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Isolation And Identification of Common Fungal Pathogens Invading Sweet Potatoes (*Ipomoea batatas*) Sold in Choba Market, Port Harcourt, Nigeria

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ABSTRACT: This research was aimed at isolating and identifying common fungal pathogens invading sweet potato tubers. Tubers were collected from Choba Market, Port Harcourt in Obio/Akpor Local Government Area of Rivers State. Fungal isolates were collected and morphologically identified. The Deoxyribonucleic acid of the most common fungal isolates, SP-1 and SP-2 were molecularly characterized using Internal Transcribed Spacer 4 and 5 molecular markers. The isolates Deoxyribonucleic acid sequence was aligned using Basic Local Alignment Search Tool for Nucleotide 2.8.0 version of National Centre for Biotechnology Information database. The molecular weight of the Deoxyribonucleic acid of the isolates were 573 base pairs for *Lasiodiplodia theobromae* and 521 base pairs for *Pichia kudriavzevii*. Based on sequence similarity, it was observed that the sweet potato tubers isolate SP-1 was 99.25% identical to *Lasiodiplodia theobromae* and SP-2 was 99.70% identical to *Pichia kudriavzevii*. These findings showed that *Lasiodiplodia theobromae and Pichia kudriavzevii* are some of the causal fungal pathogens invading sweet potato tubers. It is projected that this result will provide information for disease control approach for improving the post-harvest losses of sweet potato. This study will improve available information and aid in reducing the deteriorating activities of fungi invading Sweet Potato tubers.

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The sweet potatoes (*Ipomoea batatas*) are dicotyledonous root vegetable plants that belong to the morning glory family, *Convolvulaceae* (Woolfe, 2020). Cultivars of the sweet potato have been bred to bear tubers with flesh and skin of many colours, white, yellow and orange flesh is common with a darker skin (Rose and Vasanthakaalam, 2011). Sweet potatoes are underground tubers that have admirable quantity of beta carotene which our bodies can convert into vitamin A (Satheesh and Fanta, 2019). According to Abebe (2019), the potato crop are staple foods that are rich in carbohydrates, protein, vitamin C, vitamin A, zinc, iron and minerals which alleviate the problem of malnutrition in subsistence farmers and towns. They come in different shapes and their peels ranges from creamy white, yellow-orange, tan, reddish-purple and red colours (Leksrisompong *et al.*, 2012). They may have several health benefits, as Megan (2019) stated that they improved blood sugar regulation, maintaining healthful blood pressure levels, improving digestion, reduce risk of cancer and are a good source of provitamin A in the form of beta-carotene important for protecting eye health. Storage of sweet potato tubers after harvest is imperative, as this practice may

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prevent a surfeit of potatoes entering the food markets at any given time and prolong the period of fresh tuber availability, especially when the crop is not in season or when the economic circumstances and/or regional climates in a particular area of production dictate its production during the year. Sweet potatoes are one of the most important vegetables and they are susceptible to a variety of field and storage diseases. They are vulnerable to deterioration because of their high water and nutrient content (Oladoye *et al.,* 2016). High nutrient and water contents in the tubers make it easily susceptible to many diseases which contribute to crop losses during storage (Oladoye *et al.,* 2016). Sweet potatoes are affected by a number of diseases, both fungal and bacterial (Clark *et al.,* 2013). Commonly observed postharvest diseases caused by fungi include black rot (*Ceratocystis fimbriata*), dry rot (*A. niger* and *Diaporthe batatas*), Fusarium surface rot (*F. oxysporum*), Fusarium root and end rot (*F. solani*), foot rot (*Plenodomus destruens*), soft rot (*Rhizopus stolonifer* and *Rhizopus oryzae*), blue mold (*Penicillium* spp.), java black rot (*Botryodiplodia theobromae*), circular spot (*Sclerotium rolfsii*), charcoal rot (*M. phaseolina*) and storage rot (*Mucor* sp.) (Paul *et al.,* (2020); Sugri *et al.* (2020) & Dania and Thomas, (2019)). Species identification using quick and simple methods for determining the species composition of fungal communities have gained recognition worldwide. Stengel *et al.* (2022), argues that fungi species are diverse and that there is no single set of criteria sufficient to describe the diversity seen among their lineage. Since fungi species are diverse and morphological identification though very important to understand the evolution of morphological characters (Raja *et al.,* 2017), are not sufficient for taxonomy. Raja *et al.* (2017), recommended using both morphology and molecular data for identification. The objective of this study was to isolate and identify common fungal pathogens invading sweet potatoes (Ipomoea batatas) sold in Choba Market, Port Harcourt, Nigeria using Polymerase Chan Reaction to amplify the ITS region of fungal DNA for the purpose of sequencing.

