Molecular phylogenetics and antimicrobial potentials of selected endophytic fungi associated with *Curcuma longa*

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ABSTRACT

Background: *Curcuma longa,* popularly known as turmeric, is a rhizomatous plant belonging to the Zingiberaceae family. This study is necessitated sequel to the dearth of information on endophytic fungi associated with this medicinal plant.

Objectives: This study aimed at isolating endophytic fungi from *C. Longa* and analyzing their antimicrobial potential.

Methods: The fungal endophytes were isolated from the surface-sterilized rhizomes on potato dextrose agar (PDA) and identified through morphological, microscopical, and internal transcribed spacer (ITS) sequencing. The base calling and consensus sequence was generated using the Geneious software version 9.0.5. The sequence was blasted and submitted to the NCBI database for accession numbers. The Phylogenetic analysis was done using the Geneious software upon MUSCLE alignment. The antimicrobial potential was challenged with some Gram positives and ESKAPE organisms by agar plug and Kirby-Baurer techniques.

Results: A total number of ten (10) fungal endophytes were isolated from thirty sliced samples explored and the preliminary antimicrobial assay revealed that only two isolates, CLR1 and CLR9 could produce antimicrobial secondary metabolites. The BLAST results revealed that they are closely related to *Paecilomyces dactylethromorphus* CBS 251.55 (NR_149330.1) and *Aspergillus ochraceopetaliformis* NRRL 4752 (NR_135390.1) at percentage identity of 99.65 % and 99.43 % respectively. Phylogenetic analysis confirmed the relationship between the isolates and known species. Ethyl acetate extract showed zones of growth inhibitions between 10±0.2 mm and 28±1.2 mm in diameter against tested organisms. Their crude extract showed MIC of 1.57 mg/ml to 20 mg/ml and MBC from 2.1 mg/ml to 20.8 mg/ml.

Conclusion: These findings suggest that *Curcuma longa* hosts fungal endophytes with antimicrobial potential, warranting further biotechnological exploration.

Keywords: Fungal endophytes, *Curcuma longa*, ITS sequencing, Phylogenetics, ESKAPE organisms.

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Phylogénétique moléculaire et potentiels antimicrobiens d'une sélection de champignons endophytes associés à *Curcuma longa*

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RÉSUMÉ

Contexte: *Curcuma longa*, communément appelé curcuma, est une plante rhizomateuse appartenant à la famille des Zingibéracées. Cette étude est rendue nécessaire par le manque d'informations sur les champignons endophytes associés à cette plante médicinale.

Objectifs: Cette étude vise à isoler les champignons endophytes de *C. Longa* et à analyser leur potentiel antimicrobien.

Méthodes: Les endophytes fongiques ont été isolés des rhizomes stérilisés en surface sur gélose dextrose de pomme de terre (PDA) et identifiés par séquençage morphologique, microscopique et par séquençage de l'espaceur interne transcrit (ITS). La séquence d'appel de base et la séquence consensus ont été générées à l'aide du logiciel Geneious version 9.0.5. La séquence a été blastée et soumise à la base de données NCBI pour les numéros d'accès. L'analyse phylogénétique a été réalisée à l'aide du logiciel Geneious après alignement MUSCLE. Le potentiel antimicrobien a été testé avec certains organismes Gram positifs et ESKAPE par des techniques de bouchon d'agar et de Kirby-Baurer.

Résultats: Un nombre total de dix (10) endophytes fongiques ont été isolés à partir de trente échantillons tranchés explorés et l'essai antimicrobien préliminaire a révélé que seuls deux isolats, CLR1 et CLR9, pouvaient produire des métabolites secondaires antimicrobiens. Les résultats du test BLAST ont révélé qu'ils sont étroitement liés à *Paecilomyces dactylethromorphe* CBS 251,55 (NR_149330.1) et *Aspergillus ochraceopetaliformis* NRRL 4752 (NR_135390.1) avec des pourcentages d'identité de 99,65% et 99,43% respectivement. L'analyse phylogénétique a confirmé la relation entre les isolats et les espèces connues. L'extrait d'acétate d'éthyle a montré des zones d'inhibition de croissance entre 10±0,2 mm et 28±1,2 mm de diamètre contre les organismes testés. Leur extrait brut a montré une CMI de 1,57 mg/ml à 20 mg/ml et une CMB de 2,1 mg/ml à 20,8 mg/ml.

Conclusion: Ces résultats suggèrent que *Curcuma longa* héberge des endophytes fongiques dotés d'un potentiel antimicrobien, ce qui justifie une exploration biotechnologique plus approfondie.

Mots clés: Endophytes fongiques, *Curcuma longa,* séquençage ITS, phylogénétique, organismes ESKAPE.

