Extraction and Identification of Fungi from Different Sudanese Soil

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Abstract

The biosynthesis process utilizing fungi and other microorganisms is an essential sustainable method for the manufacture of nanomaterials, aligning with sustainable development goals. Consequently, the extraction and accurate identification of fungi from soil is strongly advised. In this study, various fungus species were collected from different Sudanese agricultural soil at various locations. Afterwards, the fungi were extracted and identified. The fungi have been isolated were then identified to the genus level and to the species according to the basis of macro morphological. Thus, the colonies were investigated for slow or for rapid growth, also based on topography and texture. Interestingly, the identification methods used for fungi isolated displayed that various fungi have been successfully identified. Thus, based on the culture method, there are fungal species of 121 species with 100% were recognized. However, based on the microscopic investigation there are 102 species of fungal species were identified. However, based on the molecular method the identified fungi were found to be 90 of the fungal species isolated.

Keywords: Fungi, Sudanese agricultural soil, Fungi identification

1 Introduction

Recently, there a progressive trend for exploitation of various microorganism such as bacteria, fungus, and plant extract for green synthesis of various metal nanoparticles for various applications [1-2]. This due to the unique features of this species for affording reduction and capping functions for production of uniform metal nanoparticles [2-6]. On the other hand, fungi are one of the most commonly utilized organisms in biogenic synthesis of nanoparticles [7-9]. This is due to their capacity to produce large quantities of species and/or biomolecules which can be used to produce nanoparticles quickly and sustainably [10-11]. In contrast, only a small percentage (less than 10%) of fungal species found in natural settings have been formally recorded, according to reported research [12]. Nevertheless, only limited knowledge about the

variety and abundance of fungi on agricultural land are existed and in turn how to be a good candidate for biosynthesis process. Thus, extraction, isolation and identification of fungi from different locations and at different depth will be good approach for this trend. Thus, in this study, there various fungi species were collected from Sudanese agricultural soil at different geographical places and different depth. Secondly, they were isolated and identified with different methods.

2 Experimental Methods

2.1 Soil sample collection

Aseptically collected soil samples from surface and sub-surface layers at four distinct depths from various locations around the plant zones, utilizing GPA coordinates in the northern (Halfia) and southern (Soba) regions of Khartoum state. The soil samples were maintained in sterile containers and transported to the laboratory, where they were held at 4°C until processing.

2.1. Isolation of Fungi

Ten grams of collected soil were suspended in 90 mL of sterile 0.9% NaCl solution and aggressively agitated on a magnetic stirrer for 20 to 30 minutes to achieve a homogenous suspension. A serial dilution up to 5-10 was performed, and 0.1 mL aliquots from each dilution were inoculated into Potato Dextrose Agar (PDA) plates, which were then incubated at 25°C for 48 to 72 hours. Isolates that exhibited morphological differences were selected, purified, and maintained on PDA slants, then stored at 4°C. The identification will be conducted through morphological and microscopic examination, including characteristics like as color, texture of mycelia, and spore development patterns [13].

2.3. Identification of Fungi

The isolates were identified based on macroscopic characteristics, including surface topography, texture, and pigmentation. The characteristics of the colony encompass colony diameter after seven days, mycelia, exudates and reverse, colony texture, form, and conidia colour [14].

2.3.1. Needle Mount with Lacto phenol Cotton Blue (LPCB) Stain

In aseptic conditions, a single drop of lacto phenol cotton blue stain was applied to a sterile glass slide. A segment of the colony was excised using a mycological needle, placed on a slide, well mixed, covered, and studied microscopically to observe the conidial head of Aspergillus [15].

2.3.2. Polymerase chain reaction PCR

2.3.2.1. DNA Extraction Method

Genomic DNA was isolated utilizing a genomic DNA miniprep purification spin kit (Qiagen, Hilden, Germany) as followed: fragment the tissue into minute sections and transfer them into a 1.5 ml microcentrifuge tube. Then, incorporate 180 ml of buffer ATL and 20 µl of proteinase K, mix thoroughly using a vortex, and incubate at 56°C until complete lysis occurs (about 24 hours).Add 200 µl of buffer AL. Blend well by vortexing for 15 seconds. Incubate at 70 °C for 10 minutes. Briefly centrifuge the tube to eliminate droplets from the lid. This followed by

