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Molecular Characterization and Phylogenetic Analysis of Some Agaricomycetes (Mushroom) Fungi from Kogi State, Central Nigeria

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Abstract

The diversity and distribution of mushroom-forming fungi of the three senatorial zones of Kogi state was studied. A multi-stage sampling technique was used to assess twelve randomly selected plots mapped out across the study area during the rainy and early dry seasons of 2014, 2015 and 2016. Identification of selected mushrooms was done through morphological and molecular characterization. Fungi description was initially based on morphometric characters. For the molecular characterization, rDNA was used in a micro satellite PCR technique using ITS1 and ITS4. Forty wild mushrooms were amplified using genomic DNA isolated from their mycelial culture. The amplified products revealed 450 to 650 base pairs. The molecular data obtained from Randomly Amplified Polymorphic DNA (RAPD) using microsatellite DNA amplification techniques characterized a total forty wild mushrooms species most of which had 99% similarity with other mushrooms when subjected to blasting.

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Mushroom sample 14S (*Trametes* sp) matched 100% *Trametes polyzona* ascension number JN848329.1 while 2S, S8, 22S, 20S, S3 and S6 mushroom samples have 99% percentage similarities with *Daldinia eschscholtzii* (KP012909.1), *Leiotrametes flavida* (KC589131.1), *Lentinus squarrosulus* (JQ428823.1 1), *Lentinus sp*. (JQ428823.1 2), *Auricularia polytricha* (FJ617296.1) and *Tricholoma matsutake* (MF037408.1) respectively. Furthermore, the result of the molecular analysis and phylogenetic relationship revealed that two (2) new species of mushrooms, never reported were been identified.

Keywords: RAPD; *Lentinus squarrosulus;* ITS1; Phylogenetic.

1. Introduction

The order Agaricales is considered one of the most important genus of edible mushrooms currently known for the great variety in the morphology of individuals. According to the author in [7], modern identification of mushrooms is quickly becoming molecular; the standard methods for identification are still used by most and have developed into a fine art dating back to medieval times and the Victorian era, combined with microscopic examination. The random amplified polymorphic DNA (RAPD) is a convenient method to detect genetic diversity [1, 2, 3,]. This method has been particularly successful when applied to check the strains of mushrooms with different origins [4].

In general, identification to genus can often be accomplished in the field using a local mushroom guide. Identification to species, however, requires more effort; one must remember that a mushroom develops from a button stage into a mature structure, and only the latter can provide certain characteristics needed for the identification of the species. However, over-mature specimens lose features and cease producing spores. Indigenous mushrooms have been named based on size of basidiocarp, structure, substrate, season they appear, or any other unique feature [5, 8]. However, scientific classification, using coloured photographs, written descriptions of the macro-morphology, microscopic examination of spore prints, tissues, and spore forming structures have been reported to be more globally acceptable [8, 9].

Molecular characterization is necessary for identifying and quantifying the fungi involved in mycorrhizal associations, since many have not yet been successfully cultured. Molecular tools will also be needed since it has been shown that there is only a poor correspondence between fungal diversity observed above ground (as fruiting bodies) and the diversity actually present on plants roots [10].

2. Methods

2.1. Sampling of macrofungi

Marcrofungi were inventoried in the rainy seasons and early dry seasons of 2014, 2015 and 2016. This was done from June to December each year. This included collection of every encountered macrofungi within each transects. These were photographed in-situ and tagged digitally with the canon camera. A few sporocarps were carefully dug out and their fertile sides (gill, tubes and spines) were exposed and macrofungi were then collected in separate waxed paper bags in order to avoid spore contamination among the different specimens. The

collection bag was correctly labeled.

Spore prints were taken and fresh sporocarps preserved in 2% formalin in glass bottles. Some were dried / preserved in air-tight containers with silica gel, others oven-dried or sun-dried. Voucher specimens of selected species were collated and sent for expert verification in North Carolina and West Wales, United Kingdom.

2.2. Production of mycelia

A total of fifty three picked sporocarp were tissue cultured in potato dextrose agar (PDA) for the purpose for mycelia production. Picked sporocarps were rinsed several times in distilled water. They were then soaked in 2% sodium hypochlorite for 5mins and rinsed several times in three petri-dishes of distilled water after which 2mm x 2mm tissue were taken from the point of attachment of the stipe to the pileus and from the gills after tearing the sporocarps into two longitudinally. These were placed on PDA in sterilized boiling tubes for transportation to the laboratory in cool box.

2.3. Preparation of fungal material for dna extraction

2.3.1. Dna extraction

The DNA of pure cultures of fifty three mushrooms samples was extracted using the ZR Fungal/Bacterial DNA MiniPrepTM (Zymo Research Corp.) according to the manufacturer's instructions: About 100mg of fungal cells/mycelia were scrapped directly from the culture plates and re-suspended in 200µl of Phosphate Buffered Saline (PBS) and the suspension was added to a ZR bashing bead lysis tube (provided in the kit). Exactly 750µl lysis solution was added to the sample in the tube and processed with a votex mixer for at least 3 minutes. The bashing bead tube was then centrifuged at 10000 rpm for 1 minute. Once removed, 400µl of the supernatant was transferred to a zymo-spin IV spin filter in a collection tube and centrifuged at 7000rpm for 1 minute. Aliquot of 1200µl of fungal DNA binding buffer was then added to the filtrate in a collection tube from the previous step. Half of this mixture was then transferred to a zymo-spin IIC column fitted in a collection tube and centrifuged for 1 minute at 10000 rpm. The flow through was discarded and the step repeated for the remaining half of the mixture. Following this, 200µl DNA pre-wash buffer was added to each column in a new collection tube and centrifuged at 10000 rpm for 1 minute. Then 500µl of fungal DNA wash buffer was added to the column and centrifuged at 10000 rpm for 1 minute. Finally, the column was transferred to a clean 1.5ml micro centrifuge tube to which 100µl DNA elution buffer was added directly to the column matrix and centrifuged at 10000 rpm for 30 seconds to elute the DNA. It was then stored at -20°C until used for amplification.