INTRODUCTION

The rise of antibiotic resistance and a dwindling antimicrobial pipeline have been recognized as emerging threats to public health.[1] The acronym ESKAPE includes six nosocomial pathogens that exhibit multidrug resistance and virulence: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter spp*. Persistent use of antibiotics has provoked the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria, which render even the most effective drugs ineffective.[2] Extended-spectrum β-lactamase (ESBL) and carbapenemase-producing Gram-negative bacteria have emerged as an important therapeutic challenge.[3] The need of the hour is to develop novel therapeutics or lead antibiotic compounds to treat drug-resistant infections, especially those caused by ESKAPE pathogens.[4]

Fungi associated with plants are diverse and constitute the second-largest organisms on earth.[5] They exist in complex communities and are well known by their high levels of species richness, turnover, and endemism.[6] To adapt to the changing environments, fungi usually evolve unique metabolic regulation mechanisms. One of the known features of fungal adaptation is the production of secondary metabolites (SMs), which are essential for survival and beneficial to the organism.[7] Many fungal secondary metabolites with a staggering variation in chemical structures and biological activities, have been developed into life-saving medicines and agrochemical as exemplified by penicillin, lovastatin, and cyclosporine.[8] Nevertheless, the discovery of SMs with novel structures and/or interesting bioactivities from conventional sources is becoming increasingly difficult, despite the technological advances in natural product separation and purification. In this circumstance, researchers are shifting their focus onto fungi that inhabit unusual biotopes or adopt uncommon survival strategies as compared with soil-dwelling fungi.[9]

Fungal endophytes refer to fungi that live asymptomatically in the inner tissue of the host plants.[10] It has been suggested that fungal endophytes are involved in intricate interactions with the host plants and suffer selective environmental stress from the hosts. These complex interactions with the host plants are thought to contribute to the evolutionary formation of unique metabolic pathways responsible for the production of novel SMs in fungal endophytes.[11] Researchers have witnessed the discovery of many SMs with intriguing structures and significant bioactivities from fungal endophytes in the last decades. Fungal endophytes have been well accepted as a promising source of antibiotics and other drug leads.[12,13]

The taxonomy of these organisms is still challenging due to a lack of reliable and advanced techniques for their identification and systematic studies. Traditional fungal systematics relying on morphological or other phenotypic characters.[14] The internal transcribed spacer regions (ITS) are used as official universal DNA barcodes for fungi.[15] The ITS1, ITS2, and ITS4 have been proven to be useful for the identification of yeasts (Cryptococcus, candida) and some fungi such as *Aspergillus, Penicillium, Talaromyces,* and *Trichosporon* species among many others. However, ITS sequences cannot be used for phylogenetic analyses of unrelated taxa due to low variability and slow evolution. Phylogenetic analyses have been used extensively to clarify species boundaries in several fungal genera.[16]

These fungal endophytes mediate induced systemic resistance in plants that is considered an important mechanism for plant protection and disease management.[17] Endophytic fungi are considered as the main source of bioactive compounds with potential applications in various fields such as agriculture, pharmaceuticals, environmental cleaning, and the food industry.[18] Some of the identified endophytic fungi produced bioactive compounds and metabolites, essential for plants to cope with biotic and abiotic stresses. Bioactive compounds may also play an important role in plant protection against disease-causing pathogens and pests.[19] Several studies have reported endophytic fungi with the ability to produce bioactive compounds and, some of these symbiotic endophytes promoted plant growth and productivity in several crop plants.[20] However, the identified fungal strains with such beneficial properties are limited in Nigeria.

Curcuma longa is a perennial herbaceous plant belonging to *zingiberaeceae*. Its rhizome is used in folk medicine for antibacterial, antifungal and antioxidant activities.[21] The endophytic fungal communities of *C. longa* from Nigeria and their antimicrobial bioactive potential have not been reported. This study investigated the endophytic fungal microbiomes of the native culinary plant *C. longa* in Nigeria as a source of novel antimicrobials to combat the ever-emerging antibiotic-resistant pathogens.

MATERIAL AND METHODS

Sampling of turmeric rhizomes

Fresh turmeric rhizomes (*Curcuma longa* L.) were collected from traditional herbal market in Mushin Lagos, Southwestern Nigeria: Mushin (6.5352°N, 3.3490°E). They were placed in sterile bags and taken to the laboratory. The samples were stored at 4 °C for further experimental analysis within 2-3 days.

Sample sterilization and fungal endophytes' isolation

Endophytic fungal isolation was done using conventional methods.[22] The rhizome samples of *Curcuma longa* were used for endophytic fungi isolation. In brief, rhizomes were rinsed with tap water until all dust particles and impurities were removed. The rhizome samples were then surface-sterilized by treating with 70% ethanol for 1 min followed by immersion in 10 % sodium hypochlorite (NaClO) solution for 20 min. The samples were then washed three times with sterile distilled water. After surface sterilization, the rhizome samples were aseptically sliced 1 cm x 1 cm using a sterile scapel. These were aseptically inoculated on potato dextrose agar (PDA) plates. Surfaces of sterilized plant tissue segments were gently pressed onto the surface of Potato Dextrose Agar (PDA), thereafter, imprinted PDA plates were incubated at 28 °C and observed for any fungal growths for a period of 7 days.[23] The absence of fungal growths on imprinted plates was taken as proof of effective surface sterilization. The PDA plates were incubated at 28 °C until fungal growth appeared on the sliced curcuma rhizome portions.[24] After 10 days of incubation, the fungal mycelia were aseptically inoculated onto fresh PDA plates. Several subculturings were done until pure culture was obtained.[25] Pure fungal culture was used to inoculate 30 % glycerol broth, and the broth culture was stored as glycerol stock at - 80 °C.[26]

Microscopic examination of fungal strains

All the endophytic fungal strains obtained were cultured on PDA and incubated at 28 °C until mycelial growth appeared and spread to the plate. The fungal strain was then investigated for morphological and microscopic features. Briefly, 2 drops of lactophenol-cotton-in blue reagent were mounted on microscopic slide, and needleful fungal mycelial was dropped on the slide and smeared.[27] Then cover slip was placed on the smear which was ready for microscopic view. Mycelial and conidial structures were observed using a light microscope at 40 X.