MATERIALS AND METHODS

Source of plant material: Diseased tubers of *Ipomoea Batatas* (Plate 1) with disease symptoms were obtained from Choba market in Port Harcourt, Rivers State in February, 2019.

Study Area: The study was conducted in Mycology/ Pathology laboratory of Plant Science and Biotechnology and Regional Centre for Biotechnology and Bio-fuel Research Laboratory where DNA extraction was carried out, University of Port Harcourt. Amplification and sequencing of the PCR products were done at the International Institute for Tropical Agriculture (IITA) Ibadan.

Plate 1: Diseased Tubers of Sweet Potato

Isolation of Fungi from Sweet potatoes using Blotter Method: According to the rules of the international seed health testing association ISTA (2016), a standard blotter method was used to isolate fungi pathogens associated with diseased tubers of *Ipomoea Batatas*. The filter paper, distilled water (placed in a conical flask) and Petri dishes (wrapped in a foil paper) used for the work were first autoclaved at 121^{o}C for 15mins. The Petri-dishes were lined with 3 layers of sterilized 9cm Whatman's filter paper, the filter paper was soaked with little water and then the petri dishes were covered immediately. Four (4) small pieces of diseased tubers of *Ipomoea Batatas* were surface sterilized in a beaker using 70% ethanol for 2-3minutes, the ethanol discarded and rinsed with sterile distilled water twice and plated per Petri-dish, the plates were then wrapped with masking tape and then labelled. After this, they were incubated at 25 $\pm$ 2^{o}C in the laboratory for 7 days. All identified fungi were sub-cultured on Potato Dextrose Agar (PDA) medium under darkness at room temperature (25$\pm$ 2^{o}C).

Extraction of Fungal DNA: Zymo Quick DNA Fungal/Bacterial Kit was used for the DNA extraction according to the manufacturers' protocol with some modifications. The fungal mycelium was scrap off from the surface of the Potatoes Dextrose Agar, froze with liquid nitrogen and homogenized with bashing bead in a sterile mortar. The homogenized mixture was centrifuge at 8,000xg for I minute. Following the manufacture's protocol, lysis, precipitation, pre-washing, washing and DNA elution was carried out. DNA quantity and concentration of pure cultures of sweet potatoes were measured using Nanodrop 2000c spectrophotometer (Thermo fisher Scientific Inc. Wilmington, Delaware, USA). The purity of the DNA was measured as a ratio of Ultraviolet (UV) light absorbance at 280nm to absorbance of 260nm. DNA quality was determined using 1% Agarose gel through gel electrophoresis.