2.4. DNA Amplification

The targeted region for amplification in the sample DNA was the internal transcribed region of ribosomal DNA (ITS of rDNA). This region consisting of 600-800 base pairs was amplified using the primers ITS 1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). The amplification was done using the protocol described by Xu and his colleagues (2002). The optimized reagent mix was made up of 37.0µl of nuclease free water, 5.0µl 10x PCR buffer (green), N 0.5µl dNTP (10mM), 1.0µl Magnesium chloride

(25mM), 1.0µl of each of the primers (working concentrations of 10µM) and 0.5µl of Taq polymerase (Dreamtaq). To this mix 4.0µl of DNA was added bringing the total reaction volume to 50.0µl. The amplification program used had an initial denaturing at 95°C for 3 minutes followed by 35 cycles of denaturing at 95°C for 78 seconds, annealing at 48°C for 22 seconds and extension at 72°C for 70 seconds, and a final extension at 72°C for 10 minutes. The amplification was done on a GeneAmp 9700 model machine (Applied Biosystems). The expected sizes of the resulting amplicons range between 500bp and 700bp.

2.5. Agarose Gel Electrophoresis

The polymerase chain reaction (PCR) products were electrophoresed at 100 volts for 40mins after staining with 5µl of 1000bp molecular weight ladder. This was loaded into wells of a 1.5% agarose gel stained with 5µl Ethidium bromide. Examination of the gel indicated the purity and length of the amplified DNA. The gel was viewed using a BioRad gel documentation system (Machine). Resulting amplicons were sent to Inqaba Biotec West Africa Laboratory, South Africa for sequencing. Resulting amplicons were purified with Care Quick PCR purification kits. The amplicons were cleaned using ExoSAP. The Exo/SAP master mix was prepared by adding 20U/ul Exonuclease I (NEB M0293) (50.0 µl) and 1U/ul Shrimp Alkaline Phosphatase (NEB M0371) (200.0 µl) to a 0.6ml micro-centrifuge tube. 10.0µl PCR mixture and 2.5µl Exo/SAP Mix reaction mixture were prepared. The prepared reaction mixture were properly mixed and incubated at 37°C for 30 min. The reaction was stopped by heating the mixture at 95°C for 5 min. The sequencing was then done with the ABI V3.1 Big dye kit according to manufacturer's instructions. The labeled products were then cleaned with the Zymo Seq clean-up kit. The cleaned products were injected on the ABI3500XL analyzers with a 50cm array, using POP7.

2.6. Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using Multiple Alignment using Fast Fourier Transform (MAFFT). The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 [22]. The bootstrap consensus tree inferred from 500 replicates as reported by the author in [11] is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method [12].

3. Results

3.1. Molecular characterization of collected mushrooms

3.1.1 Gel Electrophoresis DNA Bands

The result of the Agarose gel electrophoresis patterns showing PCR amplification products of wild mushroom isolates with the amplicon sizes range from about 480 to 650 base pairs for samples 1 to 8 (Figure 22).

3.2. Sequences, Alignments and Phylogenetic Analysis

Using genomic DNA isolated from the mycelial culture of 8 wild mushrooms in 2014, 10 wild mushrooms in 2015, and 22 wild mushrooms in 2016, the amplified products were found to have 450 to 650 base pairs (Plates 1). Figures 1 to 10 are the Genbank sequences used in the phylogenetic analysis, while Figures 11 to 20 are the edited and aligned FASTA format of the isolated mushroom species. The aligned sequences identified 10 mushrooms as shown in Tables 1 and 2 based on which it was observed that the wild mushrooms have homology score value of ranging between 94 and 100. ITS-4 sequence of most of the samples gave better result after BLASTanalysis. The evolutionary relationships between the mushroom isolates are shown in Figure 20. Two out of the ten (2/10) mushroom isolates (16S and 17S) lacked ITS region deposit in the GenBank (Table 2)