incorporation 200 µl of ethanol (96-100%). Vortex for 15 seconds. Briefly centrifuge the tube to eliminate droplets from the lid. Then, pipette the mixture onto the QIAamp Mini spin column located in the 2ml collection tube. Centrifuge at 6000 x g (8000 rpm) for 1 minute. Eliminate the flow-through and collection tube. Then, position the QIAamp Mini spin column in a fresh 2ml collection tube and introduce 500 µl of buffer AW1. Centrifuge at 6000 g (8000 rpm) for 1 minute. Dispose of the flow-through and collecting tube. This followed by putting the QIA Mini spin column in a new 2 ml collection tube (not included) and centrifuge at maximum speed for 1 minute. This mitigates the risk of potential buffer AW2 carryover. Finally, reiterate step 10 to enhance DNA yield with an additional 200 µl of buffer AE or distilled water (QIAamp DNA Mini Kit procedure).

2.3.2.2. Preparation of the primers

In accordance with the primer synthesizer company's guidelines, the lyophilized primers were reconstituted in deionized water to achieve a final concentration of 100 pM/µl, which was then stored as a stock solution at -20°C. A workable solution with a concentration of 10 pM/µl was developed. The universal 18S rRNA gene primers are: forward nu-SSU-0817-5, 5'-TTAGCATGGAATAATRRAATA-3', and reverse nu-SSU-1536-3, 5' ATTGCAATGCYCTATCCCCA-3' (Devi and Joshi, 2012). Utilized for the amplification of the 18S rRNA gene.

2.3.2.3. PCR Method

A total of 7 samples from the PCR assay test were utilized, with a total reaction volume of 25 ml comprising PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl; 0.1 mM (each) dATP, dGTP, dCTP, and dTTP; 1.5 mM MgCl2; 0.3 mM (each) primer; and 1.5 U of Platinum Taq high-fidelity DNA polymerase). Two oligonucleotide fungal primers were employed to amplify the ITS region, encompassing the 18S (ITS 1) and 18S (ITS 4) rRNA genes, along with the intervening 5.8S gene and the non-coding sections ITS 1 and ITS 2. Forty amplification cycles were conducted in a Heal Force T960 (China) thermocycler following an initial DNA denaturation at 95 °C for 4.5 minutes. Each cycle comprised a denaturation phase at 95 °C for 30 seconds, an annealing phase at 50 °C for 30 seconds, and an extension phase at 72 °C for 1 minute, concluding with a final extension at 72 °C for 3 minutes after the last cycle. Following amplification, the results were preserved at 4 °C until utilized [13].

2.3.2.3.1. Agarose gel electrophoresis

The amplified PCR product was analyzed by electrophoresis on a 2% (w/v) agarose gel stained with ethidium bromide. Each lane received 5 µL of PCR-amplified product and 1 µL of gel loading solution (Sigma). A volume of 2 µl of a 50-2000 bp molecular marker will be run concurrently to estimate the size of the PCR-amplified product .

2.3.2.3.2. DNA sequencing

PCR results from 33 random isolates (limited to 33 owing to budgetary limits) were purified utilizing QIAquick gel extraction kits (Qiagen) and dispatched for Sanger sequencing employing forward primers ITS1 and reverse primer ITS4 by BGI Company (Shenzhen, China). This was later sequenced utilizing Big Dye terminator (Applied Biosystems).

2.3.3. Bioinformatics Analysis

2.3.1. Sequences Similarity and Alignment

The BLAST sequence analysis tool was employed to identify Aspergillus species by searching and comparing sequences using nucleotide-nucleotide BLAST (BLASTn) with default settings, excluding low complexity sequence filtering. Species were identified according to the highest similarity score (100%) in the reference database sequence. Sequences exhibiting high similarity were obtained from NCBI and analyzed using multiple sequence alignment utilizing BioEdit software [Hall]. All sequences were submitted to GenBank with corresponding accession numbers.

2.3.2. Phylogenetic Tree

The phylogenetic tree of Aspergillus spp. amplified sequences was compared with reference strains from GenBank. The MEGA program, version 7 [16] was utilized to produce the neighbour-joining approach with bootstrap resampling conducted 1000 times for tree reliability assessment.