Collection of isolates

Ten multidrug-resistant pathogens (*Streptococcus agalactiae* ATCC 13813, *Klebsiella pneumoniae* ATCC 70603, Enterococcus faecalis ATCC 51299, *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 152338, *Staphylococcus aureus* ATCC 15305, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 215872, *Staphylococcus aureus* ATCC 29713, *Haemophilus influenzae* ATCC 49766) were used for the study including the 'ESKAPE' strains. They were collected on agar slants from the Department of Microbiology, University of Maiduguri Teaching Hospital, Borno State Nigeria.

Media preparation

Media used in this study were prepared following the manufacturer's instruction and were sterilized by autoclaving at 121 °C for 15 minutes. Each organism was cultured on nutrient agar for viability. Then, pathogens' cultures were adjusted to 1.5 × 108 colony-forming units (CFU) per milliliter based on 0.5 McFarland turbidity standards.[28]

Agar plug assay

The Agar plug diffusion method is often used to highlight the antagonism between microorganisms and the procedure is similar to that used in the disk-diffusion method.[29] Briefly, an agar culture of the endophytic fungi of interest was allowed to grow for up to ten days to enable secretion of secondary metabolite molecules if any.[30] After incubation, a plug of the agar is cut aseptically with a sterile cork borer and deposited on the agar surface of another plate previously inoculated with the test pathogen.[31] Then, the antimicrobial activity of the secreted molecules is detected by the appearance of the inhibition zone around the agar plug.

DNA extraction and molecular identification of the putative fungal strains

For molecular analysis, the endophytic fungal strain was inoculated in potato dextrose broth (PDB) at 28 °C for 48 h in a shaker at 150 rpm. The fungal culture was then centrifuged at 4000 rpm in a room temperature for 10 min. The supernatant was discarded, and the cell pellet was used for genomic DNA extraction using the fungal Genomic DNA Isolation Kit (Quick DNATM fungal miniprep) following the manufacturer's protocols.[32] Molecular identification was conducted through the amplification of the ITS rDNA sequences. About 544 bp sequence was amplified from genomic DNA using primers ITS1 (Forward) and ITS4 (Reverse) specific for the rDNA

genes. A 25 µL PCR reaction contained 1 µL (0.5-10.0 ng) of template DNA, 0.2 µM each primers ITS1F and ITS4R, 200 µM of each dNTP, 10x buffer, 2 mM MgSO4, and 1 U High-Fidelity KOD Taq DNA Polymerase.33 The cycle parameters were as follows: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 sec at 52 °C, and extension for 1 min at 68 °C; and a final overall extension for 7 min at 68 °C. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and was then sequenced.[34]

Gel electrophoresis

PCR products were detected by electrophoresis in 1 % agarose gel prepared in 1 × Tris Borate-EDTA (TBE) buffer, stained with 4 µL of Florosafe DNA Stain (1st BASE, Singapore), and run for 90 min at 80 V and 400 mA. After running, gels were visualized under UV light in the Bio-RAD Molecular Imager® Gel Doc™ XR + Systems (Hercules, California, USA), and photographed using the Discovery Series™ Quantity One® 1-D Analysis software.[35] The sizes of the amplified fragments were estimated by matching them with a 100 bp DNA ladder (Thermo Scientific, USA).

Molecular phylogenetic study

Base-calling of the forward and reverse sequences was done using the Geneious software version 9.0.5.[36] The consensus sequences generated were BLAST searched against homologous fungal ITS rDNA sequences using NCBI. The determined sequence was aligned in Geneious software using Neighbour-joining method, and phylogenetic trees were constructed using the Geneious software version 9.0.5. The tree was annotated using iTOL software version 6.0.[37] The nucleotide sequences of the two putative isolates CLR1 and CLR9 were then submitted to GenBank under accession numbers PQ249621 and PQ249632 respectively.