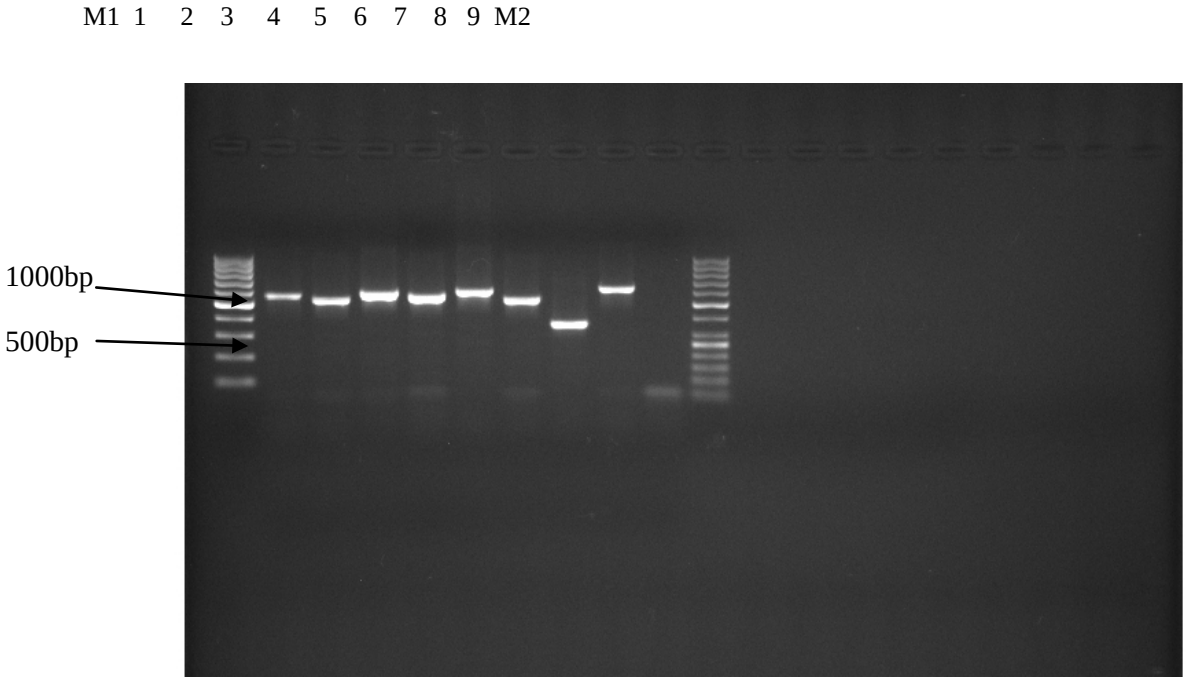

Figure 22: Agarose gel electrophoresis patterns showing PCR amplification products of eight (8) wild mushroom isolates (S1 – S8) collected in 2014

Lanes: M1 is O'GeneRuler™ 100bp DNA ladder (Fermentas Life Science); Lane 1 – S1; Lane 2 – S2; Lane 3 – S3; Lane 4 – S4; Lane 5 – S5; Lane 6 – S6; Lane 7 – S7; Lane 8 – S8; Lane 9 – Negative control; M2 is O'GeneRuler™ 50bp DNA Ladder (Fermentas Life Sciences)

Based on the phylogenetic tree (Figure 20) the evolutionary distances is in collaboration with the phylogenetic location of the ITS of 2S, 18S, 22S, 20S, 14S, 8S and S3 which were placed within the same clades and closely related to *Daldinia eschscoltzii* (KP012909.1), *Phlebiopsis crassa* (KP135394.1), *Lentinus squarrosulus* (JQ428823.1 1), *Lentinus sp* (JQ428823.1 2), *Trametes polyzona* (JN848329.1), *Tricholoma robustum* (MF037423.1) and *Auricularia polytricha* (FJ617296.1) respectively. The ITS of these sequences showed a percentage relatedness to similar species at 99% except ITS of the 14S which showed similarity to other species at 100%. The obtained ITS sequence of the mushroom coded S8 produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The ITS of the S8 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-

Cantor method were in agreement with the phylogenetic placement of the ITS of S8 within the *Leiotrametes* sp and revealed a closely relatedness to *Leiotrametes flavida strain DMC812* (gb: KC589131.1) than other *Leiotrametes* sp

Table 1: Alignment view and Distance matrix table (With Sample Mushroom sequence taken as reference sequence)

S/N	Code	NCBI Closest relative	% of closeness	Ascension number of closest relative	Location
1	S3	Auricularia polytricha	99	FJ617296.1	
2	20S	Lentinus squarrosulus	99	JQ428823.1 1	**N** $07^0$20’28.3
3	22S	Lentinus sp.	99	JQ428823.1 2	
4	S8	Leiotrametes flavida	99	KC589131.1	**E** $007^0$06’59.2
5	14S	Trametes polyzona	100	JN848329.1	
6	2S	Daldinia eschscholtzii	99	KP012909.1	to
7	18S	Phlebiopsis crassa	99	KP135394.1	**N** $07^0$46’54.3
8	8S	Tricholoma robustum	99	MF037423.1	
9	16S	Polyporales sp	94	PNigeria 1	**E** $005^0$50’25.5
10	17S	Polyporales sp.	94	PNigeria 1	

Kogi East N $07^0$20’28.3 E $007^0$06’59.2

Kogi West N $07^0$46’54.3 E $005^0$50’25.5

Table 2: Molecularly and Morphologically Characterized Mushrooms

S/N	Code	Molecularly identified mushrooms	Morphologically identified mushrooms
1	S3	Auricularia polytricha	Auricularia auricular
2	20S	Lentinus squarrosulus	Lentinus squarrosulus
3	22S	Lentinus sp.	Lentinus tigrinus
4	S8	Leiotrametes flavida	Daedaleopsis sp./Hydnum sp?
5	14S	Trametes polyzona	Lenzites sp.1
6	2S	Daldinia eschscholtzii	Daldinia cortricia
7	18S	Phlebiopsis crassa	Myco-lichen? Crust fungi
8	8S	Tricholoma robustum	Termitomyces robustus
9	16S	Polyporales sp	Lenzites elegans
10	17S	Polyporales sp.	Gilled bolete