3 Results and discussion

3.1. Isolation of the fungi from soil

The isolated fungi were identified to the genus level and, when feasible, to the species level based on macro-morphological characteristics. Consequently, the colonies were assessed for both sluggish and rapid growth, considering topology (flat, heaped, regularly or irregularly folded) and texture (e.g., powdered, granular, velvety, or cottony). This encompasses surface pigmentation, reverse pigmentation, and micro-morphological traits (hyphae, macroconidia, chlamydospores, and other specialized fungal structures) utilizing appropriate media, slide culture, and the most current identification keys. The discovered fungus was validated and corroborated using the standard reference, "Illustration of Fungi," according to the classification system as tabulated in Table S1.

3.2. The identification of fungal species using conventional by (culture, and microscopic) and molecular methods (PCR)

The identification methods employed for fungi in this study yielded varying results (Fig. 1), as detailed in the experimental section. The culture approach accurately identified all samples as fungal species, reported 121 species with a 100% success rate. Upon microscopic examination, 84.30% (102 species) of the total fungal species were identified. Similarly, the molecular approach has identified 74.38% (90) of the fungal species, as illustrated in Fig. 1.

Conversely, there was a discrepancy in fungal identification of known and unknown species (Fig. 2 and Table 1). The culture approach identified 101 (83.5%) known fungal species and 20 (16.5%) novel fungal species. The microscopic approach has effectively detected that all known fungal species, along with one new species, with a significance level of $P < 0.001$. The molecular approaches have significantly detected 81 recognized fungus species and 9 new species (P value = 0.001).

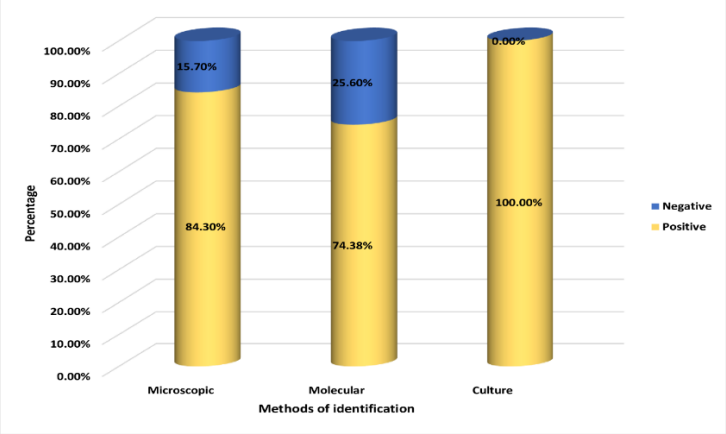


Figure 1: Displaying the identification of fungal species using different methods.

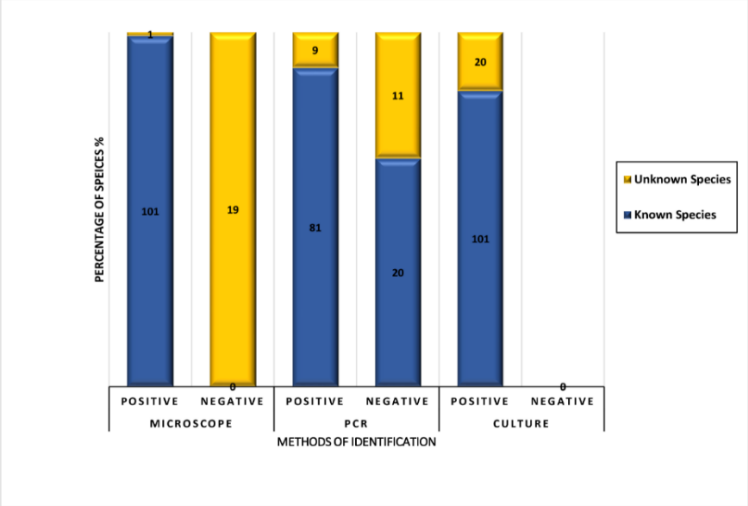


Figure 2: Comparison of fungal species identified using different methods displaying known and unknown species.

Table 1: Tabulated fungal species identified using different methods displaying known and unknown species.