Fermentation of putative fungal endophytes

Fungal endophytes were grown using a liquid-submerged fermentation technique in 1 liter Erlenmeyer's flask. They were incubated at 28 °C for two weeks with 150 rpm shaking.[38] The fungal biomass was removed by filtration using a cheesecloth. Ethyl acetate solvent was employed in the extraction of the secondary metabolites. An equal amount of the solvent was added to the filtrate and agitated for 20 min until two transparent immiscible layers appeared.[39] The solvent fraction containing the bioactive compounds was separated from the fermentation broth using a separation funnel. The solvent was then evaporated, and the extract was concentrated in a rotator vacuum evaporator to produce the crude metabolite. The crude extract was diluted with dimethyl sulphoxide (DMSO) and kept at 4 °C.[40]

Determination of minimum inhibitory concentration (MIC)

MIC was determined only for the crude extracts of the putative fungal endophytes and on the bacterial pathogens for which it showed inhibitory activity. The broth dilution technique was employed to determine the MIC of the potent extracts. Doubling dilutions of the potent extracts were prepared using peptone water (prepared) to obtain a series of dilutions containing 200, 100, 50, 25, 12.5, 6.25 mg/mL of fungal crude metabolites.[41] Standardized inoculum of the test organisms (exactly 0.02 mL) compared with the MacFarland turbidity standard was inoculated into each of the 5 sets of test tubes containing 2 mL of the mixture. Tubes containing extract-free broth were used as control, and all were incubated together at 37 °C for 24 hours. MIC was read as the least concentration that showed no growth, using turbidity as a measure.[42]

Determination of minimum bactericidal concentration (MBC)

The MBC was determined by collecting 0.2 mL of broth culture from the tubes used for the MIC determination (starting from the MIC point backward) and subculturing onto fresh extract-free and drug-free solid agar plates.[42] The plates were incubated at 37 °C for 24 hours. The least concentration that didn't show any growth after 24 hours of incubation was regarded as the MBC.

Data availability

The two putative endophytic fungal data have been submitted to the NCBI GenBank (https://www.ncbi.nlm.nih.gov/). The accession numbers for Paecilomyces sp. CLR1 and Aspergillus sp. CLR9 are PQ249621 and PQ249632 respectively.

Statistical analysis

Records of inhibition zone diameters, minimum inhibitory concentrations and minimum bactericidal concentrations were taken in triplicates and documented as average value ± standard error of the mean.

RESULTS

Ten distinct fungal endophytes were isolated from *C. longa*. They were grown on potato dextrose agar for 10

days and their morphological features were observed and documented. They have different colours like white, grey, green and dark-brown. The texture of (a) was suede; (b), (h) and (i) were powdery while (c), (d), (e), (f), (g) and (j) were very sticky on the PDA plates (Fig 1). (k - t) revealed microscopical features of these endophytic fungi showing the spores, conidia, conidiophores, and hyphae.

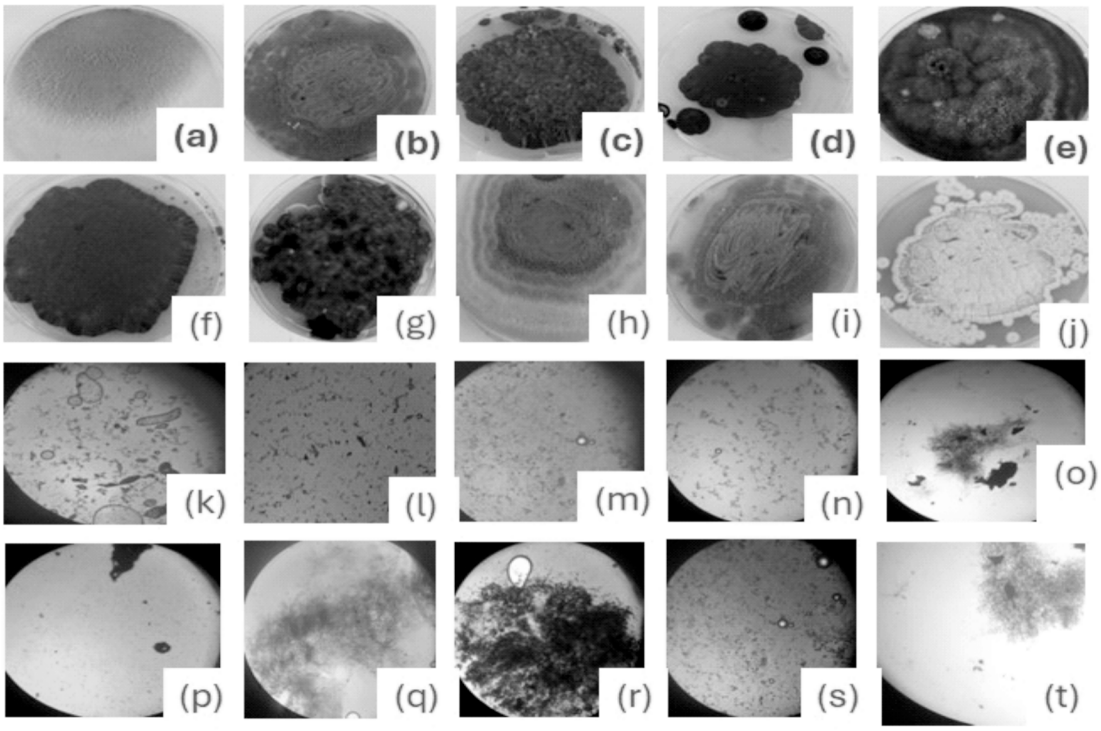

Figure 1: Morphological and microscopical features of fungal endophytes isolated from *Curcuma longa* L. (a-j) showed 10 days endophytic fungi in PDA plates incubated at 28 °C. (k-t) showed the microscopic field view of the endophytic fungi using Olympus microscope at 40X magnification.