Polymerase Chain Reaction (PCR) Product Amplification and Sequencing: The Primers used to amplify fragments of the nuclear ribosomal DNA (rDNA) are Internal Transcribed Spacer 4 (ITS4) with the sequence TCCTCCGCTTATTGATATGS and ITS5 with the sequence GGAAGTAAAAGTCGTAACAAGG. The protocol for Polymerase Chain Reaction (PCR) was carried out in a final volume of 10µL containing 2µL of genomic DNA (10ng/µL), 0.1µL of Taq polymerase, 1.0µL of 10X PCR buffer, 1.0 µL of DMSO, 0.8µL of 2.5mM DNA) DNTPs, 1.0µL of 25mM MgCl2 (Promega), 0.5µL of each primer (concentration of 5µM) and 3.1µL of Nuclease-free water. Amplifications were performed in a thermal cycler (Eppendorf) using an initial denaturation step of 94°C for 5 minutes, followed by 36 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 52°C and elongation for 45 seconds at 72°C with a final extension for 7 minutes at 72°C. The amplicon from the above reaction was subjected to gel electrophoresis in 1.5% agarose gel using TBE 1X and the gel stained with ethidium bromide (13µL/50ml). The amplicons were sequenced using the ABI 3500 capillary electrophoresis sequencer.

Phylogeny Analysis: The sequences were 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 Center for Biotechnology Information (NCBI) database for identification of species. The ITS 1-2 gene sequence in Genbank and a neighbor joining phylogenetic tree was created using maximum likelihood method.

RESULTS AND DISCUSSIONS

The result of the fungal isolation is presented in Plate 1a and 1b. The unidentified fungal organisms SP-1 and SP-2 were isolated and found to be associated with Sweet Potatoes (*Ipomoea batatas)*.

Plate 1a and b: Pure Culture of Fungus Isolated from Sweet Potatoes

DNA Extraction and Concentration Determination: The genomic DNA of the isolates SP-1 and SP-2 of sweet potatoes were successfully extracted. The NanoDrop result presented in Table 1. The Nanodrop result (Table 1) showed that the concentrations of the DNA of the isolates were 262.0 ng/µl and 51.5ng/µl respectively. While the absorption peak of the 260nm/280nm readings were 1.85 and 1.92 respectively and the 260nm/230nm readings were 1.63 and 0.67 respectively. However, to reduce the cost of sequencing, the isolates with the highest DNA concentration were selected.

Table 1: ~~Showed the~~ Concentrations of the Genomic DNA Extracted from Fungal Isolates of Sweet Potatoes SP-1 and SP-2 using Nanodrop (2000c) Spectrophotometer.

Sample ID	Nuclei Acid concentration	Unit	A260	A280	260/280	260/230	Sample Type	Factor
11a	**248.3**	ng/µl	**4.966**	**2.686**	**1.85**	**1.59**	**DNA**	**50.00**
11b	**248.7**	ng/µl	**4.975**	**2.702**	**1.84**	**1.57**	**DNA**	**50.00**
11c	**262.0**	ng/µl	**5.240**	**2.827**	**1.85**	**1.63**	**DNA**	**50.00**
12a	**50.2**	ng/µl	**1.004**	**0.532**	**1.89**	**0.65**	**DNA**	**50.00**
12b	**51.5**	ng/µl	**1.029**	**0.536**	**1.92**	**0.67**	**DNA**	**50.00**
12c	**51.5**	ng/µl	**1.030**	**0.544**	**1.89**	**0.67**	**DNA**	**50.00**

Polymerase Chain Reaction (PCR) and Gel Electrophoresis: The result of the amplified DNA or PCR band of the isolates isolates SP-1 and SP-2 are presented in Fig. 1 The amplified DNA showed a band on gel when observed under UV light. From the result, the ladder used indicated that the isolates SP-1 and SP-2 isolates sequence had over 573 and 521 base pairs respectively.

DNA Sequencing: The sequencing result after alignment are shown in Figure 2 showed the beginning while Figure 3 showed the end of the DNA sequence of the isolate SP-1. also, Figure 4 showed the beginning while Figure 5 showed the end of the DNA sequence of the isolate SP-2. Figure 6 specified that the sequence length was 573 base pairs and Figure 7 specified that the sequence length was 521 base pairs. This result authenticated the DNA amplification result

as shown in Table 1 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.