		Culture		PCR		Microscope		Total
		Positive	Negative	Positive	Negative	Positive	Negative	
Known Species	Count	101	-	81	20	101	0	101
	% within species identification	100%	-	80.2%	19.8%	100%	0%	100%
	% within Culture	83.5%	-	90%	64.5%	99%	0 %	83.5 %
Unknown Species	Count	20	-	9	11	1	19	20
	% within species identification	100%	-	45.%	55.0%	5.00%	95%	100%
	% within PCR	16.5%	-	10%	35.5%	1%	100%	16.5 %
Total	Count	121	-	-	31	102	19	121
	% within species identification	100%	-	-	25.6%	84.3%	15.7%	100%
	% within Microscope	100%	-	-	100%	100%	100%	100%

3.3. The prevalence of the isolated fungal species

The prevalence of isolated fungus species was assessed (Fig. 3). A differentiation among isolated fungal species revealed that Aspergillus niger was the most predominant (40, 33.06%), followed by Aspergillus fumigatus (10, 8.26%), Aspergillus flavus (10, 6.61%), Aspergillus spp. (8, 6.61%), Rhizopus spp. (8, 4.13%), Fusarium spp. (5, 4.13%), Alternaria spp. (4, 3.31%), Aspergillus nidulans (4, 3.31%), Aspergillus terreus (4, 3.31%), Dermatiaceae (4, 3.31%), Mucor spp. (4, 3.31%), and Penicillium spp. (2, 1.65%). Additionally, there are a significant quantity of unidentified isolated species are detected (18, 14.88%), as stated in Table 1 and illustrated in Fig. 2.

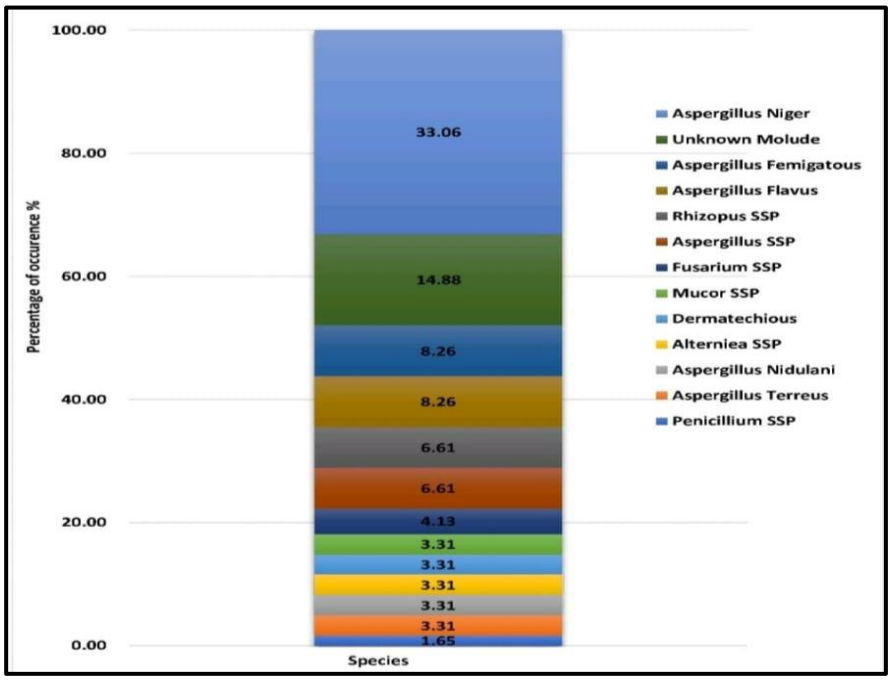


Figure 3: The Percentage of the isolated fungal species.

The prevalence of the isolated fungus species was assessed based on the regions from which they were collected. Consequently, the distribution of the isolated fungal species exhibited varying percentages between the two examined localities, Soba and Halfaia (Figs. 4). The data indicated that the Halfaia region exhibited a higher prevalence of fungal species than the Soba region. Furthermore, the species Aspergillus nidulans, Aspergillus terreus, and Penicillium spp. were exclusively identified in Halfia, whereas the species of Fusarium spp. and Mucor spp. were solely found in Soba.

