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ISOLATION AND MOLECULAR CHARACTERIZATION OF NEUROSPORA CRASSA AN ENDOPHYTIC FUNGUS ISOLATED FROM TERMINALIA ARJUNA

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ABSTRACT
Endophytic fungi are ubiquitous in nature and play an essential role in balancing the microenvironments of the host tissues. In this study, endophytic fungus Neurospora crassa was isolated from the ethnomedicinal plant Terminalia arjuna. Endophytic fungi live inside the tissues of mature plants without causing any harm to the host tissue. T. arjuna has been known as important ethnomedicinal plant employed for cardiovascular problems, high blood pressure and hypertension. For the study, the leaf and bark tissues of the host plant were selected for the isolation purpose, the pure culture obtained during the study was labelled as ANF4 and used for morphological and molecular characterization. The morphological colony characteristics study suggested the culture belonging to Neurospora sp.; The phylogenetic relationship was determined by r-DNA-based phylogenetic markers ITS (Internal Transcribed Spacers) with the help of phylogenetic tools. The present study aimed to the similarity of endophyte Neurospora crassa (sample -ANF4) isolated from Terminalia arjuna using ITS molecular marker. The dendrogram obtained from the data showed that hierarchical clustering separated the isolates into two groups. The results showed the highest similarity of Neurospora crassa. The sample that was labelled as ANF4 was found to be Neurospora crassa, and showed high similarity based on nucleotide homology and phylogenetic analysis.

KEYWORDS: Endophytic fungi, Terminalia arjuna, ITS, Molecular, Neurospora.

INTRODUCTION

Isolation of bioactive compounds from medicinal plants has been an ever-increasing need due to the rising cases of antimicrobial resistance. Endophytes are microscopic organisms that colonize living tissues of plants without causing any adverse effects to the plants, thus they form a symbiotic relationship with the plants (Soltani, 2017). Every growing plant on this planet has endophytes colonizing in their inner tissues which may be either endophytic bacteria or endophytic fungi. However, in spite of the high biological potential majority of the Indian medicinal plants have been unexplored and understudied for the investigation of endophytic diversity (Patil *et al*., 2014).

Endophytic fungi synthesize bioactive compounds including phenols, flavonoids, steroids, carbohydrates, terpenoids, tannins and saponins (Bhattacharya *et al*., 2021). Paclitaxel also known as taxol was the first identified drug isolated from the medicinal plant *Taxus brevifolia* and is highly explored as an anticancer drug. The preliminary step for isolation of endophytic fungi includes selection of host plant which has promising medicinal properties. Plants with high ethnomedicinal value are efficient candidates for the isolation and study of endophytes (Rajeswari *et al*., 2017). Endophytic mycoflora composition is influenced by environmental conditions. Endophytic assemblages inhabiting a particular host plant have demonstrated tissue specificity due to the anatomical, biochemical, morphological, and physiological characteristics of the host tissue (Rodriguez *et al*., 2009). Endophytic diversity in host plants is also strongly influenced by abiotic factors such as soil type, moisture, temperature, climatic, and environmental factors. Host species, tissue type, and abiotic factors have been shown to influence the colonization rate, diversity, and community composition of endophytic fungi (Yan *et al*., 2019).

Terminalia arjuna is an important ethnomedicinal plant belonging to family Combretaceae commonly known as Arjuna; the medicinal plant grows throughout India. The ethnomedicinal documents depict that the bark of the plant is used as a cardiotonic for heart problems, skin problems, bone fractures, fever and worm infestation (Kouipou *et al*., 2019). The present study deals with the

isolation of endophytic fungi from the aerial parts of the plant. The isolated endophytic fungi were characterized using morphological characterization and molecular markers. The diversity of fungal endophytes associated with the various plant tissues of *T. arjuna* is only sparsely reported. Therefore, the present study was designed to understand the similarity and diversity of endophytic fungi with available sequences.

MATERIALS AND METHODS

Study area and Collection of plant material

For the experimental study, the medicinal plant *Terminalia arjuna* was collected from the nearby forest regions of Pohra Forest and Melghat Forest in Amravati District. The harvested plant material was thoroughly washed with distilled water, and the cleaned plant parts were subsequently utilized for the isolation of endophytes.