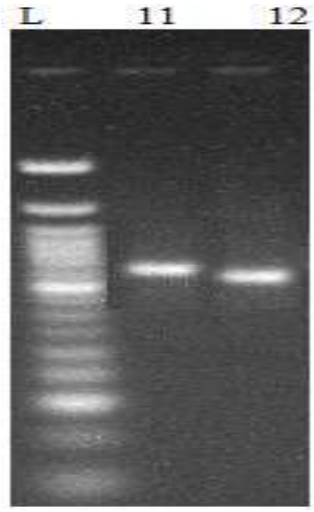

Fig 1: Amplified PCR product generated from SP-1 and SP-2 isolates

*Sequence Alignment using BLAST***:** Figures 6 and 7 indicated that the SP-1 and SP-2 isolate sequences aligned with 100 sequences deposited in the composite biological database of National Center Biotechnology Information (NCBI). The SP-1 isolate sequence was 99.25% identical to *Lasiodiplodia theobromae* (red arrow) and SP-2 was 99.70% identical to *Pichia kudriavzevii* (blue arrows). *Phylogenetic Analysis:* The phylogenetic trees constructed showed the relationship between the isolates from this study and other fungal isolates on GenBank. The phylogenetic analyses showed that *Lasiodiplodia theobromae* and *Pichia kudriavzevii* are closely related to the fungal isolates obtained from the stored sweet potatoes as presented in Figure 8 and 9.

According to Paul *et al.* (2020), sweet potatoes are the eleventh most important food crop internationally. Sweet potatoes are popular in Nigeria and during yam scarcity, many homes depend on it as substitute to yam. Sweet potato tubers are susceptible to fungi infection due to their low pH, moisture content and nutritional compositions (Paul *et al.*, 2021). The frequency of storage rots of sweet potato tubers encountered in Rivers State could be as a result of climatic factors, storage conditions, transportation and handling during harvest. Post-harvest loss of root and tubers has been of grave problem to farmers and consumers and according to Sawicka (2019), has resulted to inefficient functioning of food systems. The results of this study, are in agreement with the findings of other investigators that fungi constitute a threat to storage rots of sweet potato and many other agricultural produces.

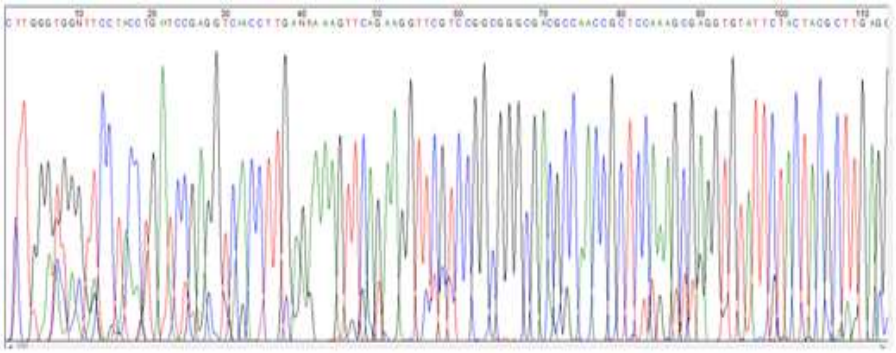

Fig 2: Beginning of Sequence Alignment of the DNA of SP-1 Isolate after Alignment

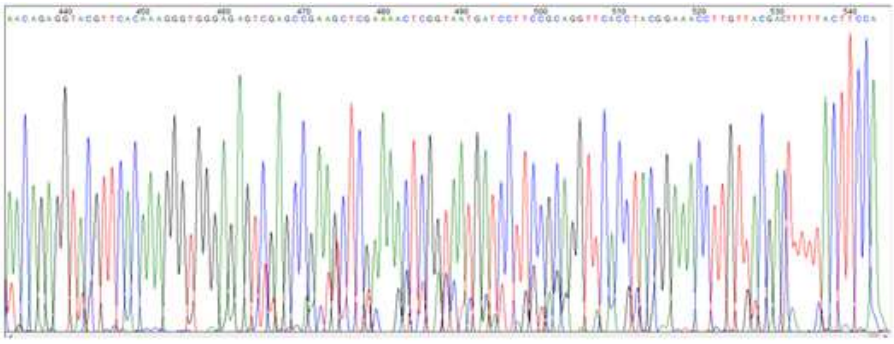

Fig 3: The End Part of Sequence Alignment of the DNA of SP-1 Isolate after Alignment

