidney. It contains sugar, dietary fiber, proteins, antioxidants, vitamins and minerals. It detoxifies the skin; helps reduce skin inflammation, rashes, fungal infections and removes stretch marks (Emojevwe, 2013).

According to Jamshidi-Kia *et al.,* (2018), the use of plants as medicines has received an extensive attention. In ancient times, plants have been - used for medicinal purposes in the treatment of various diseases - worldwide (Sofowora *et al.,* 2013). The oil extracted from the coconut, is a possible remedy to cancer (Emojevwe, 2013), because of its high selenium content and are also rich in anti-oxidants that slows down the ageing process in human cells (Wiebke, 2012). Coconut oil may help reduce the appearance of stretch marks or speed their healing. It has antimicrobial, antifungal, and antiviral properties. Coconut oil are - easily absorbed into skin, where it may prevent free radical damage (Whelan, 2018).

Coconut is rich in nutrients and as such, highly prone to microbial contamination. The accurate identification of pathogens associated with coconut rot is essential for the proper development of management strategies - but prior to this time, mycologists have relied on the use of phenotypic characters to identify fungal species, (Hyde, *et al.,* 2010) however, this method may not perform well for lower species classifications (Lutzoni, *et al.,* 2004) because they may not provide accurate groupings within an evolutionary framework, mainly at the species level. According to Geiser, (2004), morphological characters can often be misleading due to hybridization. Since identifying fungi based on morphological characters alone is challenging, DNA sequence-based methods have emerged for identifying species within the megadiverse fungi (Bridge, 2005).

Therefore, this research is aimed at identifying the causal agent of these diseases in coconuts by its morphology and molecular properties. This study combined morphological characteristics with phylogenetic analyses to investigate the diversity of *Rhizopus delemar* and *Aspergillus flavus* species that are associated with the postharvest rot of coconut seeds.

## Materials and Methods

### Study Duration

This research was carried out from 17th January 2020 to 17th September, 2021.

### Source of plant material

Diseased samples of coconuts seeds, were collected from coconut wholesalers in Rumuokoro Market, Port Harcourt, Rivers State Nigeria.

### Isolation of Fungi from Coconut using the Blotter Method

Fungal pathogens associated with coconut was isolated using the standard blotter method recommended by (ISTA, 2016). The Petri dishes were lined with 3 layers of sterile filter papers, which were soaked in distilled water. The coconut was surface sterilized, shell cracked then the diseased part scooped, plated and incubated for 7 days at a temperature of 25 $\pm$ 2°C. The organisms were isolated at the mycology/pathology laboratory of the Department of Plant Science / Biotechnology, Faculty of Science University of Port Harcourt while - the DNA –extraction -was carried out at the Regional Centre for Biotechnology and Biofuel Research Laboratory -also located at the University of Port Harcourt, Choba, Rivers State, Nigeria.

The purification and sequencing of the PCR products were carried out at the International

Institute of Tropical Agriculture (IITA) Ibadan where the most prevalent - fungal isolates were coded as -Samples 12 and 13- accordingly.

**Morphological and microscopic characterization and identification**

The morphological identification of isolates in samples 12 and 13 were conducted by visually observing the mycelium and comparing their colonies for their diameters, colours, colours of conidia, reverse colours, texture, zonation and sporulation with -Snowdon, 1990- pictorial guides. The isolates were -futher subjected to microscopic analysis for identification using an electron binocular microscope at X40.

**Molecular characterization using the Internal Transcribed Spacer (ITS) marker and identification**

The Genomic DNA of the isolate KN-01 was extracted following the protocol of Quick-DNA™ Fungal /Bacterial MiniPrepKit (Zymo Research Group, California, USA) as described by the manufacturer, with modifications at the Regional Center for Biotechnology and Bioresources (RCBB), University of Port Harcourt, Rivers State, Nigeria. The KN-01 isolate DNA quantity and concentration were measured using the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific Inc. Wilmington, Delaware, USA). The DNA purity was measured as a ratio of absorbance at 280 nanometers (nm) to that of 260 nanometers. The quality of the DNA of the isolate KN-01 was further quantified using the Agarose gel electrophoresis performed according to the modified method of (Saghai-Maroof *et al.*, 1984). The DNA sample of the KN-01 isolates shipped to the International Institute of Tropical Agriculture (IITA) Bioscience Centre, Ibadan, Nigeria for amplification and sequencing. The primers used to amplify fragments of the nuclear ribosomal DNA (rDNA) of the KN-01 isolates were the Internal Transcribed Spacer 4 (ITS4) with the sequence TCCTCCGCTTATTGATATGS and ITS5 with the sequence GGAAGTAAAAGTCGTAACAAGG. The amplicons were sequenced using the ABI 3500 capillary electrophoresis sequencer. The DNA sequence file was saved in the Bioedit file with an extension. ab1. The sequence was analyzed using the Molecular Evolutionary Genetics Analysis (MEGA) version 7.0.26 software, and aligned using the Basic Local Alignment Search Tool for nucleotide (BLASTN) 2.8.0 version of the National Centre for Biotechnology Information (NCBI) database.

