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IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF KOCHO SPOILAGE FUNGI COLLECTED FROM DIFFERENT AREA OF GURAGE ZONE, ETHIOPIA

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ABSTRACT

Enset (*Ensete ventricosum*) is a main food crop in Ethiopia on which about 20% population depends. Understanding the fermentation process is critical for optimum enset use because '*kocho'*, an important enset product, is consumed as a key energy source and serves as a staple and co-staple diet for many people. Fermented kocho is frequently stored in pits lined with enset leaves. During fermentation process, a piece of kocho can be withdrawn for consumption or sale at any moment, so it is always susceptible to contamination by a variety of microorganisms. The rotting microbes of this fermented food are little understood. The present study was aimed to identify spoilage fungi of ten kocho samples collected from various farm and market sites in Gurage zone of Ethiopia. The samples, packed in sterile Falcon tubes covered with ice, were transported to Wolkite University's Biotechnology Molecular Laboratory for morpho-biochemical characterization, as well as DNA isolation and PCR amplification of internal transcribed spacer region of genes at MRCET's Molecular Advanced Laboratory, Addis Ababa (Ethiopia). PCR results were sequenced, and each fungal isolate identified using an automated DNA sequencer. Twenty-two fungal species were identified. Molecular sequence analysis revealed that of the 22 fungal isolates identified 4 belonged to genus *Alternaria*, three to *Penicillium*, two each to *Gliotricum*, *Schizophyllum* and *Aspergillus*, and one each to *Galactomyces* and *Agaricus*. Further research on identified fungi is needed to determine the most damaging and deteriorating strains in order to properly control and manage kocho spoilage fungi.

Keywords: DNA sequencing, ITS DNA region gene, kocho spoilage organisms

INTRODUCTION

Enset (*Ensete ventricosum*) is a staple food crop used by about 20% of Ethiopian population (Genet *et al*., 2018) Although enset has a vegetative growth behaviour similar to banana (*Musa* spp.) plants, it is not grown for fruit purpose, which are largely huge and very hard seeds (Dalbato *et al*., 2016). The enset plant's corm and pseudo stem are traditionally processed into kocho, a key food product.

In the country's enset-growing regions, the method is an age-old technique that is still performed without scientific improvement. To explore the potential of this underutilized crop, efforts have been made to better understand the local production techniques and improve the conservation and utilization of enset genetic resources. The enset plant's corm and pseudo stem are traditionally processed into kocho, a key food product. The pseudo stems and tubers are removed and pulverized into a mush that is allowed to split into two portions, preferably from mature plants.

After enset fermentation is finished, a part of pit is removed and liquid squeezed out, yielding a wet fibrous kocho. Kocho, bulla, and amicho are the most common foods obtained from enset. Kocho is a fermented food made from enset crop. However, kocho has a number of restrictions that affect its quality including differences in variety selection, fermentation length and processing method. If anaerobic conditions are maintained, the fermenting product can be held in the pit for several years without spoiling (Gashe, 1987). The starter and the gradual presence of microbial flora during fermentation process determine the quality of a fermented product. During fermenting phase, however, parts of kocho can be withdrawn from the pit for consumption or sale. When kocho is withdrawn from the pit it is often susceptible to contamination from an array of microbes. Traditional identification methods of microbe are time-consuming and frequently unable to differentiate at species level that limit their application in characterization.

Fungi play vital role in nutrient cycling, plant health, organic decomposition, environmental protection, and obligate mutualistic symbiotic partnerships with plants, algae, and cyanobacteria (Vuyst *et al*., 2017). As a result, in many scientific disciplines, like taxonomy and biodiversity, precise molecular characterization of fungi at species level is crucial (Araujo *et al*., 1995). Since 1990, the molecular data has been used to identify fungal taxa based on the variability in DNA sequences of closely related species (Bruns *et al*., 1991). DNA barcoding is a valuable tool for reducing uncertainty in species identification and precisely determining their boundaries. It is a quick, accurate, and uniform procedure that has a lot of potential for species level identification. The principal DNA barcode marker for fungus is the ribosomal internal transcribed spacer (Schoch *et al*., 2014). In fungi, barcodes are typically used to identify a single DNA region that is aligned to database sequences in order to find the most comparable ones, whereas phylogenetic analyses are used to construct clades that represent species. Although the methodology and concepts behind these two tactics differ, the distinction between the two approaches in actual mycological species identification is not evident. As a result, we studied the value of regularly used genes as barcodes in the strict sense, as well as for producing highly resolved species clades, in this work.