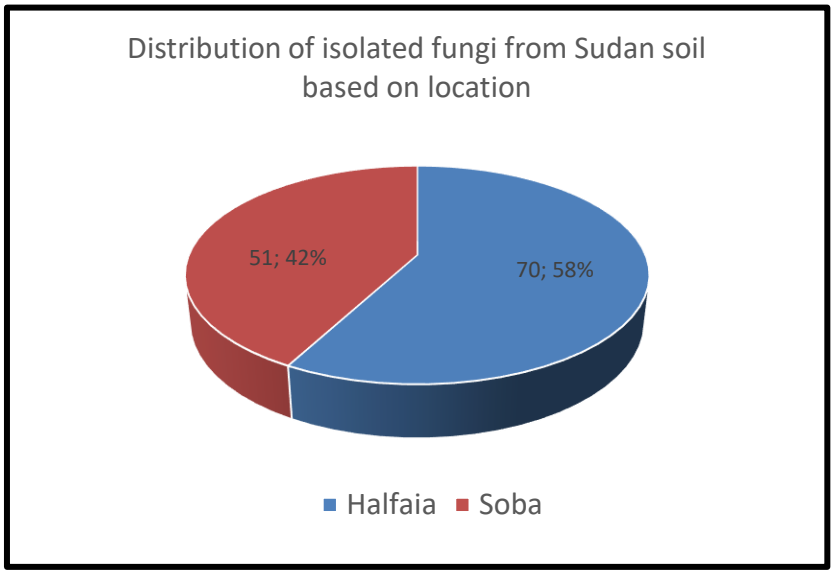


Figure 4: The Prevalence of the isolated fungal species based on locations (Soba, and Halfia).

3.4. Multiple Sequences Alignment

The multiple sequence alignment of the isolated fungus was analysed. The paired-end 25 sequences (forward and reverse) from Applied Biosystems platforms were purified and amalgamated using EMBOSS merger (https://www.bioinformatics.nl/cgi-bin/emboss/merger) to combine two overlapping sequences. Consequently, each sequence was analysed using BLASN (https://www.ncbi.nlm.nih.gov/geo/query/blast.html) to identify similar sequences, which were subsequently compared with the target sequences sharing commonalities and compiled into a single file in FASTA format for multiple sequence alignment. Consequently, MEGA v11 software was employed to generate the multiple sequence alignment and phylogenetic tree, utilizing the Clustal W procedure with default parameters (Table 2). The phylogenetic tree was constructed using the maximum likelihood approach with default parameters, encompassing all target sequences and their associated sequences.