**Results**

**Isolation, morphological and microscopic identification of fungi associated with coconut**

The result of the fungal isolation is presented in Plates 1a and 1b. The isolated fungi from samples 12 and 13 was found to be associated with coconut. The fungal isolate 12 had a white dense cottony growth at first which became yellowish-brown with sporulation while sample 13 was observed to develop an


**Plate 1a and b:** Pure Culture of Fungi Isolated from coconut on Potatoes Dextrose Agar (Sample 12) 1b: Pure Culture of Fungi Isolated from coconut on Potatoes Dextrose Agar (Sample 13)

initial whitish mass of mycelia which later turned into a powdery mass of yellowish-green spores on the upper surface of the plate and red on the reverse side. From the photomicrograph, the isolates were identified as *Rhizopus delemar* and *Aspergillus flavus*.

**Molecular Characterization using the Internal Transcribed Spacer (ITS) Marker and Identification**

The genomic DNA of the isolate samples 12 and 13 of coconut were successfully extracted. The Nanodrop result (Table 1a and b) showed - the concentrations of the DNA of the isolates. However, to reduce the cost of sequencing, the isolates with the highest DNA concentration was selected.

Table 1a: Concentration of DNA Extracted from Fungal isolates of coconut sample 12 using Nanodrop (2000c) Spectrophotometer

| Sample ID | Nucleic acid conc. (ng/µl) | Absorbance at 260 (purity) | Absorbance at 280 | 260/280 | 260/230 |
|---|---|---|---|---|---|
| **12a** | 126.9 | 2.538 | 1.387 | 1.83 | 1.10 |
| **12b** | 126.7 | 2.535 | 1.388 | 1.83 | 0.97 |
| **12c** | 126.2 | 2.524 | 1.366 | 1.85 | 1.10 |

Table 1b: Concentration of DNA Extracted from Fungal isolates of coconut sample 13 using Nanodrop (2000c) Spectrophotometer

| Sample ID | Nucleic acid conc. (ng/µl) | Absorbance at 260 (purity) | Absorbance at 280 | 260/280 | 260/230 |
|---|---|---|---|---|---|
| **13a** | 108.3 | 2.166 | 1.186 | 1.83 | 0.52 |
| **13b** | 109.1 | 2.182 | 1.178 | 1.85 | 0.53 |
| **13c** | 108.4 | 2.168 | 1.179 | 1.84 | 0.52 |

The result of the amplified DNA PCR band of the isolate samples 12 and 13 is presented in Fig. 2. The amplified DNA showed a band on gel when observed under UV light. From the result, the ladder used indicated that samples 12 isolate sequence had over 657 base pairs and samples 13 isolate sequence had over 595 base pairs.