To limit the danger of contamination and illness from kocho handling and consumption, it is important to identify these spoilage microorganisms, particularly the one contaminating the kocho products and those pathogenic to people. For the fungal studies based on morphological traits, traditional cultivation and microscopic identification have frequently been used (Al-Hindi *et al*., 2011). Molecular identification of fungus isolates up to species and strain levels is a powerful and reliable method. Therefore, the objective of this study was to identify and characterize the undesirable kocho spoilage fungal species that need to be controlled during fermentation.

MATERIALS AND METHODS

Ten spoilage kocho samples (five from farm and five from local market) were collected from different Wereda of Gurage zone, Fereze farm (FSPS1), Terehogn farm (FSPS2)), Endeber market (FSPS3), Wolkite farm (FSPS4), Wolkite market (FSPS5), Agena farm (FSPS6), Agena market (FSPS7), Gubrea market (FSPS8), Hole farm (FSPS9), Hole market (FSPS10). The samples were collected in sterile Falcon tubes and transported to Wolkite University's Biotechnology Molecular Laboratory. The Wolkite University Biotechnology Lab performed morphological and biochemical characterization. DNA isolation and PCR amplification (ITS region of gene) was done at MRCET

Molecular Advanced Laboratory, Addis Ababa (Ethiopia). The sequencing of PCR products and identification of fungal isolates were performed by automated DNA sequencer MRC-Holland, Amsterdam, Netherlands.

Isolation of fungi

Fermented spoilage kocho (5 g) were suspended in 5 mL sterile double-distilled water. To inhibit bacterial growth, 0.1 mL aliquots of serially diluted samples were placed in culture plates containing sterile Sabouraud dextrose agar (SDA) containing chloramphenicol @ 0.05 mg mL^{-1} (Holt *et al.*, 1994). The samples were uniformly distributed across the medium's surfaces by using a sterile glass rod. The Petri-plates were incubated in inverted position at 28°C for 5 days. The fungal colonies were observed, and pure cultures were maintained (Gaddeyya *et al.*, 2012). Serial dilution method was used to isolate kocho spoilage fungi. Serial dilutions of 10^{-1} to 10^{-5} was done. The rotting kocho (5 g) was suspended in 50 mL sterile distilled water and then 1 mL of each dilution was put on SDA containing 1% chloramphenicol and incubated in dark at 28°C (Gaddeyya *et al*., 2012).

Macro- and microscopic examination of isolated fungi

The fungal characteristics such as colony shape, septa presence, spore share, and colony coloration were observed after lactophenol cotton blue staining of the isolates (Gaddeyya *et al*., 2012). The stained slides were examined using a compound microscope (AmScope 40X-2000X biological compound LED microscope) equipped with a digital camera.