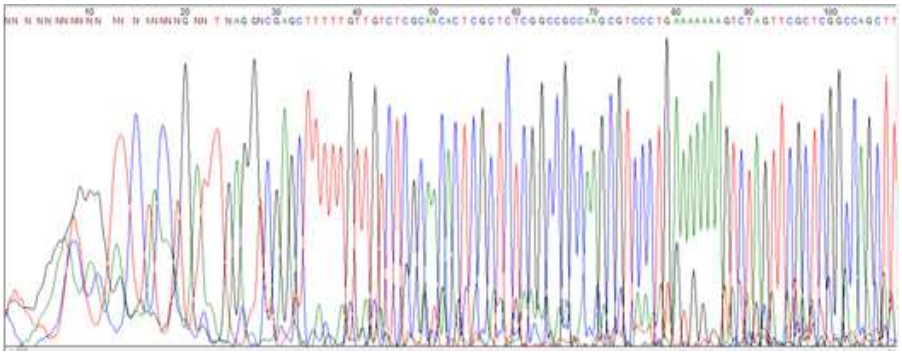

Fig 4: The Beginning Part of Sequence Alignment of the DNA of SP-2 Isolate after Alignment

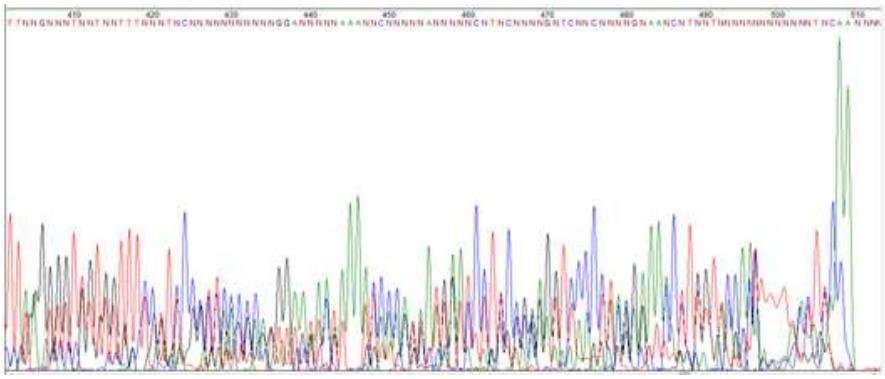

Fig 5: The End Part of Sequence Alignment of the DNA of SP-2 Isolate after Alignment