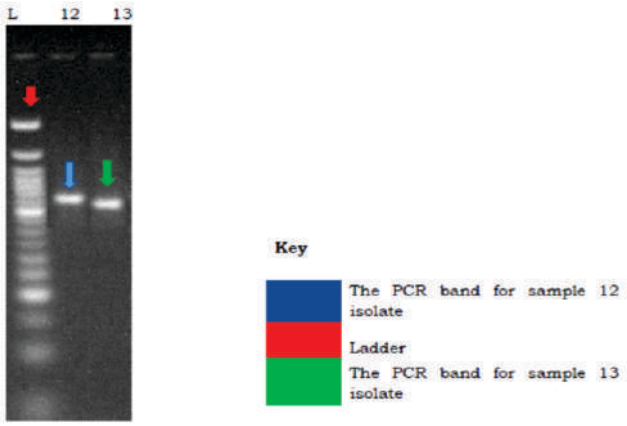


**Figure 2: Amplified PCR Product Generated from KN-01 Isolate**

**DNA Sequencing**

The sequencing result after alignment presented -in Figure 3.1 showed the beginning while Figure 3.2 showed the end of the DNA sequence of the isolate sample 12. -Figure 3.3 showed the beginning while Figure 3.4 showed the end of the DNA sequence of the isolate sample 13. This result authenticated the DNA amplification result as shown in Tables 1a and 1b above -. Also, from the results, it was noticed that the colours of the bases of the nucleotides were existing in four colours [green: adenine (A), red: thymine (T), blue: cytosine (C), black: guanine (G). These diverse colours allow for easy interpretation of the sequence.