The selection of endophytic fungal strains CLR1 and CLR9 for further molecular identification was based on their positive results upon the preliminary antimicrobial assay by agar plug technique. Both strains demonstrated antimicrobial potential by creating zones of inhibition when their 6 mm plug of 10-day-old culture came in contact with bacterial pathogens for 24 hours at 37 °C (Fig. 2).

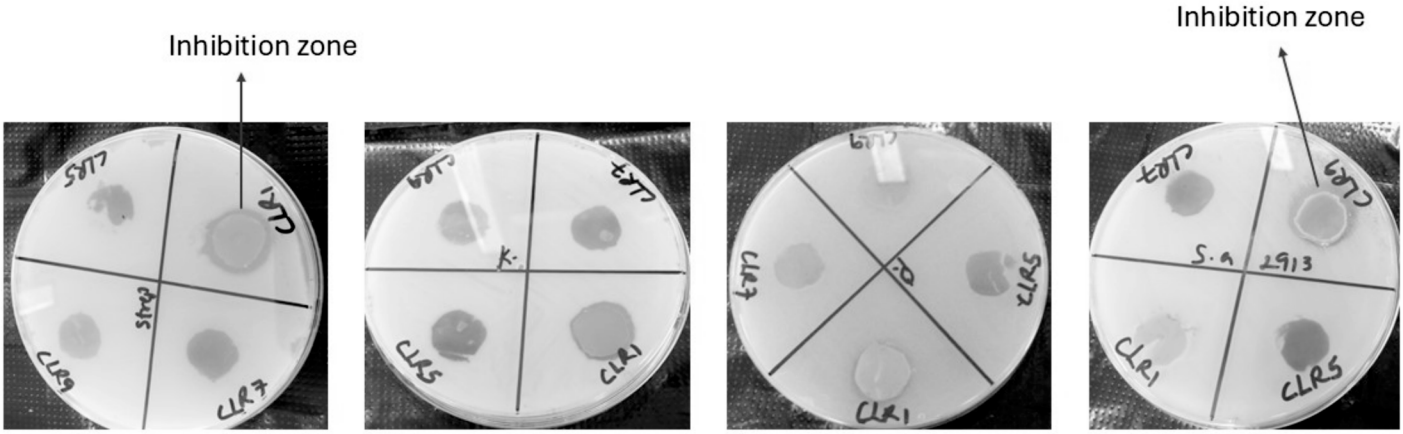

Figure 2: Agar plates showing the preliminary antimicrobial screening of CLR1 and CLR9 isolates against several pathogens. Strep - *Streptococcus agalactiae* ATCC 13813; K- *Klebsiella pneumoniae* ATCC 70603; *P- Pseudomonas aeruginosa* ATCC 152338 and S.a - *Staphylococcus aureus* ATCC 15305.

The sequence blast prediction based on ITS sequence for strains CLR1 and CLR9 identified them as *Paecilomyces dactylethromorphus* and *Aspergillus ochraceopetaliformis* at percentage identity values of 99.65 % and 99.43 % respectively.

Table 1: Sequence BLAST prediction report for the putative fungal isolates

Sample ID	Organism	sequence length (bp)	% identity	Accession no of BLAST hit	E-value	Alignment score	Query coverage (%)
CLR1	*Paecilomyces dactylethromorphus*	606	99.65%	NR_149330.1	0.0	=200	99%
CLR9	*Aspergillus ochraceopetaliformis*	555	99.43%	NR_135390.1	0.0	=200	99%

bp – base pair, **E-value** (expect value).

The ITS consensus sequence was generated by aligning forward and reverse reads of the ITS1-ITS4 regions for the two putative endophytic fungal isolates and the construction was done using the Geneious software (Fig. 3).

>CLR1

TTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGCGGGTCCCTCGTGGCCCAACCTCCCATCCGTGTTGACCGACACCTGTTG
CTTCGGCGGGCCCGCCAGGGCTCCCGCCCCGGCCGCCGGGGGGCATTGCCCCCGGGCCCGCGCCCGCCGAAGACCCCTCGAACGC
TGCCTTGAAGGTTGCCGTCTGAGTATCAAATCAAATCGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACG
CAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGCATTCCGGGG
GGCATGCCTGTCCGAGCGTCATTGCTAACCCTCCAGCCCGGCTGGTGTGTTGGGCCGTCGTCCCCTCCGGGGGACGGGCCCGAAAG
GCAGCGGCGGCGCCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACGCGCTCTAGTAGGCCCGGCCGGCTTGCTGGCCAACGAC
CTCACGGTCACCTAACTTCTCTCTTAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