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc Len	Accession
☑	Lasiodiplodia theobromae isolate MGSD091 small subunit ribosomal RNA gene, partial sequence; internal transcri	Lasiodiplodia the	961	961	97%	0.0	99.25%	673	MW138056.1
☐	Lasiodiplodia theobromae isolate MGSD071 small subunit ribosomal RNA gene, partial sequence; internal transcri	Lasiodiplodia the	961	961	97%	0.0	99.25%	672	MW138057.1
☐	Lasiodiplodia theobromae isolate MGSD045 small subunit ribosomal RNA gene, partial sequence; internal transcri	Lasiodiplodia the	961	961	97%	0.0	99.25%	672	MW138056.1
☐	Lasiodiplodia theobromae isolate MGSD028 small subunit ribosomal RNA gene, partial sequence; internal transcri	Lasiodiplodia the	961	961	97%	0.0	99.25%	674	MW138055.1
☐	Lasiodiplodia theobromae isolate MGSD017 small subunit ribosomal RNA gene, partial sequence; internal transcri	Lasiodiplodia the	961	961	97%	0.0	99.25%	672	MW138054.1
☐	Lasiodiplodia theobromae isolate MGSD006 small subunit ribosomal RNA gene, partial sequence; internal transcri	Lasiodiplodia the	961	961	97%	0.0	99.25%	672	MW138053.1
☐	Lasiodiplodia theobromae isolate MGSD005 small subunit ribosomal RNA gene, partial sequence; internal transcri	Lasiodiplodia the	961	961	97%	0.0	99.25%	673	MW138052.1
☐	Lasiodiplodia theobromae isolate MGSD004 small subunit ribosomal RNA gene, partial sequence; internal transcri	Lasiodiplodia the	961	961	97%	0.0	99.25%	672	MW138051.1
☐	Lasiodiplodia theobromae isolate MGSD003 small subunit ribosomal RNA gene, partial sequence; internal transcri	Lasiodiplodia the	961	961	97%	0.0	99.25%	674	MW138050.1
☐	Lasiodiplodia theobromae isolate MGSD002 small subunit ribosomal RNA gene, partial sequence; internal transcri	Lasiodiplodia the	961	961	97%	0.0	99.25%	673	MW138049.1
☐	Lasiodiplodia theobromae isolate MGSD001 small subunit ribosomal RNA gene, partial sequence; internal transcri	Lasiodiplodia the	961	961	97%	0.0	99.25%	673	MW138048.1
☐	Fungal sp. ARIZ B370 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal R	fungal sp. ARIZ B	961	961	97%	0.0	99.25%	568	FJ612958.1
☐	Fungal sp. ARIZ B648 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal R	fungal sp. ARIZ B	961	961	97%	0.0	99.25%	569	FJ612649.1
☐	Botryosphaeria rhodina strain Br1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S r	Lasiodiplodia the	961	961	97%	0.0	99.25%	533	EF150929.1

Fig 6: The Sequence Alignments of SP-1 Isolate Sequence with NCBI Database Sequences

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc Len	Accession
☑	Pichia kudriavzevii isolate L-012 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1	Pichia kudriavzevii	619	619	54%	8e-173	99.70%	521	MT599316.1
☐	Pichia kudriavzevii isolate BAL sample internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene a	Pichia kudriavzevii	619	619	54%	8e-173	99.70%	523	MT539201.1
☐	Pichia kudriavzevii isolate BAL sample internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene a	Pichia kudriavzevii	619	619	54%	8e-173	99.70%	521	MT539200.1
☐	Pichia kudriavzevii isolate BAL sample internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene a	Pichia kudriavzevii	619	619	54%	8e-173	99.70%	520	MT539198.1
☐	Pichia kudriavzevii isolate BAL sample internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene a	Pichia kudriavzevii	619	619	54%	8e-173	99.70%	521	MT539197.1
☐	Pichia kudriavzevii strain SD1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 a	Pichia kudriavzevii	619	619	54%	8e-173	99.70%	531	MT221645.1
☐	Pichia kudriavzevii isolate 48 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and interna	Pichia kudriavzevii	619	619	54%	8e-173	99.70%	426	MT443939.1
☐	Pichia kudriavzevii isolate OM10 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inte	Pichia kudriavzevii	619	619	54%	8e-173	99.70%	471	MT136539.1
☐	Pichia kudriavzevii strain TTG-S1-1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i	Pichia kudriavzevii	619	619	54%	8e-173	99.70%	480	MT321265.1
☐	Pichia kudriavzevii strain TTG-S1-2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i	Pichia kudriavzevii	619	619	54%	8e-173	99.70%	516	MT321167.1
☐	Pichia kudriavzevii isolate 4Y149 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer	Pichia kudriavzevii	619	619	54%	8e-173	99.70%	473	MT239534.1
☐	Pichia kudriavzevii isolate 3Y3 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 a	Pichia kudriavzevii	619	619	54%	8e-173	99.70%	486	MT234092.1
☐	Pichia kudriavzevii isolate 1Y12 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inter	Pichia kudriavzevii	619	619	54%	8e-173	99.70%	468	MT233404.1
☐	Pichia kudriavzevii strain DS8-1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inter	Pichia kudriavzevii	619	619	54%	8e-173	99.70%	424	MT157508.1
☐	Pichia kudriavzevii isolate CUMB VP - KGA-03 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA	Pichia kudriavzevii	619	619	54%	8e-173	99.70%	479	MT138866.1