4.5. Phylogenetic tree analysis

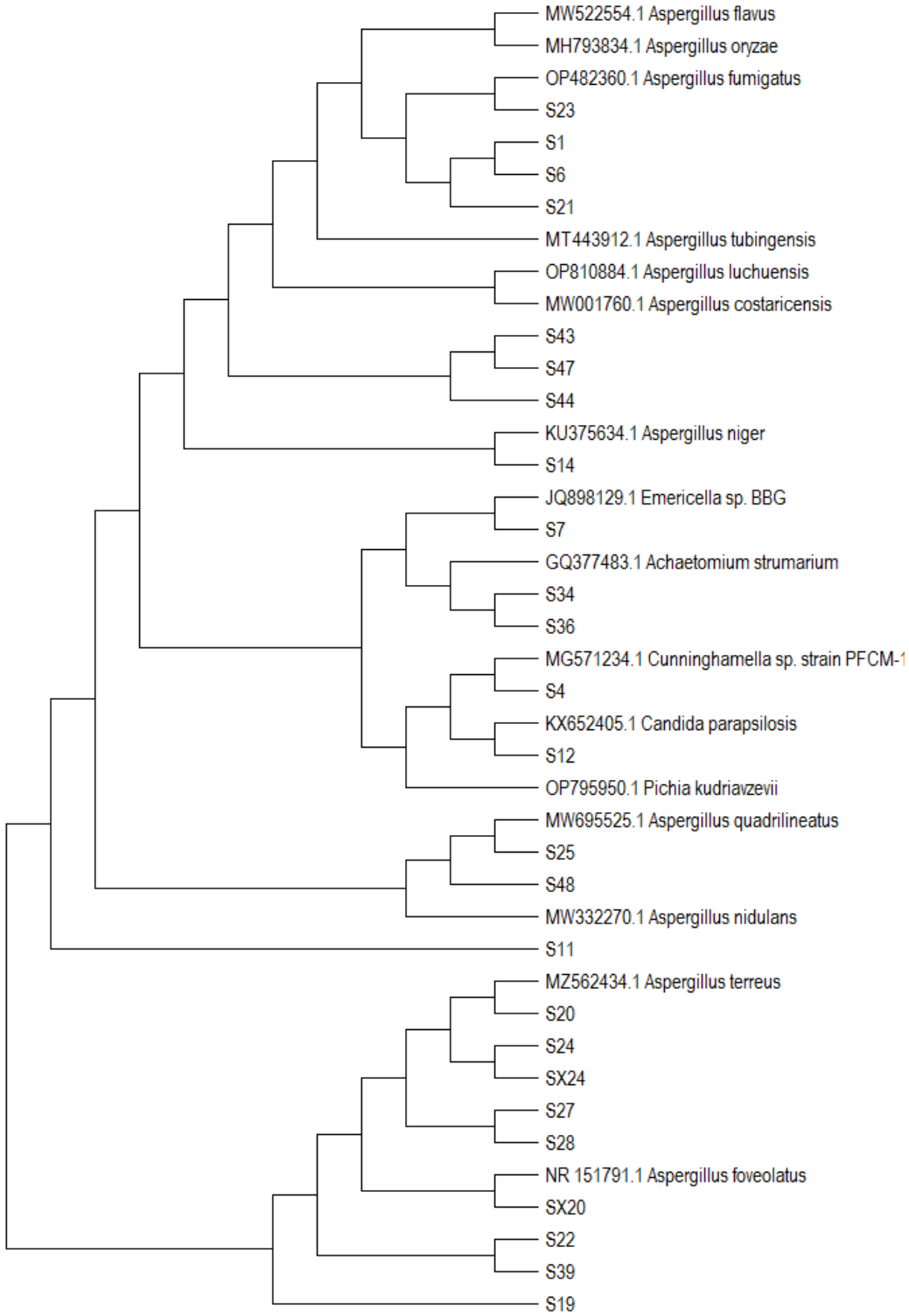
The phylogenetic tree illustrates current yeast strains alongside closely related species, derived from nucleotide sequences obtained from the BLASTN database of yeast species. The target yeasts designated S (Sample), accompanied by sample numbers and corresponding accession numbers and species names, were utilized to create the phylogenetic tree using the maximum likelihood technique (Fig. 5). Consequently, the phylogenetic trees of the fungal isolates indicated that the isolates were clustered in patterns of close similarity. Sequences from this investigation are designated as S1-S48, whereas sequences from BLAST are identified by their species and accession number. The S23 was identified as closely linked to Aspergillus fumigatus within the same clade, while S1, S6, and S21 were classified in distinct clades. S43, S44, and S47 were associated with Aspergillus costaricensis and Aspergillus luchuensis in distinct clades. S14 was closely associated with Aspergillus niger within the same clade. S7 was closely associated with Emericella sp. BBG within the same clade. Nonetheless, S4 exhibited a close phylogenetic relationship with Cunninghamella sp. strain PFCM-1 within the same clade, whereas S34 and S36 were strongly associated with Achaetomium strumarium in the same clade. S12 was closely associated with Candida parapsilosis within the same clade.

Nonetheless, S25 was associated with Aspergillus quadrilineatus within the same clade. S11 and S48 were associated with Aspergillus nidulans in distinct clades. Conversely, S24, XS24, S27, and S28 were associated with Aspergillus terreus in distinct clades, although S20 was analogous to it within the same clade. Furthermore, S19, S22, SX20, and S39 were associated with Aspergillus foveolatus in several clades (Fig. 5).

Protein accession No.	Species	Identity	Country
MW522554.1	Aspergillus flavus	90.58%	Egypt
OP482360.1	Aspergillus fumigatus	93.26%	China
JQ898129.1	Emericella sp. BBG	72.29%	Mexico
MW695525.1	Aspergillus quadrilineatus	91.94%	Egypt
MW332270.1	Aspergillus nidulans	86.31%	Iraq
KU375634.1	Aspergillus niger	87.67%	Iran
OP810884.1	Aspergillus luchuensis	98.99%	India
MT443912.1	Aspergillus tubingensis	77.38%	India
MG571234.1	Cunninghamella sp. strain PFCM-1	77.02%	Thailand
KX652405.1	Candida parapsilosis	97.89%	Turkey
GQ377483.1	Achaetomiumstrumarium	95.78%	Mexico
OP795950.1	Pichia kudriavzevii	85.05%	Mexico
MH793834.1	Aspergillus oryzae	90.48%	Nigeria
MZ562434.1	Aspergillus terreus	90.29%	India
NR_151791.1	Aspergillus foveolatus	100.00%	Netherlands
MW001760.1	Aspergillus costaricensis	100.00%	India

Table 2. Displays similar species to the target from BLASTN database with its identity and country region.

Figure 5: Phylogenetic trees of the fungal isolates.