>CLR9

TCCGTAGGTGAACCTGCGGAAGGATCATTACTGAGTGAGGGTTCCTTCGGGGCCCAACCTCCCACCCTTGTATACTGTACCAAGTTG
CTTCGGCGGGCCCGCCGTTCGCGCGGCCGCCGGGGGGGAACCCCTCCCCCCGGGCGAGCGCCCGCCGGAGACCCCAACGTGAACA
CTGTCTGAAGTTTTGTCGTCTGAGTTCGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAA
GAACGCAGCGAAATGCGATAATTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCACCCCCTGGTATTC
CGGGGGGTATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCCTCGTCCCCCCCGGGGGACGGGCCCG
AAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACACCGCTCTCGTAGGCCCGGCCGGCGCTGGCCGAC
GCTGAAAAGCAACCATTATTCTCCCAGGGTTGACC

Figure 3: Consensus ITS sequences of the two putative endophytic fungi *Paecilomyces* sp. CLR1 and *Aspergillus* sp. CLR9 generated from base calling using Geneious software version 9.0.5

The crude extract of the two putative endophytic fungal isolates showed inhibitory activities against all the pathogenic isolates tested. *Paecilomyces* sp. CLR1 showed higher inhibitory zone diameter (IZD) values for all pathogens when compared to the IZD values shown by *Aspergillus* sp. CLR9 (Table 2).

Table 2: Antimicrobial potential of putative endophytic fungal strains from Curcuma longa

Fungi strain	Inhibition zone diameters									
	S. aga	K. pneumo	E. faeca	A. bauma	P. aeru	S. aur (a)	E. coli	B. sub	S. aur (b)	H. infl
CLR1	18±1.2	20±1.4	26±0.4	12±2.3	18±0.7	24±1.5	18±0.1	28±1.8	24±2.2	22±0.2
CLR9	14±0.6	16±2.1	12±0.6	15±1.3	12±0.8	20±1.3	10±0.5	22±0.3	18±0.6	16±1.2
Gentamicin	12±1.2	18±0.3	20±1.6	0	0	20±0.2	15±0.6	20±1.8	16±2.4	14±1.6

S. aga - *Streptococcus agalactiae* ATCC 13813, K. pneumo - *Klebsiella pneumoniae* ATCC 70603, E. faeca - *Enterococcus faecalis* ATCC 51299, A. bauma - *Acinetobacter baumannii* ATCC 19606, P. aeru - P*seudomonas aeruginosa* ATCC 152338, S. aur (a) - *Staphylococcus aureus* ATCC 15305, E. coli - *Escherichia coli* ATCC 25922, B. sub - *Bacillus subtilis* ATCC 215872, S. aur (b) - *Staphylococcus aureus* ATCC 29713, H. infl - *Haemophilus influenzae* ATCC 49766.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in parenthesis for each of the tested pathogen showed the antimicrobial potential of *Paecilomyces* sp. CLR1 and *Aspergillus* sp. CLR9 (Table 3).

Table 3: MIC and MBC of putative endophytic fungal strains from *Curcuma longa*

Fungi strain	MIC and MBC									
	S. aga	K. pneumo	E. faeca	A. bauma	P. aeru	S. aur (a)	E. coli	B. sub	S. aur (b)	H. infl
CLR1	6.25±0 (8.3±1.7)	16.67±3.40 (25.0±0)	10.42±2.1 (12.5±0)	12.5±0 (25±0)	20±4.16 (16.7±4.2)	4.2±1.04 (8.3±2.0)	8.3±2.04 (10.4±2.1)	5.2±1.04 (6.25±0)	4.2±1.04 (5.17±1.04)	16.7±4.16 (20.8±4.2)
CLR9	3.13±0 (3.13±0)	2.6±0.50 (4.2±0.5)	10.4±2.1 (12.5±0)	10.4±2.1 (10.4±2.1)	16.7±4.1 (20.8±4.2)	2.1±0.5 (2.6±0.5)	5.2±1.04 (6.25±0)	6.25±0 (6.25±0)	1.57±0. (2.1±0.5)	12.5±0 (16.7±4.2)

S. aga - *Streptococcus agalactiae* ATCC 13813, K. pneumo - *Klebsiella pneumoniae* ATCC 70603, E. faeca - Enterococcus faecalis ATCC 51299, A. bauma - *Acinetobacter baumannii* ATCC 19606, P. aeru - *Pseudomonas aeruginosa* ATCC 152338, S. aur (a) - *Staphylococcus aureus* ATCC 15305, E. coli - *Escherichia coli* ATCC 25922, B. sub - *Bacillus subtilis* ATCC 215872, S. aur (b) - *Staphylococcus aureus* ATCC 29713, H. infl - *Haemophilus influenzae* ATCC 49766. The values at the upper part are the MIC (mg/mL) while the values in brackets are the MBC (mg/mL). MIC stands for Minimum Inhibitory Concentration; MBC stands for Minimum Bactericidal Concentration. Records were taken in triplicates and documented as average value ± standard error of the mean.