Genbank sequences (FASTA Format) used in the phylogenetic analysis

GAACTTACCGCCGTTGCCTCGGCGGGCCGCGTTCGCCCTGTAGTTTACTACCTGGCGGCGCGCTAC
AGGCCCGCCGGTGGACTGCTAAACTCTGTTATATATACGTATCTCTGAATGCTTCAACTTAATAAG
TTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATT
CTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTGGGAATCT
AGGTCTCCAGGGCCTAGTTCCCCAAAGTCATCGGCGGAGTCGGAGCGTACTCTCAGCGTAGTAATA
CCATTCTCGCTTTTGCAGTAGCCCCGGCGGCTTGCCGTAAAACCCCTATATCTTTAGTGGTTGACCT
CGAATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAG

Figure 1: *Daldinia eschscholtzii* (KP012909.1) voucher MEL: 2382728 (2S)

CCCTAATAAGGCTACGGCGGAGGGACATTACCGAGTTTACAACTCCCAAACCCATGTGAACATAC
CTACTGTTGCTTCGGCGGGATTGCCCCGGGCGCCTCGTGTGCCCCGGATCAGGCGCCCGCCTAGGA
AACTCAACTCTTGTTTTATTTTGGAATCTTCTGAGTAGTTTTTACAAATAAATAAAAACTTTCAACA
ACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCA
GAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCT
GTCTGAGCGTCATTTCAACCCTCATGCCCCTAGGGCGTGGTGTTGGGGATCGGCCAAAGCCCGCGA
GGGACGGCCGGCCCCTAAATCTAGTGGCGGACCCGTCGTGGCCTCCCCTGCGAAGTAGTGATATTC
CGCATCGGAGAGCGACGAGCCCCTGCCGTTAAACCCCCAACTTTCCAAGGTTGACCTCAGATCAG
GTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

Figure 2: *Auricularia polytricha* (FJ617296.1) voucher_Cui6021 (S3)

TTCCGTAGGTGAACCTGCGGAAGGATCATTAACGAGTTTTGAAACGGGTTGTAGCTGGCCTTCCGA
GGCATGTGCACACCCTGCTCATCCACTCTACACCTGTGCACTTACTGTAGGTTGGCGTGGGCTTCG
GACCTCCGGGTTCGAGGCATTCTGCCGGCCTATGTACACTACAAACTCCGAAGTAACAGAATGTAA
ACGCGTCTAACGCATCTTAATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAA
CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA
CCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGGAATTCTCAACCCATAGAT
CCTTGTGGTCTACGGGCTTGGATTTGGAGGCTTGCCGGCCCTTACACGGGGTCGGCTCCTCTTGAA
TGCATTAGCTTGATTCCGTGCGAATCGGCTCTCAGTGTGATAATTGTCTACGCTGTGGCCGTGAAG
CGTTTGGCGAGCTTCTAACCGTCCGTTAGGACA

Figure 3: *Tricholoma robustum* isolate (MF037423.1) F17_M (8S)

CTGGCCTTACGAGGCATGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTTACTGTAGGTTTG
GCGTGGGCTTCGGGGGCCTTCGCGGGCTTTCGAGGCATTCTGCCTGCCTATGTATCACTACAAACA
CTATAAAGTAACAGAATGTAATCGCGTCTAACGCATCTTAATACAACTTTCAGCAACGGATCTCTT
GGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGA
ATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCA
TGGTATTCTCAACCCACACATCCTTGTGATGTTCGTAGGGCTTGGACTTGGAGGCTTGCTGGCCGTC
GCGGTCGGCTCCTCTTGAATGCATTAGCTTGGTTCCTTGCGGATCGGCTCTCAGTGTGATAATTGTC
TACGCTGTGACCGTGAAGCGTTTGGCGAGCTTCTAACCGTCCTGCTAGGGACAAACTTACTTGACA
TCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATA

Figure 4: *Leiotrametes flavida* strain (KC589131.1) DMC812 (S8)

GGGTTGTAGCTGGCCTTTCGAGGCATGTGCACACCCTGCTCATCCACTTTACACCTGTGCACTTACT
GTAGGTTGGCGTGGGCTTCGGACCTCCGGGTTCGAGGCATTCTGCCGCCCTATGTACAATATAAAC
TCCGAAGTAACAGAATGTAAACGCGTCTAACGCATCTTAATACAACTTTCAGCAACGGATCTCTTG
GCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA
TCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCAT
GAAATTCTCAACCCATAGATCCTTGTGGTCTACGGGCTTGGATTTGGAGGCTTGCCGGCCCTTACA
CGGGGTCGGCTCCTCTTGAATGCATTAGCTTGATTCCGTGCGAATCGGCTCTCAGTGTGATAATTGT
CTACGCTGTGGCCGTGAAGCGTTTGGCGAGCTTCTAACCGTCCGTTAGGACAACTTCTTGACATCT
GACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGGCGGAGGAAGATCATT
AACGAGTTTTGAAACGGGTTGTAGCTGGCCTT

Figure 5: *Trametes polyzona* strain (JN848329.1) WR710-1 (14S)

ATATAAAATACAAATTAAAAAAAAAAAAAAAATCCTCCGTAGGTGAACCTGCGGAGGGATCATTAA
AGAGTTGCAAAACTCCCAAACCATTTGTTTATCTACCTGTTTCGTTGCTTCGGCAGGCGGCTCTTCG
GGGCCTTTGCTACCTGTAGTGCCTGCCGGAGTATCTAAAACTCTTGTTATTTTTGTTCTGTCTGAGT
AAACTTTTAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC
GAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG
CCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTCTTGCTTGGT
GTTGGGGCTTCTGCGGCTTCGGCCGCAGGCCCTGAAAAACAGTGGCGGGCTCGCTATAACTCCGA
GCGTAGTAATCTCTCTCG