Fungal DNA extraction

Qiagen's DNA extraction kit was used to extract genomic DNA from fungal isolates (Janda and Abbott, 2007). The genomic DNA were extracted using DNeasy cell culture mini kit from a one-week-old SDB culture. For this, 1 mL overnight suspension culture (5 x 10^6) taken from each sample was centrifuged at 1640 rpm for 5 min, re-suspended the pellets in 200 µL phosphate buffered saline (PSB 1X, pH 7.4**)** and vortexed. Then 20 µL proteinase K was added to each centrifuged sample. The pellets were mixed thoroughly by vortex with 200 µL buffer AL (lysis buffer without ethanol) supplied by Qiagen kit and incubated at 56°C for 2 h. Then 200 µL ethanol (97%) was added and contents thoroughly stirred to get a homogeneous solution. The upper aqueous phase having DNA from the above step was pipetted into fresh DNeasy mini spin column, placed in 2 mL collection tube and centrifuged at 8000 rpm for 1 min. The supernatant was thrown away. The DNeasy mini spin column was placed in a new 2 mL collection tube and 500 µL AW1 buffer (wash buffer1) added to samples to wash DNA bound to the silica columns. After centrifugation at 8000 rpm the supernatant was discarded; the pellet washed by 500 µL buffer AW2 (wash buffer 2) by placing into a DNeasy mini-spin column in a new 2 mL collection tube and centrifuged for 3 min at 14,000 rpm to dry the DNeasy membrane. This centrifugation ensured that no residual ethanol was carried over during subsequent elution. Since flow-through contained either buffer AL or buffer AW1, it was incompatible with bleach. Following centrifugation, the DNeasy mini spin column was carefully removed. It was ensured that it did not come in contact with flow-through, as this would result in ethanol carryover. To remove ethanol, the collection tube was emptied and centrifuged at 14,000 rpm for 1 min. A clean 1.5 mL micro-centrifuge tube was used to hold DNeasy mini spin column. To elute, 200 µL AE buffer was pipetted directly onto DNeasy membrane and incubated at room temperature for 1 min before centrifuging at 8000 rpm for 1 min. The final DNA concentration in elute was increased by 100:l instead of 200:l, but the overall DNA yield was decreased. To avoid dilution of first-elute, the elution procedure was repeated many times to get maximal DNA yield using a new micro-centrifuge tube. Nano-drop technology (García-Alegría *et al.,* 2020) was used to determine the purity and concentration of isolated fungal DNA (Nano-drop 2000 user manual).

PCR amplification

PCR amplification was done using universal primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') to amplify ribosomal internal transcribed spacer (ITS) of fungi. The PCR products were purified using QIA quick PCR purification kit (Bao *et al*., 2012). The PCR reaction mixtures (50 µL) used comprised of 5 µL PCR buffer, 2 µL dNTPs, 1 µL each primer, 1 µL Taq DNA polymerase (Fermentas, St. Leon- Rot, Germany), 5 µL template DNA and reverse osmosis purified water. The 15 µL was used from each mixture for PCR (Tilahun *et al.*, 2018). The PCR reaction was set up as follows: initial denaturation at 95°C for 60 sec, 35 cycles of denaturation at 94°C for 60 sec, primer annealing at 62°C for fungal DNA for 30 sec, primer extension at 72°C for 60 sec, and final extension at 72°C for 60 sec. The PCR products were separated by gel electrophoresis using 1% agarose gel and 1 µL loading dye with 5 µL PCR products and stained with ethidium bromide for gel documentation and visualization (Tilahun *et al.*, 2018).

Nucleotide sequencing and phylogenetic analyses

The ribosomal internal transcribed spacer (ITS) of fungal DNA PCR product of each isolate was sequenced by automated DNA sequencer (ABI model 377; Applied Biosystems), MRC-Holland, Amsterdam (Tilahun *et al.*, 2018). Both fungal DNA isolates and raw DNA sequences were edited with FinchTV package to obtain consensus sequences. Using BLAST search, the sequences were compared with NCBI DNA database (Tamura and Nei, 1993). The sequences were aligned with Clustalx 2.1 and a phylogenetic tree was created with MEGA X (Kumar *et al*., 2018).

RESULTS AND DISCUSSION

Morpho-biochemical characterization and identification of spoilage kocho

The Table 1 and 2 show the culturo-morphological and some biochemical characteristics and tentative identification of kocho spoilage fungi, isolated from various spoiled fermented samples (brown discoloration, soft slimy, black mark, dark, greyish discoloration, moldy, and blue green).

Table 1: Characterization of fungal isolates by colony morphology and microscopic analysis