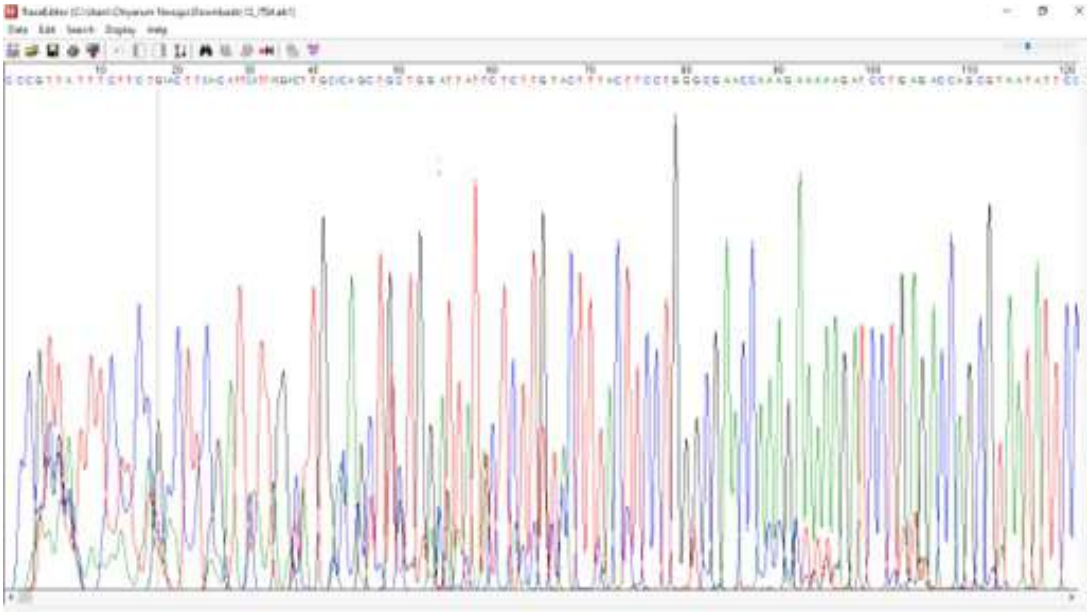

**Figure 3.1: Beginning of Sequence Alignment of the DNA of sample 12 Isolate after Alignment**

**Figure 3.2: The End Part of Sequence Alignment of the DNA of sample 12 Isolate after Alignment**

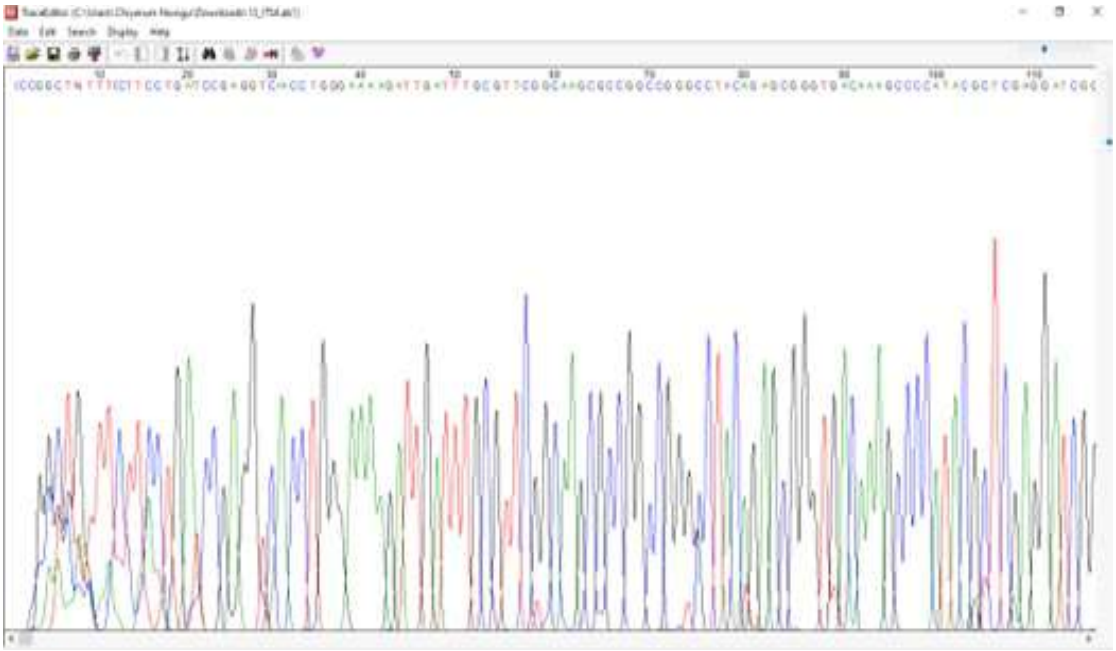

**Figure 3.3: The Beginning Part of Sequence Alignment of the DNA of sample 13 Isolate after Alignment**

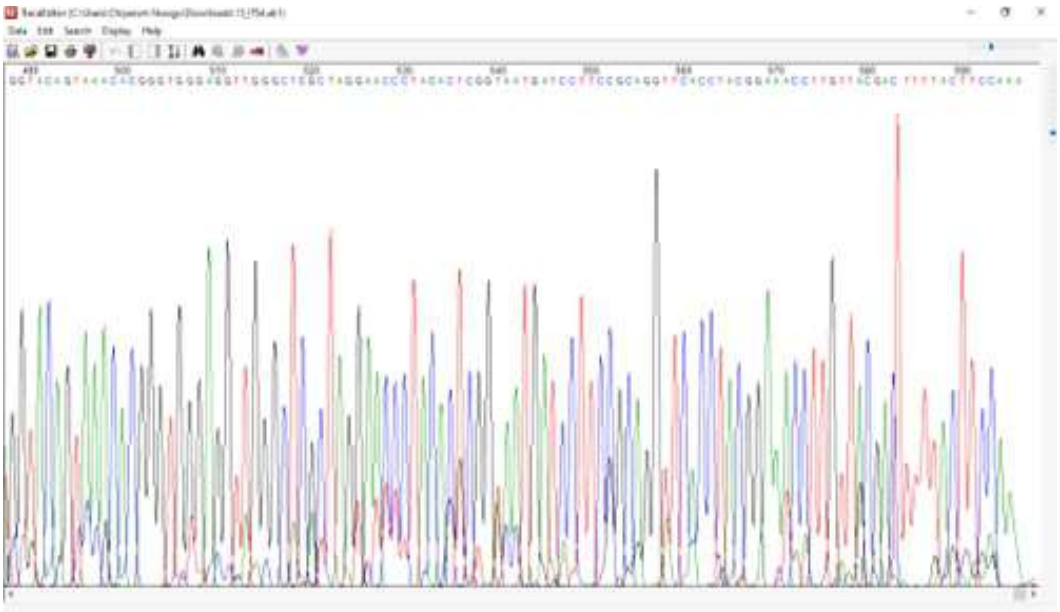

**Figure 3.4: The End Part of Sequence Alignment of the DNA of sample 13 Isolate after Alignment**

**Sequence Alignment using BLAST**

Figures 3.5 and 3.6 indicated that the sample 12 and sample 13 isolates sequences aligned with 100 sequences deposited in the composite biological database of National Center Biotechnology Information (NCBI). The sample 12 isolate sequence was 99.83% identical to *Rhizopus delemar* (red arrow) and sample 13 was 99.83. % identical to *Aspergillus flavus* (blue arrows).