Figure 6: *Polyporales sp* strain (16S) E9220a

GGATCATTACAGAGTTCATGCCCTTCGGGGTAGACCTCCCACCCTGTGTATTTATACCTTTGTTGCT
TTGGCAGGCCGTCAGGCTTCGGCTCGACTACCGGCTCCGGCTGGTAAGCGCCTGCCAGAGGAAAT
CAAACTCTGAATATCATTATTGTCTGAGTAAAACATAATAAGTTAAAACTTTCAACAACGGATCTC
TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT
GAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTCGAGCG
TCATTACAACCCTCAAGCTCTGCTTGGTATTGGGCAGCACCAGCGATGGTGTGCCTTAAAATCAGT
GGCGGTGCCATCTGGCTCTAAGCGTAGTAATTCTCTCGCTATAGACGTCCGGTGGATGCTTGCCAG
CAACCCCTAATTTTTTAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCA
ATAAGC

Figure 7: *Aphyllophorales sp* strain (17S) EXP0530F

GTAGCTGGCCTCAATACGGGGCATGTGCACGCCTGGCTCATCCACTCCTTAACCTCTGTGCACTTTT
TGTAGGCTGGTTGAAAGGCGTTGCTTCACTTCGGTGTTGTTATCGCTGGAAGACCTGGTCTATGTTT
TACTACAAACGCTTCAGTTATAGAATGTTTATCTGCGTATAACGCATTTATATACAACTTTCAGCAA
CGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG
AATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCCTGGTATTCCGGGGAGCATGCCTG
TTTGAGTGTCATGGAATTCTCAACTTCTAATGCTTTTTTGTATTGGAAGCTTGGATTTGGAGGCTTG
TGCTGGCTCCTTTGTTGAGTCGGCTCCTCTTAAATGGATTAGCGTGAATCACTATGGATCGCTTCGG
TGTGATAATTATCTGCGCCGTGGTCGTGAAGTATCGATAAGTTCGCGCTTCTAACCGTCCTTCACG
GGACAATTTACCCTGACTTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAA
T

Figure 8: *Phlebiopsis crassa* strain (KP135394.1) KKN-86 (18S)

AGCTGGCCTTCCGAGGCATGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTTACTGTGGGTT
TCAGGAGCTTCGAAAGCGAGAAAAGGGGCCTTCACGGGCTTTTTTCTTGCCTAGTTGTTACTGGGC
CTACGTTTCACTACAAACACTTATAAAGTATCAGAATGTGTATTGCGATGTAACGCATCTATATAC
AACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA
TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGA
GGAGCATGCCTGTTTGAGTGTCATGAAATTCTCAACCTAACGGGTTCTTAACGGGACTTGCTTTAG
GCTTGGACTTGGAGGTTCTTGTCGGCTTGCTTCAATGTCAAGTCGGCTCCTCTTAAATGCATTAGCT
TGGTTCCTGTGCGGATCGGCTCACGGTGTGATAATTGTCTACGCCGCGACCGTTGAAGCGTTTTTAT
AGGCCAGCTTCTAGTCGTCTCTTTACGAGACAATAATCATCGAACTCTGACCTCAAATCAGGTAGG
ACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

Figure 9: *Lentinus squarrosulus* strain (JQ428823.1) VKGJ05 (20S)

TAGCTGGCCTTCCGAGGCATGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTTACTGTGGGT
TTCAGGAGCTTCGAAAGCGAGAAAAGGGGCCTTCACGGGCTTTTTTCTTGCCTAGTTGTTACTGGG
CCTACGTTTCACTACAAACACTTATAAAGTATCAGAATGTGTATTGCGATGTAACGCATCTATATA
CAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTA
ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCG
AGGAGCATGCCTGTTTGAGTGTCATGAAATTCTCAACCTAACGGGTTCTTAACGGGACTTGCTTTA
GGCTTGGACTTGGAGGTTCTTGTCGGCTTGCTTCAATGTCAAGTCGGCTCCTCTTAAATGCATTAGC
TTGGTTCCTGTGCGGATCGGCTCACGGTGTGATAATTGTCTACGCCGCGACCGTTGAAGCGTTTTTA
TAGGCCAGCTTCTAGTCGTCTCTTTACGAGACAATAATCATCGAACTCTGACCTCAAATCAGGTAG
GACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGG

Figure 10: *Lentinus squarrosulus* strain (JQ428823.1) VKGJ05 (22S)

Aligned and Edited Sequence Data

GAACTTACCGCCGTTGCCTCGGCGGGCCGCGTTCGCCCTGTACTTTACTACCTGGCGGCGCGCTAC
AGGCCCGCCGGTGGACTGCTAAACTCTGTTATATATACGTATCTCTGAATGCTTCAACTTAATAAG
TTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATT
CTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTGGGAATCT
AGGTCTCCAGGGCCTAGTTCCCCAAAGTCATCGGCGGAGTCGGAGCGTACTCTCAGCGTAGTAATA
CCATTCTCGCTTTTGCAGTAGCCCCGGCGGCTTGCCGTAAAACCCCTATGTCTTTAGTGGTTGACCT
CGAATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGRARRAATCATTACTGA
GTTATCTAAACTCCAACCCTATGTGAACTTACCGCCGTTGCCT