The phylogenetic analysis of the two putative strains showed their taxonomic position compared to the strains on the gene bank. Strain CLR1 had a close phylogenetic relationship with *Paecilomyces dactylethromorphus* CBS 251.55 at a bootstrap support of 80% (Figure 4) while strain CLR9 had most recent relationship with *Aspergillus ochraceopetaliformis* NRRL 4752 and *Aspergillus flocculosus* CBS 112785 at a bootstrap support of 60% (Figure 5)

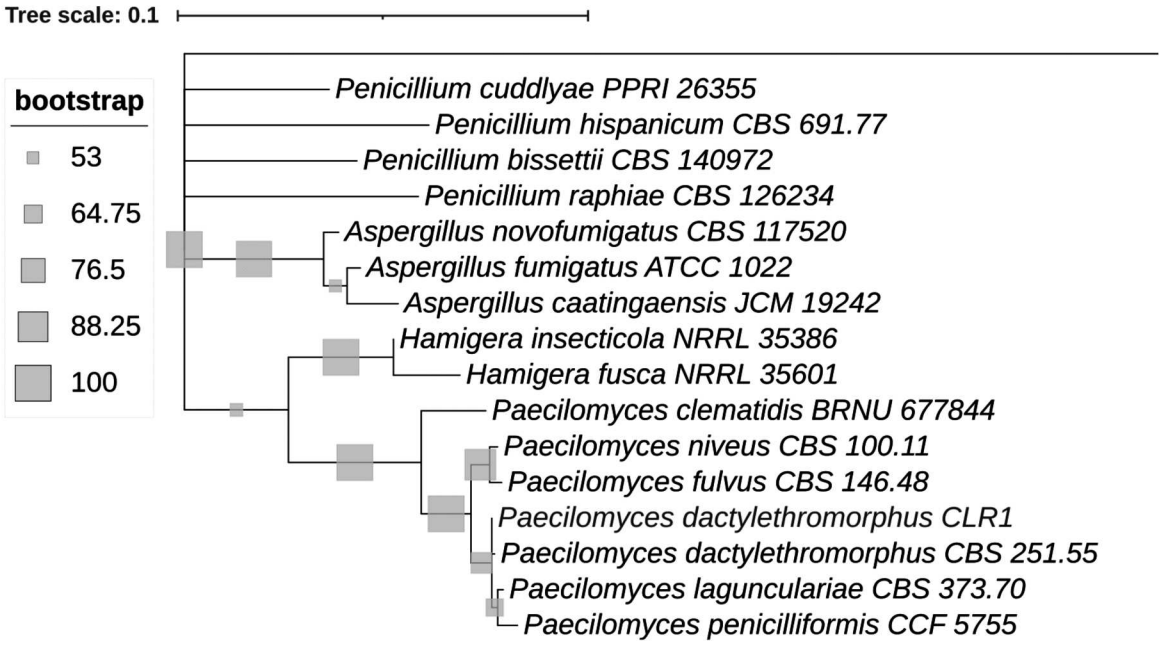

Figure 4: phylogenetic tree indicating the position of *Paecilomyces dactylethromorphus* CLR1 among related isolates obtained from NCBI database. The tree was drawn using the Geneious software and annotated using iTOL v.6 (https://itol.embl.de). Our isolate was highlighted in red while others are related strains from NCBI database. The scale 0.1 represents the number of substitutions per site.