Fig 7: The Sequence Alignments of SP-2 Isolate Sequence with NCBI Database Sequences

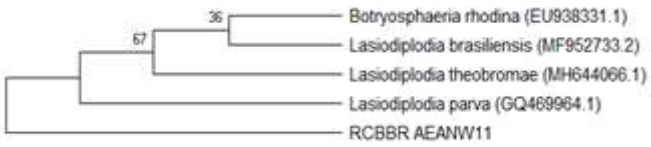

Fig 8: Phylogenetic tree of sample 11 (Sample 11; Strain number – RCBBR_AEANW11; *Lasiodiplodia theobromae; Assertion no:* MW898113)

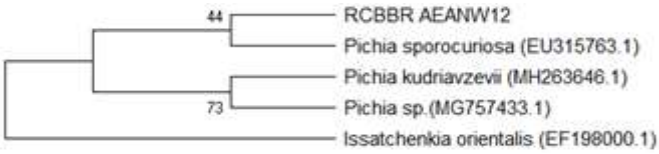

Fig 9: Phylogenetic tree of sample 12 (Sample 12; Strain number – RCBBR_AEANW12; *Pichia kudriavzevii;* Assertion no:MW898114)

Despite the popularity of sweet potato, several fungal pathogens attack them during growing season, harvesting, packing, transport, storage, marketing, or after purchasing by consumer Abebe (2020); Etefa *et al.* (2022) and the use of traditional method of identification, is not as fast and adequate as the molecular method that according to Liu *et al.* (2020), provides high specificity to distinguish between the species and subspecies of fungi. Molecular identification procedures based on total fungal DNA extraction provide a unique barcode for characterization of diverse fungal isolates up to a species level (Landeweert *et al.,* 2003). This study revealed the identity of the unknown fungal cultures to be *Lasiodiplodia theobromae* and *Pichia kudriavzevii*. A study conducted by De Silva and Dania and Thomas (2019), also indicated that *Lasiodiplodia theobromae* is associated with rot disease of sweet potato. The pathogen has been reported on sweet potato causing java black rot, which can be one of the most destructive post-harvest diseases of sweet potato, and also can infect sprouts produced in plant beds, but did not attack the vines in the field (Clark *et al.,* 2013). Agarose gel was successful in showing the different fragment size of the PCR products of the two organisms (*Lasiodiplodia theobromae* and *Pichia kudriavzevii*). The internal Transcribed Spacer (ITS region has been reported for its importance in biodiversity study and has been reported as more reliable Zhang *et al.* (1999) in distinguishing between fungal species.

Conclusions: Based on this study, *Lasiodiplodia theobromae and Pichia kudriavzevii* cause tubers to rot, reducing the postharvest quality and selling price of the tubers despite its economic and nutritive values. This study will promote knowledge of the fungal species associated with sweet potato and also enhance disease control which will increase the production yield. Infestation by weevils of *C. formicarius* contributes to major problems during post-harvest storage of sweet potato tuber. Following infestation, fungal infection takes place that make the tuber prone to many diseases. Based on this study, *F. oxysporum* was highly pathogenic to healthy sweet potato tuber compared to *Penicillium sp*. and *Aspergillus spp*. Rotten tubers due to fungal infection will reduce the post-harvest quality thus reduce the selling price of the marketable produce. Therefore, study on lifecycle of *C. formicarius* can help in control strategy of this population. Identification of fungal pathogens is important to avoid epidemic diseases which cause big loss of economic return. Farmers should apply integrated pest management control to ensure continuous production of sweet potato for food supply and economic growth development.

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