Figure 11: *Daldinia eschscholtzii*

CCAWTCCRCMTGATCCGAGGTCACCATTAAAAATTGGGGGGTTTAGCGGCTAAAGACGCTGCAAC
TCCMGTCAAAAGCGAGATAAAATTACTACGCTCAGAGGATATCGCAGATCCGCCGTTGTATTTCA
GGAGCTACCGCTAGCAAAAGCAGTAGGCTCCCAACACTAAGCTAGGCTTAAGGGTTGAAATGACG
CTCGAACAGGCATGCCCACTAGAATACTAATGGGCGCAATGTGCGTTCAAAGATTCGATGATTCAC
TGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGA
TCCGTTGTTGAAAGTTTTGACTTATTAAAATAAGACGCTCAGATTACAATAAAATAACAAGAGTTT
AGTAGTCCACCGGCAGCCGTTGCAGGGTAAGCCGTTCCAGGGTAAGGCGCTACAGGGTAGGCCGT
TCCAAGGTAGGGTATACCGAGCAGCTTCTGCCGAGGCAACAATGGTAAGTTCACATGGGTTGGGA
GTTTAGAAAACTCTATAATGATCCCTCCGCAGGTTCACCCCTACGGAAG

Figure 12: *Auricularia polytricha*

CAGGTGTAGAGTGGATGAGCAGGGTGTGCACATGCCTCGGAAGGCCAGCTACAACCCGTTTCAAA
ACTCGTTAATGATCCTTCCGTAGGTGAACCTGCGGAAGGATCATTAACGAGTTTTGAAACGGGTTG
TAGCTGGCCTTCCGAGGCATGTGCACACCCTGCTCATCCACTCTACACCTGTGCACTTACTGTAGGT
TGGCGTGGGCTTCGGACCTCCGGGTTCGAGGCATTCTGCCGGCCTATGTACACTACAAACTCCGAA
GTAACAGAATGTAAACGCGTCTAACGCATCTTAATACAACTTTCAGCAACGGATCTCTTGGCTCTC
GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCG
AATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGGAATT
CTCAACCCATAGATCCTTGTGGTCTACGGGCTTGGATTTGGAGGCTTGCCGGCCCTTACACGGGGT
CGGCTCCTCTTGAATGCATTAGCTTGATTCCGTGCGAATCGGCTCTCAGTGTGATAATTGTCTACGC
TGTGGCCGTGAAGCGTTTGGCGAGCTTCTAACCGTCCGTTAGGACA

Figure 11: *Tricholoma robustum*

CTGGCCTTACGAGGCATGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTTACTGTAGGTTTG
GCGTGGGCTTCGGGGGCCTTCGCGGGCTTTCGAGGCATTCTGCCTGCCTATGTATCACTACAAACA
CTATAAAGTAACAGAATGTAATCGCGTCTAACGCATCTTAATACAACTTTCAGCAACGGATCTCTT
GGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGA
ATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCA
TGGTATTCTCAACCCACACATCCTTGTGATGTTCGTAGGGCTTGGACTTGGAGGCTTGCTGGCCGTC
GCGGTCGGCTCCTCTTGAATGCATTAGCTTGGTTCCTTGCGGATCGGCTCTCAGTGTGATAATTGTC
TACGCTGTGACCGTGAAGCGTTTGGCGAGCTTCTAACCGTCCTGCTAGGGACAAACTTACTTGACA
TCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATA

Figure 14: *Leiotrametes flavida*

GGGTTGTAGCTGGCCTTCCGAGGCATGTGCACACCCTGCTCATCCACTCTACACCTGTGCACTTACT
GTAGGTTGGCGTGGGCTTCGGACCTCCGGGTTCGAGGCATTCTGCCGGCCTATGTACACTACAAAC
TCCGAAGTAACAGAATGTAAACGCGTCTAACGCATCTTAATACAACTTTCAGCAACGGATCTCTTG
GCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA
TCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCAT
GGAATTCTCAACCCATAGATCCTTGTGGTCTACGGGCTTGGATTTGGAGGCTTGCCGGCCCTTACA
CGGGGTCGGCTCCTCTTGAATGCATTAGCTTGATTCCGTGCGAATCGGCTCTCAGTGTGATAATTGT
CTACGCTGTGGCCGTGAAGCGTTTGGCGAGCTTCTAACCGTCCGTTAGGACAACTTCTTGACATCT
GACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAGGATCATTA
ACGAGTTTTGAAACGGGTTGTAGCTGGCCTT

Figure 15: *Trametes polyzona*

ATTGGGGGATTTAGCGGCTAAAGACGCTGCAACTCCAGTCAAAAGCGAGATAAAAATTACTACGC
TCAGAGGATATCGCAGATCCGCCGTTGTATTTCAGGAGCTACAGCTAGCAAAAGCAGTAGGCTCC
CAACACTAAGCTAGGCTTAAGGGTTGAAATGACGCTCGAACAGGCATGCCCACTAGAATACTAAT
GGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCAT
TTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGACTTATTAAAAT
AAGACGCTCAGATTACAATAAAATAACAAGAGTTTGGTAGTCCACCGGCAGCCGCTACAGGGTAA
GCCGTTCCAGGGTAAGGCGCTACAGGGTAGGCCGTTCCAAGGTAAGGTGCACCGAGCAGCTTCTG
CCGAGGCAACAATGGTAAGTTCACATGGGTTGGGAGTTTAGAAAACTCTATAATGATCCCTC