Fungal isolate	Cultural and morphological characteristics	Tentative identification
FSPS1	Colonies of large fluffy white milky colonies that turn black as the culture ages. Non-septate hyphal with erect sporangiophore and black pear-shaped rhizoids joined by stolon.	*Alternaria* spp *Aspergillus* spp
FSPS2	Large fluffy white colonies, covered the whole surface of cream. Sporangium emerges from the hyphal without the use of a stolon or rhizoids columella.	*Alternaria* spp
FSPS3	Lemon and yellow flowers grow quickly from woolly to cottony. Multicellular macro conciliates with a sickle form.	*Alternaria* spp
FSPS4	Colonies of large fluffy white milky colonies that turn black as the culture ages. Dark pear-shaped non-septate hyphal with upright sporangiophore joined by stolon and rhizoids.	*Alternaria* spp
FSPS5	Wooly to cottony lemon and yellow in appearance. Multicellular macro conciliates with a sickle form.	*Penicillium* spp *Aspergillus* spp
FSPS6	Large fluffy white colonies cover virtually the entire surface. Non-septate branched hyphal create brownish black cardia in chains by enlarging at the apex to form conidiophore.	*Geotrichum* spp *Galactomyces* spp
FSPS7	Colony colors that are very common (black and white). Conidia are borne in 360^{o} configurations that covered the upper 2/3rd of conidiophores.	*Pencillium* spp
FSPS8	Large fluffy white milky colonies that turned black as the culture aged. The sporangium emerged from hypha without the use of stolon or rhizoids columella	*Pencillium* spp
FSPS10	Almost the entire surface is covered in cream white fluffy white colonies. Sporangium emerges from the hyphal without the use of a stolon or rhizoids columella.	*Anthostomella* spp.

Table 2: Spore formation, motility and biochemical characters of fungi isolated from spoilage Kocho

Isolates	Carbohydrate assimilation	Spore formation	Amino acid assimilation	Motility	Hydrolysis of starch	Lipase activities
FSPS1	+	_	+	+	+	_
FSPS2	+	+	_	+	_	+
FSPS3	+	_	+	_	_	+
FSPS4	+	+	+	_	_	_
FSPS5	+	_	+	+	_	+
FSPS6	+	_	+	+	+	+
FSPS7	+	_	+	+	_	+
FSPS8	+	+	+	+	_	+
FSPS9	+	+	+	+	_	_
FSPS10	+	+	+	+	+	+

+ = Positive; - = Negative

Concentration and purity evaluation of DNA extraction of fungal isolates

Prior to the purification studies, DNA quantification (nucleic acid quantification) is widely used to measure the average concentration of DNA in a sample. While calculating the amount of DNA in a sample, sample purity is also considered. Nano-drop (username Nanodrop2000) measurement was used in this experiment to assess the samples, process, and throughput, which resulted in accurate DNA quantification and saves time and money by preventing downstream experimental failures. DNA was extracted from overnight grown culture and dissolved in TE buffer (100 mM tris hydrochloride, 1 mM EDTA, pH8. At 260 and 280 nm, the concentration of DNA was measured using Thermo Scientific™ Nano Drop™ 2000/2000c spectrophotometer. DNA purity was determined by scanning the absorbance of DNA samples between 200 and 400 nm and monitoring the absorbance ratios at 260/280 nm. On Nano drop machine, 1µL isolated DNA from each sample extract was dropped. To proceed to the next phase in sequencing process, the concentration measurement must be more than nine and the purity must be between 1.5 and 2.03 ng µL^{-1}.