Isolation of Endophytic fungi

For isolation, the collected fresh plant parts were washed thoroughly under running tap water thrice to remove any debris attached and disinfected. Further the collected plant parts were excised using scalpel/Sterile scissors in 0.5 to 1.0 cm pieces. The plant pieces were surface sterilized using 70% alcohol, 2% sodium hypochlorite solution and sterile distilled water. The disinfected plant segments were place on a sterile filter paper for drying and later placed on Malt Extract Agar (MEA) plates for growth. The petri plates were incubated at 27±2°C for 3-5 days and checked regularly for growth (Patil *et al*., 2014).

Morphological identifications

The endophytic fungi were morphologically observed using the lactophenol cotton blue reagent and examined under the Leica DM 750 microscope at various magnifications. The fungal isolates were identified based on their macroscopic and microscopic characteristics, including colony color, hyphal structure, fruiting structures, spore morphology, and reproductive structures, in accordance with standard taxonomic manuals (Bhattacharya *et al*., 2021).

Extraction of genomic DNA

DNA was isolated from the ANF4 endophyte isolated from *Neurospora crassa*. Its quality was evaluated on % agarose gel, a single band of high-molecular-weight DNA was observed. The fragment of 18S rRNA gene was amplified by NS1 and NS4 primers. A single discrete PCR amplicon band of 1050 bp was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with NS1 and NS4 primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The consensus sequence of the 18S rRNA gene was generated from forward and reverse sequence data using aligner software. The 18S rRNA gene sequence was used to carry out BLAST with the 'nr' database of NCBI GenBank database. Based on the maximum identity score first ten sequences were selected and aligned using the multiple alignment software program Clustal W. Distance matrix and phylogenetic tree were constructed using MEGA 10.

Evolutionary analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura *et al.*, 2004). The tree with the highest log likelihood (-1542.26) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1040 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Kumar *et al.*, 2018).

RESULTS AND DISCUSSIONS

During the study, endophytic fungus was isolated in the form of pure culture through leaves, and bark tissues of *Terminalia arjuna*. Based on the morphological observation, the colony characteristics of isolate ANF4 was isolated on malt extract agar (MEA) and maintained as pure cultures. They colony and microscopic morphology of the isolate was observed for the identification purpose. The colony morphology observation of the AF4 isolate in the flask culture showed similarity with *Neurospora* species. Filamentous hyphae were observed with axons woven into a closed mycelium.

Figure 1: Colony morphology of Isolate ANF4.

Figure 2 shows the highest similarity of endophytes isolated from *Terminalia arjuna Neurospora crassa*. with available sequences. Table 1 shows sequences producing significant alignments. The similarity of *Neurospora crassa* was found *99.50*% to 99.81% and Table 2 shows the distance matrix.

The sample labeled as ANF4 was found to be Neurospora crassa, showing high similarity based on nucleotide homology and phylogenetic analysis. The number of base substitutions per site from between

sequences is shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Maximum Composite Likelihood model.[1] This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1040 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

The screening of endophytic fungi in some medicinal plants provides knowledge about their fungal symbionts. Also, it is possible to speculate on the role of these fungi through mutual interactions.

Selker (2011) explained the morphological characteristics of *Neurospora crassa*. The author observed that the haploid vegetative filaments/hyphae, were like spindle threads intertwined to form mycelium. The fungus is "heterosporous," meaning that it has different subtypes ("reproductive types") that must be found to enter the sexual stage of its life cycle; about 10 days later, its fruiting bodies ("perithecia") push the conidia toward the light. Choudhary *et al*. (2024) reported isolation of twenty Neurospora strains from spore samples collected during the study. The reproductive types and culture species of Neurospora were determined during the study. The results showed that the 20 strains all belonged to *Neurospora intermedia* and had large (11-19 µm) yellow spores.

Similar results were found by Tripura et al., (2024) studied endophytic fungal diversity in Terminalia arjuna (Roxb.) Wight & Arn. of Tripura, Northeast India at different sampling sites and plant organs. Tejasvi et al., (2024) demonstrated endophytic Fungal Assemblages from the Inner Bark and Twig of Terminalia arjuna W. & A. (Combretaceae).