Figure 16: *Polyporales sp*.1

ATTGGGGGGTTTAGCGGCTAAAGACGCTGCAACTCCAGTCAAAAGCGAGATAAAAATTACTACGC
TCAGAGGATATCGCAGATCCGCCGTTGTATTTCAGGAGCTACAGCTAGCAAAAGCAGTAGGCTCC
CAACACTAAGCTAGGCTTAAGGGTTGAAATGACGCTCGAACAGGCATGCCCACTAGAATACTAAT
GGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCAT
TTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGACTTATTAAAAT
AAGACGCTCAGATAACCATAAAATAACAAGAGTTTGGTAGTCCACCGGCAGCCGTTGCAGGGTAA
GCCGTTCCAGGGTAAGGCGCTACAGGGTAGGCCGTTCCAAGGTAAGGTGCACCGAGCAGCTTCTG
CCGAGGCAACAATGGTAAGTTCACATGGGTTGGGAGTTTAGAAAACTCTATAATGATCCCTCCGCA
GGTTCACCTACGGA

Figure 17: *Polyporales sp*.2

GTAGCTGGCCTCAATTCGGGGCATGTGCACGCCTGGCTCATCCACTCCTTCACCTCTGTGCACTTTT
TGTAGGCTGGTTGAAAGGCGTCTTCACTCCGGTGTTGTTCGCTGGAAGACCTGGTCTATGTTTTACT
ACAAACGCTTCAGTTATAGAATGTTTATCTGCGTATAACGCATTTATATACAACTTTCAGCAACGG
ATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATT
CAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCCTGGTATTCCGGGGAGCATGCCTGTTTG
AGTGTCATGGAATTCTCAACTTCTAATGCTTTTTTGTATTGGAAGCTTGGATTTGGAGGCTTGTGCT
GGCTCCTAGTTGAGTCGGCTCCTCTTAAATGAATTAGCGTGAATCACTATGGATCGCTTCGGTGTG
ATAATTATCTGCGCCGTGGTCGTGAAGTATCGATAAGTTCGCGCTTCTAATCGTCCTTCACGGGAC
AATTAACCCTGACTTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAG
CGG

Figure 18: *Phlebiopsis crassa*

AGCTGGCCTTCCGAGGCATGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTTACTGTGGGTT
TCAGGAGCTTCGAAAGCGAGAAAAGGGGCCTTCACGGGCTTTTTTCTTGCCTAGTTGTTACTGGGC
CTACGTTTCACTACAAACACTTATAAAGTATCAGAATGTGTATTGCGATGTAACGCATCTATATAC
AACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA
TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGA
GGAGCATGCCTGTTTGAGTGTCATGAAATTCTCAACCTAACGGGTTCTTAACGGGACTTGCTTTAG
GCTTGGACTTGGAGGTTCTTGTCGGCTTGCTTCAATGTCAAGTCGGCTCCTCTTAAATGCATTAGCT
TGGTTCCTGTGCGGATCGGCTCACGGTGTGATAATTGTCTACGCCGCGACCGTTGAAGCGTTTTTAT
AGGCCAGCTTCTAGTCGTCTCTTTACGAGACAATAATCATCGAACTCTGACCTCAAATCAGGTAGG
ACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAG

Figure 19: *Lentinus squarrosulus*

GGTTGTAGCTGGCCTTCCGAGGCATGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTTACTG
TGGGTTTCAGGAGCTTCGAAAGCGAGAAAAGGGGCCTTCACGGGCTTTTTTCTTGCCTAGTTGTTA
CTGGGCCTACGTTTCACTACAAACACTTATAAAGTATCAGAATGTGTATTGCGATGTAACGCATCT
ATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATA
AGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTAT
TCCGAGGAGCATGCCTGTTTGAGTGTCATGAAATTCTCAACCTAACGGGTTCTTAACGGGACTTGC
TTTAGGCTTGGACTTGGAGGTTCTTGTCGGCTTGCTTCAATGTCAAGTCGGCTCCTCTTAAATGCAT
TAGCTTGGTTCCTGTGCGGATCGGCTCACGGTGTGATAATTGTCTACGCCGCGACCGTTGAAGCGT
TTTTATAGGCCAGCTTCTAGTCGTCTCTTTACGAGACAATAATCATCGAACTCTGACCTCAAATCAG
GTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGGAGGATCATTATCGAGTTTTGAA
ACGGGTTGTAGCTGGCCTTCCG

Figure 20: *Lentinus sp*

3.3. Molecular characterization of collected mushrooms

Molecular characterization of mushrooms encompasses use of genetic markers for identification. The ITS partial nucleotide sequences of the mushrooms samples were searched against available sequences on GenBank data for molecular identification [14]. The nucleotide sequences of 40 mushroom samples were blasted against available sequences from the Genbank database for identification (Table 1; Figures 1-10). One sample 14S (*Trametes* sp) matched 100% *Trametes polyzona* ascension number JN848329.1 as reported by the authors in [16]. On the other hand 2S, S8, 22S, 20S, S3 and S6 have 99% percentage similarities with *Daldinia eschscholtzii (*KP012909.1), *Leiotrametes flavida* (KC589131.1), *Lentinus squarrosulus* (JQ428823.1 1), *Lentinus* sp. (JQ428823.1 2), *Auricularia polytricha* (FJ617296.1) and *Tricholoma matsutake* (MF037408.1) respectively [6, 13, 15, 18, 21, 23].