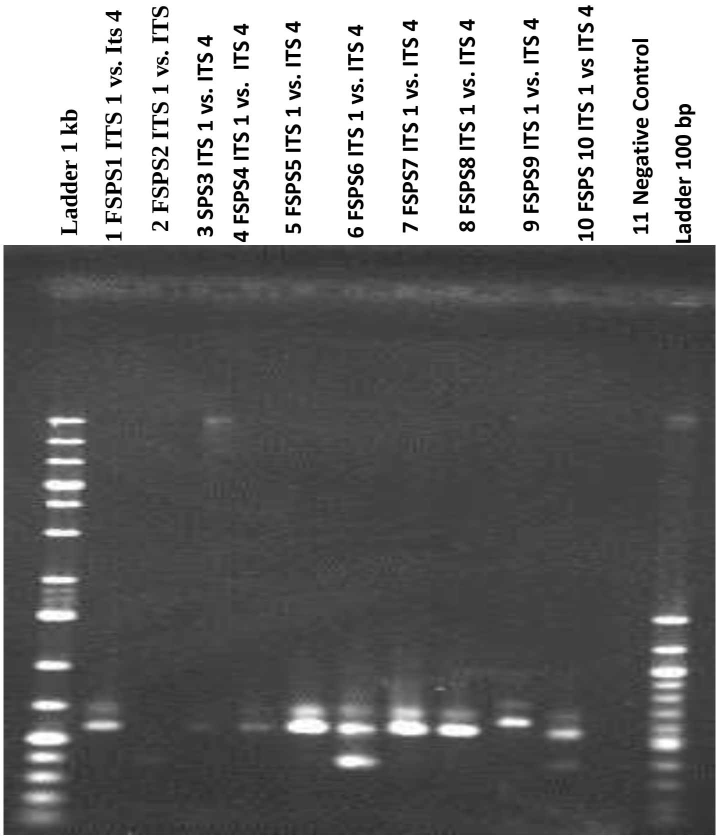

Fig. 1: Amplification of ribosomal internal transcribed spacer (ITS) of fungal DNA gene isolates

PCR analysis of isolates

The PCR amplification products of 10 fungal isolates of ITS DNA region is shown on Fig. 1. After all the raw ITS region gene sequences of 10 fungal isolates were edited using the FinchTV package, consensus sequences (edited sequence) were blasted in GenBank of NCBI. The samples which showed 89-99.81% homology were identified, except FSPS10 which showed 81.01% homology (Table 3). Of the fungal isolates identified 9 belonging to the genera *Alternaria* (*A. aletrnata, , A. tenuissima, A. tamaricis, A.alstroemeriae,A. solani* and *Alternaria* sp. S20*)* which were isolated from WKUE03, WKUF03, WKUG03 and WKUH03, 5 to genra *Pencillium* (*P. glabrum*, *P. spinulosum*, *P. thomii*, *P. expansum* and *Penicillium* sp.,) which were isolated from WKUA04, WKUC04 and WKUD04. The 2 fungi identified belongs to *Geotrichum* genera (*Geotrichum* sp. strain DTO and *G. candidum* isolate ObaCOsho_16) which were isolated from WKUB04. *Schizophyllum commune* and *S. radiatum* fungi identified were also isolated from WKUE04. In addition to this, *Aspergillus proliferans* and *A. niger,* from WKUE03 and WKUA04, respectively, were identified. From WKUB04 and WKUE04 *Galactomyces* sp. and *Agaricus* sp. were identified. WKUA04, WKUC04 and WKUD04 were identified as *Pencillium* genera with 98.40, 99.81 and 99.81% homology, respectively. *Geotrichum* sp. strain DTO and *Geotrichum candidum* which were identified from WKUB04 showed 93.95% homology similarity. *Schizophyllum commune* and *S. radiatum* fungi also isolated from WKUE04 depicted 98.40% homology similarity. *Galactomyces* spp. (WKUB04) and *Agaricus* spp. (WKUE04) showed 93.95 and 99.06% homology similarity, respectively. *Alternaria alternata* was prevalent among all isolates. Molds such as *Alternaria* spp., *Botrytis* spp., and *Penicillium* spp. can grow on a wide range of starchy foods and fruits, spoiling them (Tournas and Stack, 2001).

Table 3: Identification of fungal isolate from spoilage kocho as revealed by ITS region gene sequence BLAST search