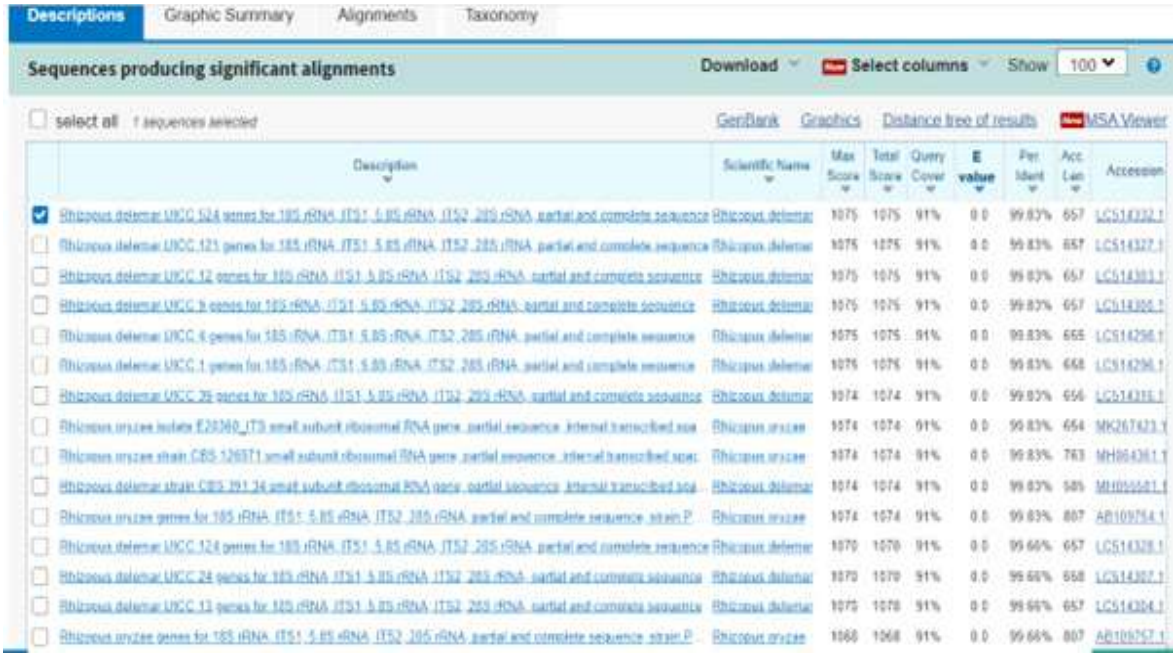


**Figure 3.5: The Sequence Alignments of sample 12 Isolate Sequence with NCBI Database Sequences**

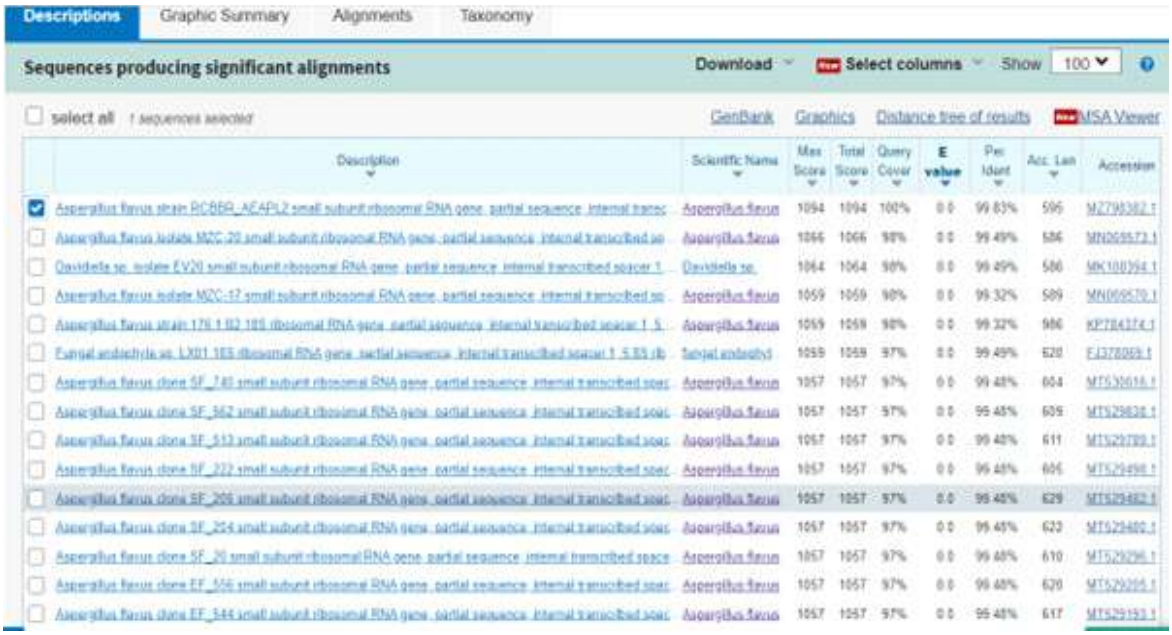


**Figure 3.6: The Sequence Alignments of sample 13 Isolate Sequence with NCBI Database Sequences**

## Phylogenetic Analysis

The phylogenetic tree constructed showed the relationship between the isolates from this study and other fungal isolates on GenBank. The phylogenetic analysis showed that *Rhizopus delemar* and *Aspergillus flavus* - are closely related to the fungal isolates obtained from the Coconut as presented in Figure 4a and b.