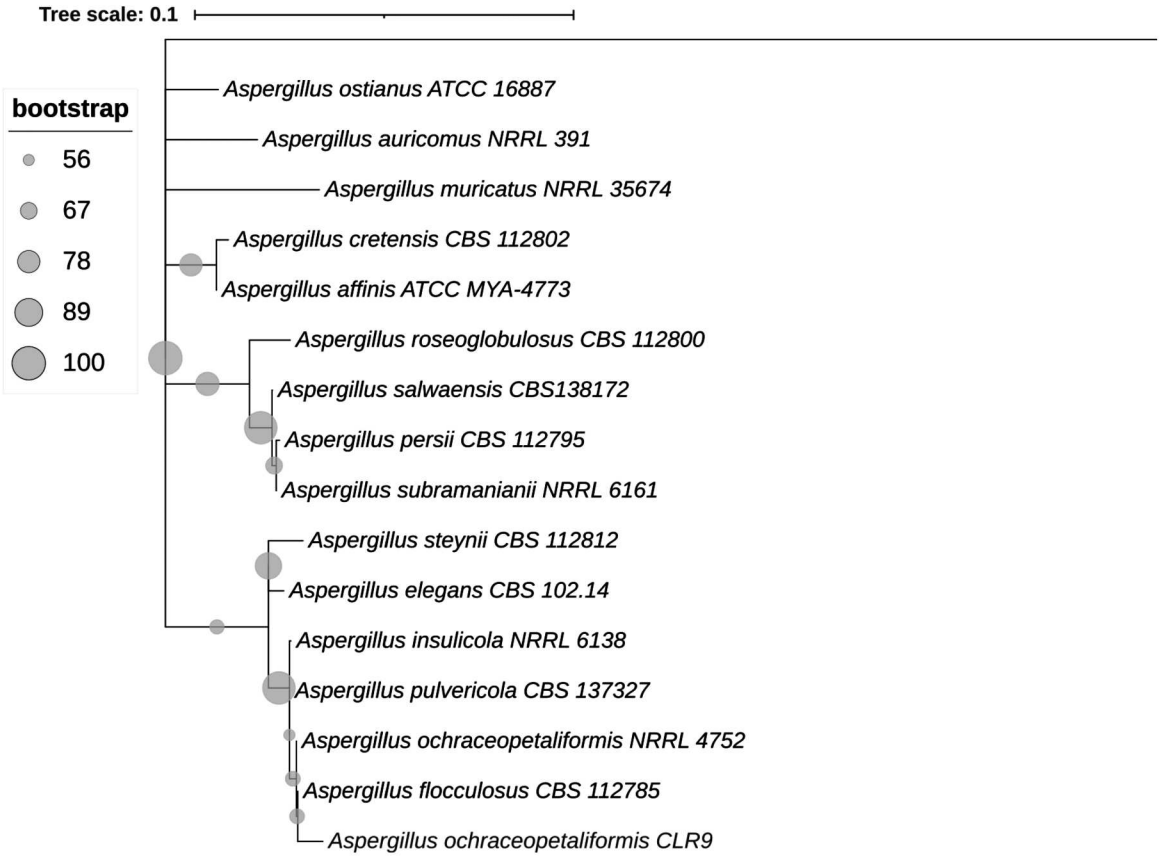

Figure 5: Phylogenetic tree indicating the position of *Aspergillus ochraceopetaliformis* CLR9 among related isolates obtained from NCBI database. The tree was drawn using the Geneious software and annotated using iTOL v.6 (https://itol.embl.de). Our isolate was highlighted in red while others are related strains from NCBI database. The scale 0.1 represents the number of substitutions per site.

DISCUSSION

Ten fungal endophytes were isolated from turmeric (*Curcuma longa*) rhizomes. Various endophytic fungi within *C. longa* suggest a rich microbial diversity, potentially contributing to the plant's known medicinal properties. Each endophyte displayed unique morphological characteristics, indicating a variety of species. Surface-sterilized plant tissue segments that were gently pressed onto the surface of potato dextrose agar showed that the sterilization was effective, confirming only true endophytes were obtained.

Of the 10 isolates, only CLR1 and CLR9 exhibited notable antimicrobial properties. Molecular analysis through ITS sequencing identified these strains as closely related to *Paecilomyces dactylethromorphus* and *Aspergillus ochraceopetaliformis*, respectively, with high similarity percentages (99.65% for CLR1 and 99.43 % for CLR9). These findings are significant because members of these genera are known for producing secondary metabolites, which can have pharmacological applications.[43,44] The identification through ITS sequencing strengthens the reliability of these isolates as distinct strains with potential antimicrobial utility, with phylogenetic analysis further supporting the taxonomic placement of these isolates.

Both CLR1 and CLR9 showed antimicrobial activity against a spectrum of pathogens, including ESKAPE group members, known for their resistance to conventional antibiotics.[45] The zone of growth inhibition in diameter for CLR1 and CLR9 ranged from 10 mm to 28 mm across different tested pathogens, comparable to or exceeding those achieved by gentamicin in some cases. This suggests the crude extracts from these endophytes contain bioactive compounds capable of inhibiting multidrug-resistant organisms.

CLR1 elicited more potent inhibition than CLR9, particularly against Bacillus subtilis, *Staphylococcus aureus*, and *Klebsiella pneumoniae*, highlighting CLR1's potential for further study in the development of novel antimicrobials. CLR9's activity, although somewhat lower, also demonstrated substantial effectiveness against *Acinetobacter baumannii* and *Haemophilus influenzae*. The broad antimicrobial properties of Paecilomyces species agree with the report of many authors. They are also known for antibiofilm and wound healing effects.[46,47] The MIC values for the fungal crude extracts ranged from 1.57 to 20 mg/ml, indicating varying degrees of potency across different pathogens. Notably, lower MIC values against certain pathogens (like *K. pneumoniae* and *S. aureus*) reinforce the isolates' potential as sources of effective antimicrobial agents. The MBC results showed a similar trend, with concentrations needed for bactericidal effects varying by organism. These MIC and MBC values suggest that while the extracts show promise, further fractionation and purification could enhance potency and yield active components with potentially lower MIC and MBC values.

The study highlights that *C. longa* serves as a host to endophytic fungi with antibiotic potential. This discovery opens new avenues for the exploration of *C. longa*-associated fungi in search of bioactive compounds to combat antibiotic-resistant pathogens. Further studies should aim at the purification and structural elucidation of the active metabolites in CLR1 and CLR9. Techniques such as high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), and mass spectrometry (MS) could help isolate specific compounds responsible for the observed antimicrobial effects.[48,40] The findings underscore the potential of using endophytic fungi from ethnomedicinal plants to discover new antibiotics. With the rise of multidrug-resistant pathogens, exploring underutilized sources like endophytic fungi is increasingly crucial.[49,50] Identifying and developing these secondary metabolites could significantly impact healthcare by providing alternative treatments to address the global antibiotic resistance crisis.[51]

In conclusion, this study provided evidence that endophytic fungi isolated from C. longa possess potent antimicrobial properties. The remarkable antimicrobial activity of CLR1 and CLR9 against selected isolates which is suggestive of their potential for antibiotic production. Further investigations into the active compounds could be of therapeutic values for effective management of infections caused by multi drug resistant pathogens.

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