S. No.	Identified organisms/ Species	NCBI Accession No.	%Identity	Bp	Sample code	Bacterial isolated code
1.	*Alternaria alternata* isolate Aa41L-20GAa	MW090865.1	99.22	1018	FSPS-1	WKUE03
	Alternaria tamaricis strain A028	MW449616.1	99.22			
	Alternaria tenuissima isolate 33L1	MZ151368.1	99.22			
	Aspergillus proliferans isolate DR9	MT106900.1	99.22			
2.	*Alternaria alternata* strain EX2019-M2	MT495455.1	98.42	1045	FSPS-2	WKUF03
	Alternaria alstroemeriae isolate 1-92	OK011827.1	98.42			
	Alternaria tenuissima strain 090716NER21U5	MZ374047.1	98.42			
3.	*Alternaria* sp. S20	MG190024.1	89.56	1045	FSPS-3	WKUG03
	Alternaria alternata isolate N14R2	MT134992.1	89.56			
	Alternaria solani clone 185	MN871616.1	89.56			
4.	*Alternaria alternata* isolate SWFU 000063	MK850391.1	98.40	998	FSPS-4	WKUH03
	Alternaria sp. isolate PYCC 8691	OK094898.1	98.40			
	Alternaria alstroemeriae isolate 1-4	OK011825.1	98.40			
5.	*Penicillium glabrum* isolate VP41/3E.2	MN956892.1	98.40	998	FSPS-5	WKUA04
	Penicillium sp. isolate TW_JN17_93	MN634686.1	98.40			
	Penicillium spinulosum strain 2S.115	KY439036.1	98.40			
	Aspergillus niger isolate OLTO579	MW423981.1	98.39			
6.	*Geotrichum* sp. strain DTO 400-C2	MT316348.1	93.95	928	FSPS-6	WKUB04
	Geotrichum candidum isolate ObaCOsho_16	MN861070.1	93.95			
	Galactomyces sp. isolate TSE04	MT481758.1	93.95			
7.	*Penicillium* sp. isolate TW_JN17_93	MN634686.1	99.81	1058	FSPS-7	WKUC04
	Penicillium thomii strain CBS 130194	MH865743.1	99.81			
	Penicillium glabrum strain MS/2/5	JF327812.1	99.81			
8.	*Penicillium* sp. isolate TW_JN17_93	MN634686.1	99.81	1058	FSPS-8	WKUD04
	Penicillium glabrum strain S5-M-2-1	KP216902.1	99.81			
	Penicillium expansum isolate R11	KX243327.1	99.81			
9.	*Schizophyllum commune* strain BL1	MT466518.1	99.06	1059	FSPS-9	WKUE04
	Schizophyllum radiatum isolate DO90	KP050645.1	99.06			
	Agaricus sp. CtFE 16	KM388608.1	99.06			
10	Uncultured fungus clone SW016_F01	KP889701.1	81..01	928	FSPS-10	WKUF04
	Anthostomella rubicola isolate IT1630	KP297407.1	84.40			
	Purpureocillium lilacinum culture MUT	MK053582.1	81.90			