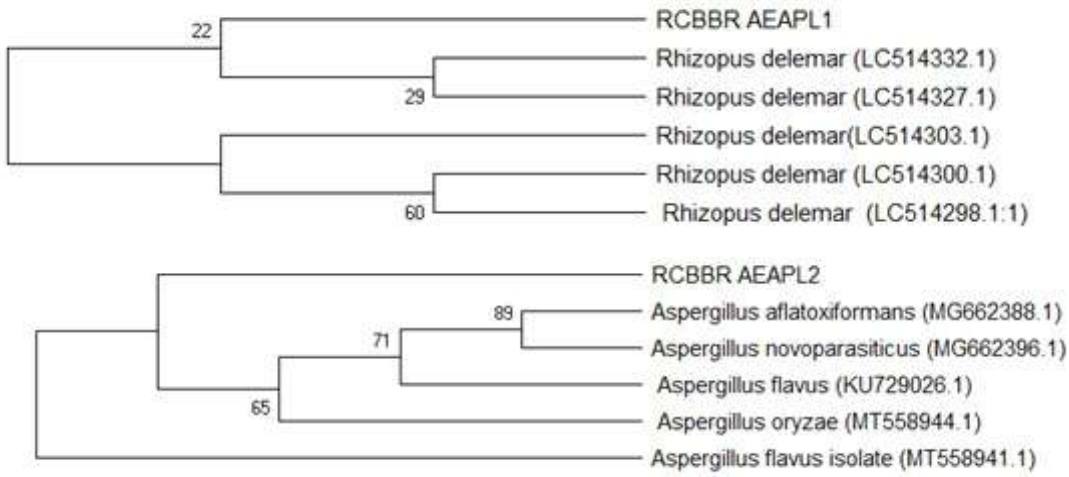


**Figure 4a and b: Phylogenetic tree generated by maximum composite likelihood analysis based on the ITS 4 and ITS 5**

## Discussion

The current study on the isolate and the identity -of fungi associated with the rot of coconut, - revealed that these fungi used the existing nutrient in the coconut and render them unfit for human consumption.

As reported in the present investigation, the fungal species associated with the rot of coconut were identified as *Rhizopus delemar* and *Aspergillus flavus* by molecular characterization. The sequence of the isolated fungi has shown up to 99.83% identical to other *Rhizopus delemar* species and 69.06% identical to other *Aspergillus flavus* species. The two fungal strains are known as plant pathogenic fungi that infect several plant species.

Our results are in agreement with previous reports of Chuku *et al.* (2007) who - stated that - *Aspergillus* and *Rhizopus* species as pathogenic fungi associated with coconut disease. Gupta, (2002), also isolated *Aspergillus flavus* from naturally infected leaf-eating caterpillar (*Opisina arenosella* W.), lace bug (*Stephanitis typica* D.) and plant hopper (*Proutista moesta* Westwood), insect pests of the coconut palm. Some other researcher such as Okolie, (2011), reported *Aspergillus flavus* as one of the principal fungal agents associated with coconut spoilage. Three fungal organisms namely: *Aspergillus niger*, *Rhizopus* sp and *Fusarium oxysporum* were isolated from spoilt fruits of coconut (Chuku, *et al.,* 2022).

According to Labuza and Erdman, (1993), microbial spoilage of foods is quicker when such foods contain growth-promoting elements that enhance the well-being of the invading microbes.

## Conclusion

Coconut seed is - -highly nutritional and contains antioxidants that support human health. However, -it is prone to spoilage due to fungal contamination. Spoilt coconut fruits are easily detected as the water inside would have changed odour. In order to maintain -the good quality of the abundant nutrient in the coconut seed, the need for hygienic and proper handling of the product during harvest and processing is advocated.

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