CONCLUSIONS

In current scenario, researchers are increasingly interested in isolating and characterizing endophytic fungi found in medicinal plants, because they produce a wide variety of chemical entities of biotechnological and pharmaceutical importance. In addition, some of them are capable of producing metabolites with biological activities similar to those of their host plants. The present study reported isolation of endophytic fungi from the leaf and bark tissues of *T. arjuna*. The isolated endophytic sample ANF4 was found to be *Neurospora crassa*, and showed high similarity based on nucleotide homology and phylogenetic analysis.

Sanger Seq Chromatogram data file Data

>Forward Seq data

TTAAGCAATTAAACCGCGAAACTGCGAATGGCTC
ATTAAATCAGTTATAGTTTATTTGATAGTACCTTA
CTACATG
GATAACCGTGGTAATTCTAGAGCTAATACATGCT
AAAAACCCCGACTTCGGAAGGGGTGTATTTATTA
GATTAAA
AACCAATGCCCTTCGGGGCTAACTGGTGATTCAT
AATAACTTCTCGAATCGCATGGCCTTGCGCTGGC
GATGGTT
CATTCAAATTTCTGCCCTATCAACTTTCGACGGCT
GGGTCTTGGCCAGCCATGGTGACAACGGGTAAC
GGAGGG
TTAGGGCTCGACCCCGGAGAAGGAGCCTGAGAA
ACGGCTACTACATCCAAGGAAGGCAGCAGGCGC
GCAAATT
ACCCAATCCCGACACGGGGAGGTAGTGACAATA
AATACTGATACAGGGCTCTTTTGGGTCTTGTAAT
TGGAATG
AGTACAATTTAAATCCCTTAACGAGGAACAATTG
GAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAAT
TCCAGCT
CCAATAGCGTATATTAAAGTTGTTGAGGTTAAAA
AGCTCGTAGTTGAACCTTGGGCTCGGCCCGTCGG
TCCGCCT
CACCGCGTGCACTGACTGGGTCGGGCCTTTTTTC
CTGGAGAACCGCATGCCCTTCACTGGGTGTGTCA
GGGAAC
CAGGACTTTTACCGTGAACAAATCAGATCGCTCA
AAGAAGGCCTATGCTCCAATGTACTAGCATGGA
ATAATAG AATAGGACGTGTGGTTCTA

>Reverse Seq Data

CAAACATTTTGATTTATCGTAAGGTGCCGAACGG
GTCAAAAAATAACACCGTCCGATCCCTAATCGGC
ATAGTTT
ATGGTTAAGACTACGACGGTATCTGATCGTCTTC
GATCCCCTAACTTTCGTTCCTGATTAATGAAAAC
ATCCTTGG
CAAATGCTTTCGCAGTAGTTAGTCTTCAATAAAT
CCAAGAATTTCACCTCTGACAATTGAATACTGAT
GCCCCCGA
CTGTCCCTATTAATCATTACGGCGGTCCTAGAAA
CCAACAAAATAGAACCACACGTCCTATTCTATTA
TTCCATGC
TAGTACATTCGAGCATAGGCCTTCTTTGAGCGAT
CTGATTTGTTCACGGTAAAAGTCCTGGTTCCCCG
ACACACCC
AGTGAAGGGCATGCGGTTCTCCAGGAAAAAAGG
CCCGACCCAGTCAGTGCACGCGGTGAGGCGGAC
CGACGG
GCCGAGCCCAAGGTTCAACTACGAGCTTTTTAAC
CTCAACAACTTTAATATACGCTATTGGAGCTGGA
ATTACCG
CGGCTGCTGGCACCAGACTTGCCCTCCAATTGTT
CCTCGTTAAGGGATTTAAATTGTACTCATTCCAA
TTACAAGA
CCCAAAAGAGCCCTGTATCAGTATTTATTGTCAC
TACCTCCCCGTGTCGGGATTGGGTAATTTGCGCG
CCTGCTG
CCTTCCTTGGATGTAGTAGCCGTTTCTCAGGCTCC
TTCTCCGGGGTCGAGCCCTAACCCTCCGTTACCC
GTTGTCA
CCATGGCTGGCCAAGACCCAGCCGTCGAAAGTT
GATAGGGCAGAAATTTGAATGAACCATCGCCAG
CGCAAGG