4 Conclusion

This paper details the collection of several fungal species from diverse agricultural soils across multiple locations in Sudan. Subsequently, they were extracted and identified. The isolated fungi were subsequently recognized to the genus and species levels based on macro-morphological characteristics. Consequently, the colonies were examined for both sluggish and

quick growth, considering topography and texture. The identification methods employed for fungi revealed several identification methodologies. Consequently, according to the culture method, there are 121 fungal species identified with a 100% success rate. Microscopic research revealed the identification of 102 fungus species. Nonetheless, the molecular technique revealed that the discovered fungi comprised 90 distinct fungal species.

5 References

[1] N. F. Attia, E. M. Abd El-Monaem, H. G. El-Aqapa, S. E. A. Elashery, A. S. Eltaweil, M. El Kady, S. A. M. Khalifa, H. B. Hawash, H. R. El-Seedi. Iron oxide nanoparticles and their pharmaceutical applications. Applied Surface Science Advances, 11, 100284 (2022).

[2] L. A. Alshehri, N. F. Attia. Reinforcement and antibacterial properties of hand embroidery threads based on green nanocoatings. Coatings, 13, 747 (2023).

[3] N. Durán, P. D. Marcato, M. Durán, A. Yadav, A. Gade, M. Rai. Mechanistic aspects in the biogenic synthesis of extracellular metal nanoparticles by peptides, bacteria, fungi, and plants. Applied microbiology and biotechnology, 90, 1609-1624 (2011).

[4] C. Guilger, L. Mariana. Synthesis of silver nanoparticles mediated by fungi: a review. Frontiers in bioengineering and biotechnology 22, 7, 287 (2019).

[5] N. F. Attia, M. Moussa, A. M. F. Sheta, R. Taha, H. Gamal. Effect of different nanoparticles-based coating on the performance of textile properties. Prog. Org. Coat. 106, 41-49 (2017).

[6] N. F. Attia, M. S. Morsy. Facile synthesis of novel nanocomposite as antibacterial and flame-retardant material for textile fabrics. Mater. Chem. Phys. 180, 364-372 (2016).

[7] A. K. Gade, P. P. Bonde, A. P. Ingle, P. D. Marcato, N. Duran, M. K. Rai. Exploitation of Aspergillus niger for synthesis of silver nanoparticles. Journal of Biobased Materials and Bioenergy, 2, 243-247(2008).

[8] P. Azmath, S. Baker, D. Rakshith, S. Satish. Mycosynthesis of silver nanoparticles bearing antibacterial activity. Saudi Pharm. J. 24, 140–146 (2016).

[9] V. Ahluwalia, J. Kumar, R. Sisodia, N. A. Shakil, S. Walia. Green synthesis of silver nanoparticles by Trichoderma harzianum and their bio-efficacy evaluation against Staphylococcus aureus and Klebsiella pneumonia. Industrial Crops and Products, 55, 202-206 (2014).

[10] K.Vahabi, S. K. Dorcheh. Biosynthesis of silver nano-particles by Trichoderma and its medical applications. In Biotechnology and biology of Trichoderma.3,393-404 (2013).

[11] M. A. Alghuthaymi, H. Almoammar, M. Rai, E. Said-Galiev, K. A. Abd-Elsalam. Myconanoparticles: synthesis and their role in phytopathogens management. Biotechnology & Biotechnological Equipment, 29, 221-236 (2015).

[12] H. K. Wang, K. D. Hyde, K. Soytong, F. C. Lin. Fungal diversity on fallen leaves of Ficus in northern Thailand. Journal of Zhejiang University Science B 9, 835-841 (2008).

[13] L. S. Devi, S. R. Joshi. Antimicrobial and synergistic effects of silver nanoparticles synthesized using soil fungi of high altitudes of eastern Himalaya. Mycobiology 40, 27-34 (2012).

[14] H. Afzal, S. Shahzad, S. Qamar, U. Nisa. Morphological identification of Aspergillus species from the soil of larkana district (Sindh, Pakistan). Asian J. Agric. Biol. 1, 105-117 (2013).

[15] M. Cheesbourgh. Medical Laboratory Manual for Tropical Countries, 2nd ed.; Cambridge University Press: New York, NY, USA, 2006.

[16] E. J. Klemm, V. K. Wong, G. Dougan. Emergence of dominant multidrug-resistant bacterial clades: lessons from history and whole-genome sequencing. Proc Natl Acad Sci USA. 115, 12872-12877 (2018).