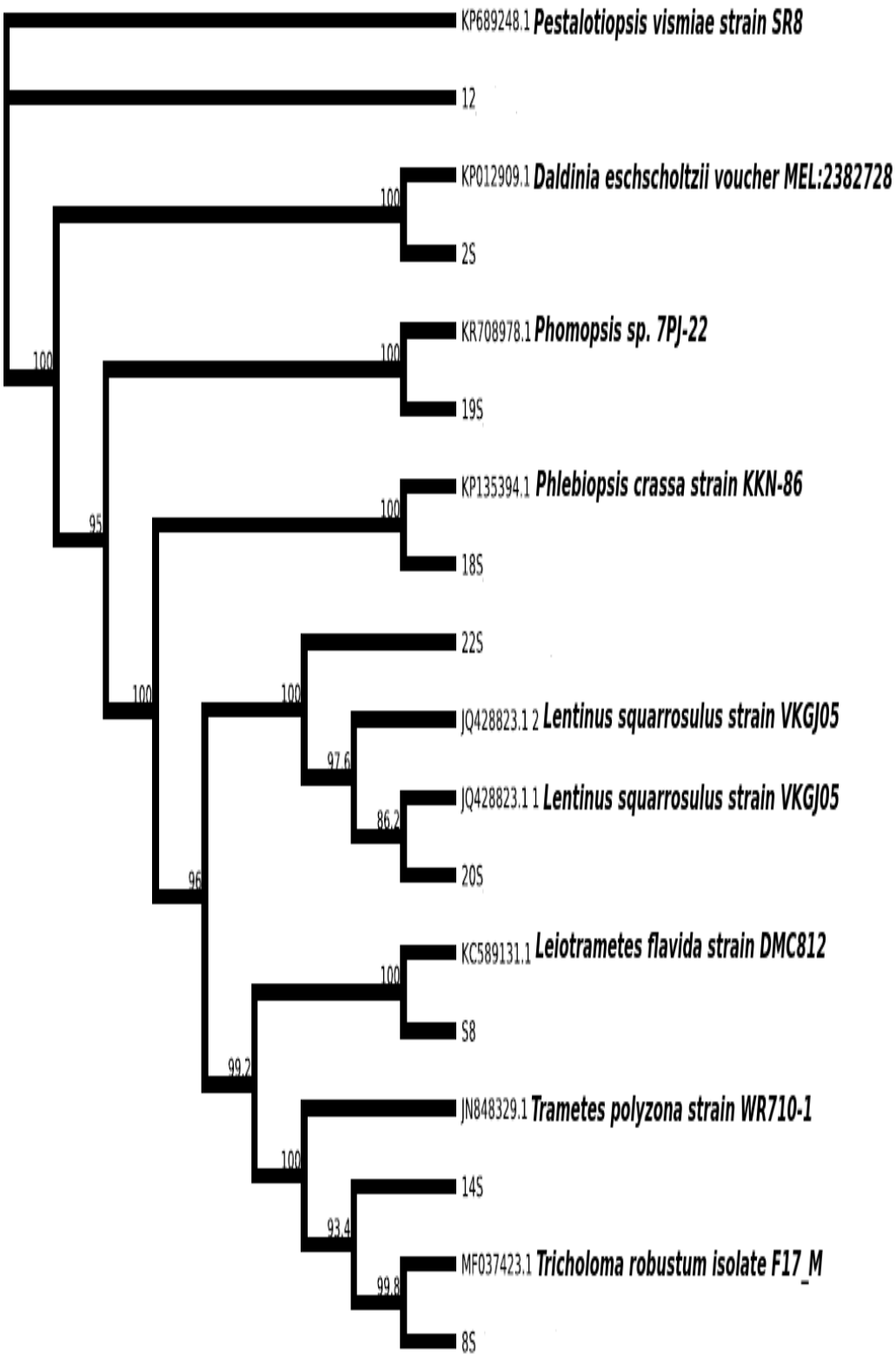

Figure 21: A Neighbour-Joining phylogenetic tree showing evolutionary relationships of the ITS gene sequences. The tree was constructed based on evolutionary distances computed using the Maximum-Composite Likelihood Method.

The findings of this research study corroborate the report of the authors in [24] on molecular characterization of wild mushrooms. They revealed that molecular identification has proven to be efficient in taxonomic identification to the species level. Molecular identification results of the ITS sequences of the rDNA and published mushroom record validates that Polyporus species Nigeria 1 and 2 (16S and 17S) may be new additions to Nigerian Agaricomycetes. The bootstrap consensus tree was inferred from 500 replicates sequences retrieved from the GenBank [17, 19, 20] as earlier mentioned. Accession numbers of ITS sequences used in the phylogenetic analysis are given in Table 2. However, relationships between some of the ITS groups (S4, S7, 1, 2, 6, 12, 17, 16, 1S, 2S, 5S, 7S, 9S, 16S, 17S and 21S) and the placement of other species are unresolved in the analyses as similarly observed by the authors in [19]. This could also be attributed to inability to attain complete sterilization of the basiodiocaps in situ. The closest relatives of the isolates used were placed in 1 to 3 clades as shown on the phylogenetic tree (Figure 21). Ten (10) aligned sequenced mushrooms were identified with homology score between 94 and 100, while two of the mushroom isolates seemed to be new additions as they lacked ITS region deposit in the Genbank. The phylogenetic tree constructed showed ITS relatedness with *Daldinia eschscoltzii, Phlebiopsis crassa , Lentinus squarrosulus, Lentinus sp, Trametes polyzona, Tricholoma robustum, Polyporales* and *Auricularia polytricha* generated from the Genbank..

4. Conclusion

The analysis of the molecular characterization in this work detected the existence of genetic variability among the agaricomycetes evaluated. This variability makes possible future studies in relation to their agronomic characteristics. In general, ITS barcoding is thus an effective tool in identification of mushroom species. The molecular characterization of collected mushrooms in the course of this study provides additional information enriching GenBank database.

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