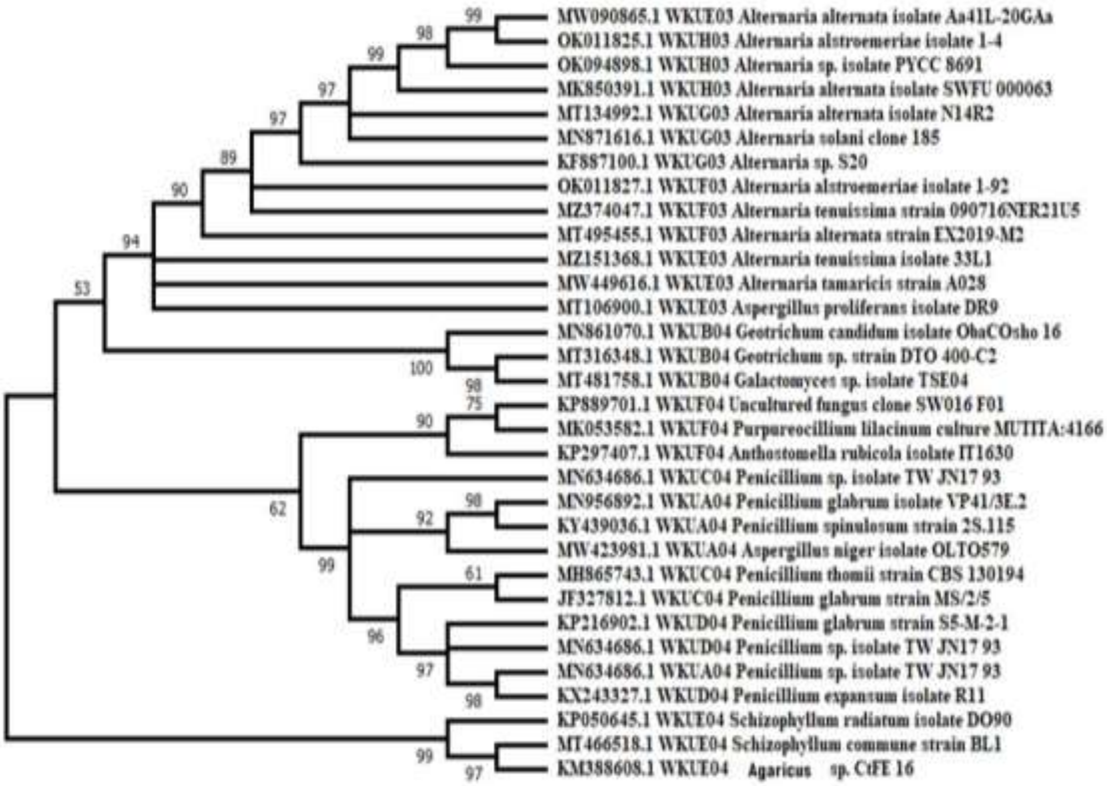

Fig. 2: Phylogenetic analysis based on ITS region gene sequences of fungal strains isolated from spoilage kocho; NCBI search accession number, followed the isolate code as WKU followed by capital letter and numbers and bacterial strains using the Maximum Likelihood method

Aspergillus spp. and *Penicillium* spp. are the most common genera that impact many types of foods, including fruits (Abdullah *et al*., 2016). In many parts of the world, *A. alternata* can affect a wide range of agricultural crops (Bashar *et al*., 2012: Harteveld *et al*., 2013). Gashe, 1987, also mentioned the fungus species linked to kocho rotting. Genet *et al*. (2018) observed *Galactomyces geotrichum* in kocho which agrees with this investigation.

Evolutionary analysis by Maximum Likelihood method

To infer the evolutionary history, the Tamura and Nei, 1993, model and the Maximum Likelihood approach were used. The tree with the highest log likelihood is displayed (-1958.26). The proportion of trees in which the related taxa clustered together is shown next to the branches. To generate the initial tree(s) for the heuristic search, the Maximum Parsimony approach was used. This study included 32 nucleotide sequences. The final dataset had a total of 567 places. MEGA X was used for evolutionary analysis (Kumar *et al*., 2018).

The results of this study revealed that *Alternaria alternata* was the most frequently isolated fungus species across all the four sample collection sites (Fereze farm, FSPS1), Terehogn farm (FSPS2), Endeber Market (FSPS3), and Wolkite farm (FSPS4). *Penicillium glabrum* and *Pencillium* sp., on the other hand, were isolated from Wolkite Market (FSPS5), Wolkite Farm (FSPS4), and Gubre Market (FSPS8. Fatima *et al.*, 2012, found *Aspergillus niger* and *Aspergillus flavus* in vegetables and fruits, spoiling the food; this finding is consistent with our current investigation. According to (Al-Hindi *et al*., 2011), *Aspergillus* spp. were the most frequent spoilage fungi among all examined spoilage fruits. This finding was also consistent with the findings of (Adebayo-Tayo *et al*., 2012), who reported that *Aspergillus* and *Rhizopus* species were the most common, though their frequency of occurrence was slightly lower than that obtained in this study. *Alternaria alternata* was the most widely distributed spoilage fungi. These findings back up the findings of (Barry *et al.*, 2003), who discovered that fungal isolates recovered from *Alternaria alternata* lesions matched 96 percent of the fungi responsible for *Alternaria* rot.

Conclusion: The decorticated leaf sheaths and grated corm make up the bulk of the fermented delicacy known as kocho. However, kocho has a number of limitations that affect its qualities due to the differences in variety selection, fermentation length, and processing method. When fermenting kocho is withdrawn from the pit for mixing or usage, it is susceptible to fungi contamination. Based on the morphological characteristics, ten different varieties of spoiled kocho samples (showing brown discoloration, soft slimy, black spot, dark, greyish discoloration, moldy and bluish green) were collected from different sites in Gurage zone. Based on colony morphology and macroscopic inspection, the isolated fungi were identified as *Penicillium* spp., *Aspergillus* spp., *Alternaria* spp., and *Geotrichum* spp. *Penicillium* spp. and *Alternaria* spp. were the most common genera in all the samples examined. The sequencing of ITS DNA region of fungal isolate revealed that molecular identification of fungi is the most accurate method, especially for recognized organisms. As a result, more research should be done to identify the most common and potentially spoilage-causing fungi from the ones found in this study. Furthermore, chemical, biological, or physical treatment should be used to prevent koch's product from deteriorating.

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