CCATGCGATTCGAGAAGTTATTATGAATCACCAG
TTAGCCCCGAAGGGCAT

>Reverse complement

ATGCCCTTCGGGGCTAACTGGTGATTCATAATAA
CTTCTCGAATCGCATGGCCTTGCGCTGGCGATGG
TTCATTC
AAATTTCTGCCCTATCAACTTTCGACGGCTGGGT
CTTGGCCAGCCATGGTGACAACGGGTAACGGAG
GGTTAGG
GCTCGACCCCGGAGAAGGAGCCTGAGAAACGGC
TACTACATCCAAGGAAGGCAGCAGGCGCGCAAA
TTACCCA
ATCCCGACACGGGGAGGTAGTGACAATAAATAC
TGATACAGGGCTCTTTTGGGTCTTGTAATTGGAA
TGAGTAC
AATTTAAATCCCTTAACGAGGAACAATTGGAGG
GCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAG
CTCCAAT
AGCGTATATTAAAGTTGTTGAGGTTAAAAAGCTC
GTAGTTGAACCTTGGGCTCGGCCCGTCGGTCCGC
CTCACCG
CGTGCACTGACTGGGTCGGGCCTTTTTTCCTGGA
GAACCGCATGCCCTTCACTGGGTGTGTCGGGGAA
CCAGGA
CTTTTACCGTGAACAAATCAGATCGCTCAAAGAA
GGCCTATGCTCGAATGTACTAGCATGGAATAATA
GAATAG
GACGTGTGGTTCTATTTTGTTGGTTTCTAGGACCG
CCGTAATGATTAATAGGGACAGTCGGGGGCATC
AGTATTC
AATTGTCAGAGGTGAAATTCTTGGATTTATTGAA
GACTAACTACTGCGAAAGCATTTGCCAAGGATGT
TTTCATT
AATCAGGAACGAAAGTTAGGGGATCGAAGACGA
TCAGATACCGTCGTAGTCTTAACCATAAACTATG
CCGATTA
GGGATCGGACGGTGTTATTTTTTGACCCGTTCGG
CACCTTACGATAAATCAAAATGTTTG

> Consensus data

TTAAGCAATTAAACCGCGAAACTGCGAATGGCTC
ATTAAATCAGTTATAGTTTATTTGATAGTACCTTA
CTACATG
GATAACCGTGGTAATTCTAGAGCTAATACATGCT
AAAAACCCCGACTTCGGAAGGGGTGTATTTATTA
GATTAAA
AACCAATGCCCTTCGGGGCTAACTGGTGATTCAT
AATAACTTCTCGAATCGCATGGCCTTGCGCTGGC
GATGGTT
CATTCAAATTTCTGCCCTATCAACTTTCGACGGCT
GGGTCTTGGCCAGCCATGGTGACAACGGGTAAC
GGAGGG
TTAGGGCTCGACCCCGGAGAAGGAGCCTGAGAA
ACGGCTACTACATCCAAGGAAGGCAGCAGGCGC
GCAAATT
ACCCAATCCCGACACGGGGAGGTAGTGACAATA
AATACTGATACAGGGCTCTTTTGGGTCTTGTAAT
TGGAATG
AGTACAATTTAAATCCCTTAACGAGGAACAATTG
GAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAAT
TCCAGCT
CCAATAGCGTATATTAAAGTTGTTGAGGTTAAAA
AGCTCGTAGTTGAACCTTGGGCTCGGCCCGTCGG
TCCGCCT
CACCGCGTGCACTGACTGGGTCGGGCCTTTTTTC
CTGGAGAACCGCATGCCCTTCACTGGGTGTGTCA
GGGAAC
CAGGACTTTTACCGTGAACAAATCAGATCGCTCA
AAGAAGGCCTATGCTCCAATGTACTAGCATGGA
ATAATAG
AATAGGACGTGTGGTTCTATTTTGTTGGTTTCTAG
GACCGCCGTAATGATTAATAGGGACAGTCGGGG
GCATCA
GTATTCAATTGTCAGAGGTGAAATTCTTGGATTT
ATTGAAGACTAACTACTGCGAAAGCATTTGCCAA
GGATGTT
TTCATTAATCAGGAACGAAAGTTAGGGGATCGA
AGACGATCAGATACCGTCGTAGTCTTAACCATAA
ACTATGC
CGATTAGGGATCGGACGGTGTTATTTTTTGACCC
GTTCGGCACCTTACGATAAATCAAAATGTTTG

Table 1: Sequences producing significant alignments.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Neurospora crassa OR74A	1897	1897	100%	0.0	99.81%	XR_898033.1
Neurospora crassa 18S ribosomal RNA	1897	1897	100%	0.0	99.81%	FJ360521.1
Neurospora crassa 18S ribosomal RNA	1897	1897	100%	0.0	99.81%	AY046271.1
Neurospora crassa DNA ribosomal RNA	1890	1890	100%	0.0	99.71%	X04971.1
Fungal sp. J21 ZM-2014	1884	1884	99%	0.0	99.71%	KP148842.1
Neurospora crassa voucher SABS	1875	1875	100%	0.0	99.42%	MK418236.1
Neurospora crassa strain ZK01	1873	1873	99%	0.0	99.51%	OM009248.1
Neurospora crassa isolate AKS-11	1866	1866	100%	0.0	99.23%	OQ551171.1
Thermocarpiscus australiensis SYAC	1810	1810	97%	0.0	99.01%	OP107015.1
Neurospora crassa strain TM2	1808	1808	96%	0.0	99.50%	OR807405.1

Table. Estimates of Evolutionary Divergence between Sequences.

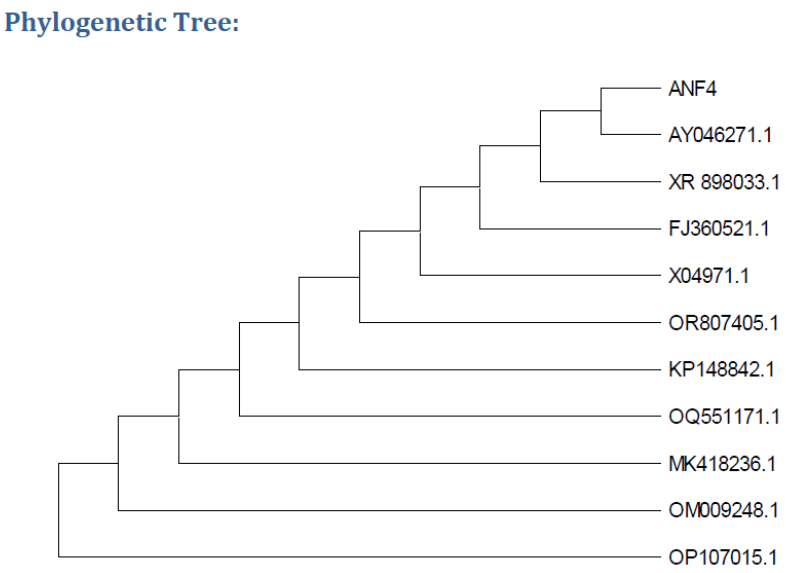

Figure 2: Phylogenetic tree.

Table 2: Distance Matrix.

ANF4		0.001	0.001	0.001	0.001	0.001	0.003	0.002	0.002	0.003	0.001
XR_898033.1	0.002		0.000	0.000	0.000	0.000	0.002	0.002	0.001	0.002	0.000
FJ360521.1	0.002	0.000		0.000	0.000	0.000	0.002	0.002	0.001	0.002	0.000
AY046271.1	0.002	0.000	0.000		0.000	0.000	0.002	0.002	0.001	0.002	0.000
X04971.1	0.002	0.000	0.000	0.000		0.000	0.002	0.001	0.001	0.002	0.000
KP148842.1	0.002	0.000	0.000	0.000	0.000		0.002	0.002	0.001	0.002	0.000
MK418236.1	0.006	0.004	0.004	0.004	0.003	0.003		0.002	0.002	0.003	0.002
OM009248.1	0.004	0.002	0.002	0.002	0.001	0.002	0.003		0.002	0.002	0.001
OQ551171.1	0.004	0.002	0.002	0.002	0.002	0.001	0.004	0.003		0.002	0.001
OP107015.1	0.008	0.006	0.006	0.006	0.005	0.006	0.007	0.005	0.007		0.002
OR807405.1	0.002	0.000	0.000	0.000	0.000	0.000	0.003	0.001	0.001	0.006	

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